Module 2

Cell Signalling Pathways

Synopsis

Cells use a large number of clearly defined signalling pathways to regulate their activity. In this module, attention is focused on the On mechanisms responsible for transmitting information into the cell. These signalling pathways fall into two main groups depending on how they are activated. Most of them are activated by external stimuli and function to transfer information from the cell surface to internal effector systems. However, some of the signalling systems respond to information generated from within the cell, usually in the form of metabolic messengers. For all of these signalling pathways, information is conveyed either through protein–protein interactions or it is transmitted by diffusible elements usually referred to as second messengers. Cells often employ a number of these signalling pathways, and cross-talk between them is an important feature. In this section, attention is focused on the properties of the major intracellular signalling pathways operating in cells to regulate their cellular activity.

During the processes of development, specific cell types select out those signalling systems that are suitable to control their particular functions. One of the aims of this website is to understand how these unique cell-specific signalsomes function to regulate different mammalian cell types.

Intracellular signalling pathways

There are a large number of intracellular signalling pathways responsible for transmitting information within the cell. They fall into two main categories. The majority respond to external stimuli arriving at the cell surface, usually in the form of a chemical signal (neurotransmitter, hormone or growth factor), which is received by receptors at the cell periphery that function as molecular antennae embedded in the plasma membrane. These receptors then transfer information across the membrane using a variety of transducers and amplifiers that engage a diverse repertoire of intracellular signalling pathways (Pathways 1–16 in Module 2: Figure cell signalling pathways). The phosphoinositide signalling and Ca^{2+} signalling systems (Pathways 2–6) have been grouped together because they contain a number of related signalling pathways that often interact with each other. The other categories are the pathways that are activated by signals generated from within the cell (Pathways 17 and 18). There are a number of metabolic messengers that act from within the cell to initiate a variety of signalling pathways. GTP-binding proteins often play a central role in the transduction process responsible for initiating many of these signalling pathways.

All of these signalling pathways generate an internal messenger that is responsible for relaying information to the sensors that then engage the effectors that activate cellular responses. The main features of the signalling pathways summarized in Module 2: Figure cell signalling pathways are outlined below:

1. Cyclic AMP signalling pathway. One of the first signalling systems to be characterized was the cyclic AMP signalling pathway, which led to the second messenger concept that applies to many other signalling systems. The idea is that the external stimulus arriving at the cell surface is the first messenger, which is then transformed at the cell surface by adenylyl cyclase (AC) into a second messenger, cyclic AMP, which is part of the signalling cascade that then activates downstream effectors.
2. Cyclic ADP-ribose (cADPR) signalling and nicotinic acid–adenine dinucleotide phosphate (NAADP) signalling systems function in Ca^{2+} signalling. An ADP–ribosyl cyclase (ADP-RC) is responsible for generating these two second messengers.
3. Voltage-operated channels (VOCs) contribute to Ca^{2+} signals by controlling the entry of external Ca^{2+} in excitable cells.
4. Receptor-operated channels (ROCs) contribute to Ca^{2+} signals by controlling Ca^{2+} entry in both excitable and non-excitable cells.
5. Stimuli that activate phospholipase C (PLC) to hydrolyse PtdIns4,5P_2 (also known as PIP_2) generate a number of signalling pathways:
   - Inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette
   - Diacylglycerol (DAG)/protein kinase C (PKC) signalling cassette
   - PtdIns4,5P_2 signalling cassette
   - Multipurpose inositol polyphosphate signalling pathway
6. PtdIns 3-kinase signalling is activated by stimuli that stimulate PtdIns 3-kinase to phosphorylate PIP_2

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to form the lipid second messenger PtdIns3,4,5P_3 (PIP_3).

7. Nitric oxide (NO)/cyclic GMP signalling pathway. Nitric oxide (NO) synthase (NOS) generates the gas NO that acts both through cyclic GMP and nitrosylation reactions. NO has a particularly important role in modulating the activity of other pathways such as Ca^{2+} signalling.
8. Redox signalling. Many receptors act through NADPH oxidase (NOX) to form reactive oxygen species, such as the superoxide radical ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2), which act to regulate the activity of specific signalling proteins such as tyrosine phosphatases, transcription factors and ion channels. The $O_2^{-\bullet}$ participates in the nitrosylation reaction in Pathway 7.
9. Mitogen-activated protein kinase (MAPK) signalling. This is a classical example of a protein phosphorylation cascade that often begins with Ras and consists of a number of parallel pathways that function to control many cellular processes and particularly those related to cell proliferation, cell stress and apoptosis.
10. Nuclear factor κB (NF-κB) signalling pathway. This signalling system has a multitude of functions. It is particularly important in initiating inflammatory responses in macrophages and neutrophils as part of an innate immune response to invading pathogens.
11. Phospholipase D (PLD) signalling pathway. This is a lipid-based signalling system that depends upon the hydrolysis of phosphatidylcholine by phospholipase D (PLD) to give phosphatidic acid (PA), which functions as a second messenger to control a variety of cellular processes.
12. Sphingomyelin signalling pathway. Certain growth factors and cytokines hydrolyse sphingomyelin to generate two second messengers that appear to have opposing effects in the cell. Ceramide seems to promote apoptosis, whereas sphingosine 1-phosphate (S1P) stimulates cell proliferation. S1P may also release Ca^{2+} from internal stores. The action of S1P is complicated in that it is released from the cell, where it can act as a hormone to stimulate external receptors.
13. Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway. This is a fast-track signal transduction pathway for transferring information from cell-surface receptors into the nucleus. The Janus kinases (JAKs) are tyrosine kinases that phosphorylate the signal transducers and activators of transcription (STATs), which carry the information into the nucleus.
14. Smad signalling pathway. This pathway mediates the action of the transforming growth factor β (TGF-β) superfamily, which controls transcription through the Smad transcription factors.
15. Wnt signalling pathways. These pathways play an important role in both development and cell proliferation.
16. Hedgehog signalling pathway. This pathway resembles the Wnt signalling pathway in that it also functions to regulate development and cell proliferation. The ligand Hedgehog (Hh) acts through the transcription factor GLI.
17. Hippo signalling pathway. This pathway has a core protein kinase cascade that has some similarities to the MAP kinase cascade in that a series of serine/threonine protein kinases function to regulate the transcription of a number of genes that function in cell growth, proliferation and apoptosis.
18. Notch signalling pathway. This is a highly conserved signalling system that functions in developmental processes related to cell-fate determination particularly in stem cells. The notch receptor generates the transcription factor NICD (Notch intracellular domain).
19. Endoplasmic reticulum (ER) stress signalling. The endoplasmic reticulum (ER) stress signalling pathway concerns the mechanisms used by the ER to transmit information to the nucleus about the state of protein processing within the lumen of the ER.
20. AMP signalling pathway. This pathway is regulated by adenosine monophosphate (AMP), which functions as a metabolic messenger to activate an important pathway for the control of cell proliferation.

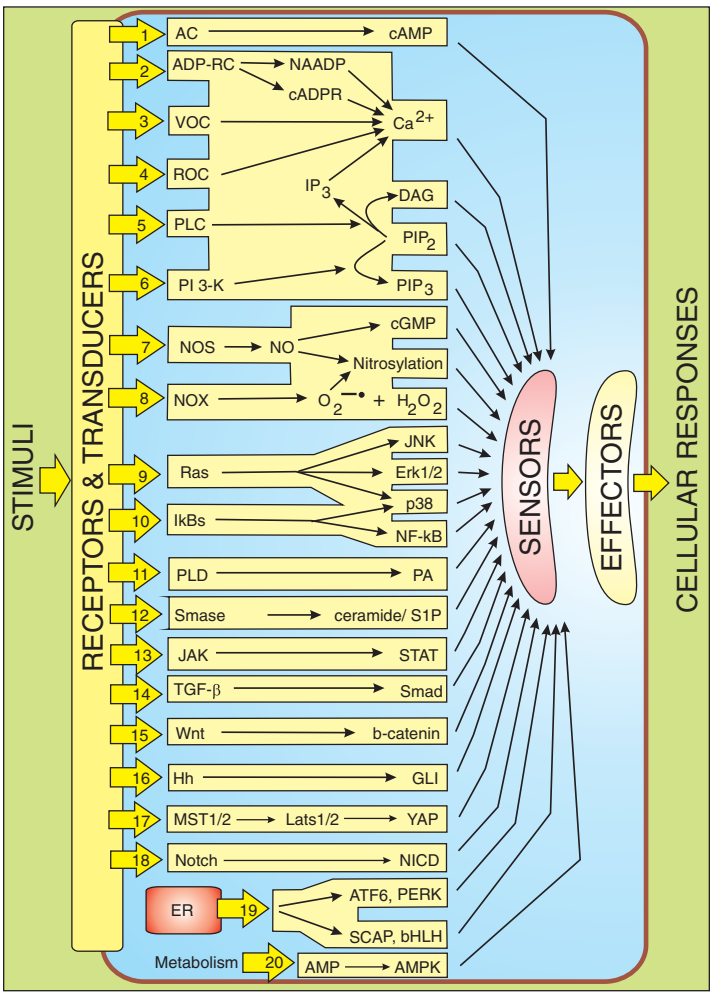
Not included in Module 2: Figure cell signalling pathways are some additional signalling pathways that have specific functions in regulating various aspects of cell metabolism, such as sterol sensing and cholesterol biosynthesis, that control the level of cholesterol in cell membranes. Another example is found in the NAD signalling pathways, where NAD^+ functions to regulate a number of cellular processes, including energy metabolism, gene transcription, DNA repair and perhaps ageing as well.

These cassettes then engage a variety of effectors that are responsible for activating cellular responses. All of these mechanisms (Module 1: Figure signal transmission mechanisms) depend upon information transfer mechanisms whereby information is transferred along an orderly sequence of events to activate the internal effectors responsible for inducing a great variety of cellular responses.

Cyclic AMP signalling pathway

Cyclic AMP is a ubiquitous second messenger that regulates a multitude of cellular responses. Cyclic AMP formation usually depends upon the activation of G-protein-coupled receptors (GPCRs) that use heterotrimeric G proteins to activate the amplifier adenylyl cyclase (AC), which is a large family of isoforms that differ considerably in both their cellular distribution and the way they are activated. There are a number of cyclic AMP signalling effectors such as protein kinase A (PKA), the exchange proteins activated by cyclic AMP (EPACs) that activate the small GTP-binding protein Rap1 and the cyclic nucleotide-gated channels (CNGCs). These various effectors are then responsible for carrying out the cyclic AMP signalling functions that include control of metabolism, gene transcription and ion channel activity. In many cases, these functions are modulatory in that cyclic AMP often acts to adjust the activity of other signalling pathways and thus has a central role to play in the cross-talk

Module 2: | Figure cell signalling pathways



Summary of the major signalling pathways used by cells to regulate cellular processes.

Cells have a number of signalling systems that are capable of responding either to external stimuli or to internal stimuli. In the case of the former, external stimuli acting on cell-surface receptors are coupled to transducers to relay information into the cell using a number of different signalling pathways (Pathways 1-17). Internal stimuli derived from the endoplasmic reticulum (ER) or from metabolism activate signalling pathways independently of external signals (Pathways 18 and 19). All of these pathways generate an internal messenger that then acts through an internal sensor to stimulate the effectors that bring about different cellular responses. As described in the text, the names of these signalling pathways usually reflect a major component(s) of the pathway.

between signalling pathways. This modulatory function is particularly evident in the case of Ca^{2+} signalling in both neuronal and muscle cells. Many of the functions of cyclic AMP depend upon the precise location of PKA relative to both its upstream and downstream effectors. A family of A-kinase-anchoring proteins (AKAPs) determines this cellular localization of PKA as well as a number of other signalling components. The OFF reactions responsible for removing cyclic AMP are carried out by either cyclic AMP hydrolysis or by cyclic AMP efflux from the cell.

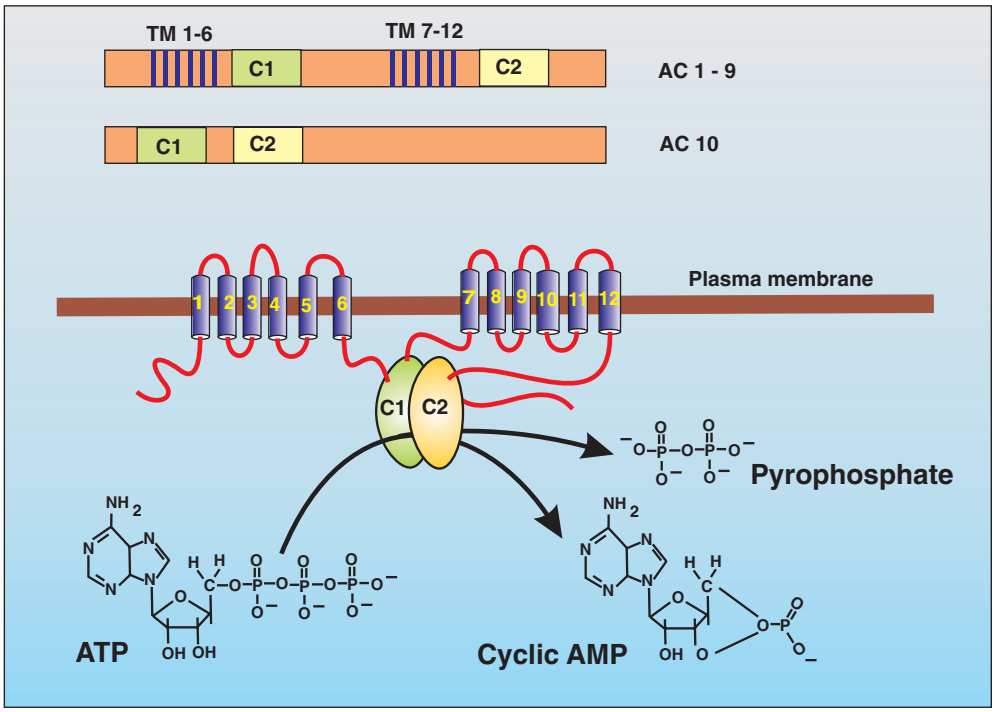
Cyclic AMP formation

The formation of cyclic AMP can be activated by a very large number of cell stimuli, mainly neurotransmitters and hormones (Module 1: Figure stimuli for cyclic AMP signalling). All these stimuli are detected by G-protein-coupled receptors (GPCRs) that use heterotrimeric G proteins, which are the transducers that are responsible for either activating or inhibiting the enzyme adenylyl cyclase (AC) (Module 2: Figure heterotrimeric G protein signalling). In the case of AC stimulation, the external stimulus binds to the GPCR that functions as a guanine nucleotide exchange factor (GEF) to replace GDP with GTP, which dissociates the heterotrimeric complex into their Gβγ and Gα subunits (Module 2: Figure cyclic AMP signalling). The $G\alpha_S$·GTP complex activates AC, whereas $G\alpha_i$·GTP inhibits AC. The Gα subunits have GTPase activity that hydrolyses GTP to GDP, thus terminating their effects on AC. The endogenous GTPase of $G\alpha_S$·GTP is inhibited by cholera toxin and this causes the persistent activation of the intestinal fluid secretion that results in the symptoms of cholera.

Adenylyl cyclase (AC)

The adenylyl cyclase (AC) family is composed of ten isoforms: nine of them are membrane-bound (AC1–AC9), while one of them is soluble (AC10) (**Module 2: Table adenylyl cyclases**). The domain structure of AC1–AC9 is

Module 2: | Figure adenylyl cyclase structure



Domain structure of adenylyl cyclase (AC).
The nine membrane-bound adenylyl cyclases (AC1-AC9) have a similar domain structure. The single polypeptide has a tandem repeat of six transmembrane domains (TM) with TM1-TM6 in one repeat and TM7-TM12 in the other. Each TM cassette is followed by large cytoplasmic domains (C1 and C2), which contain the catalytic regions that convert ATP into cyclic AMP. As shown in the lower panel, the C1 and C2 domains come together to form a heterodimer. The ATP-binding site is located at the interface between these two domains. The soluble AC10 isoform lacks the transmembrane regions, but it retains the C1 and C2 domains that are responsible for catalysis.

characterized by having two regions where there are six transmembrane regions (**Module 2: Figure adenylyl cyclase structure**). The large cytoplasmic domains C1 and C2, which contain the catalytic region, form a heterodimer and co-operate with each other to convert ATP into cyclic AMP.

Cyclic AMP signalling effectors

Cyclic AMP is a highly versatile intracellular messenger capable of activating a number of different effectors (Module 2: Figure cyclic AMP signalling). An example of such effectors is the exchange proteins activated by cyclic AMP (EPACs), which act to stimulate Rap. Another group of effectors are the cyclic nucleotide-gated channels (CNGCs) (Module 3: Figure Ca^{2+} entry mechanisms) that play a particularly important role in the sensory systems responsible for smell and taste. Most of the actions of cyclic AMP are carried out by protein kinase A (PKA).

Protein kinase A (PKA)

Many of the actions of cyclic AMP are carried out by protein kinase A (PKA), which phosphorylates specific sites on downstream effector processes (Module 2: Figure cyclic AMP signalling). PKA is composed of two regulatory (R) subunits and two catalytic (C) subunits. The way in which cyclic AMP activates PKA is to bind to the R subunits, which then enables the C subunits to phosphorylate a wide range of different substrates [Module 2: Figure protein kinase A (PKA)]. Of the two types of PKA, protein kinase A (PKA) I is found mainly free in the cytoplasm and has a high affinity for cyclic AMP, whereas protein kinase A (PKA) II has a much more precise location by being coupled to the A-kinase-anchoring proteins (AKAPs). The AKAPs are examples of the scaffolding proteins that function in the spatial organization of signalling pathways by bringing PKA into contact with its many substrates. The scaffolding function of the AKAPs is carried out by various domains such as the conserved PKA-anchoring domain [yellow region in Module 2: Figure protein kinase A (PKA)], which is a hydrophobic surface that binds to an extended hydrophobic surface on the N-terminal dimerization region of the R subunits. At the other end of the molecule, there are unique targeting domains (blue) that determine the way AKAPs identify and bind specific cellular targets in discrete regions of the cell.

Protein kinase A (PKA) I

Type I protein kinase A (PKA) associates with the RI isoforms. As for all isoforms, the R subunits form dimers through their N-terminal dimerization/docking domains [yellow bar in Module 2: Figure protein kinase A (PKA)]. In addition to holding two R subunits together, this N-terminal region is also responsible for docking to the A-kinase-anchoring proteins (AKAPs), as occurs for PKA II. However, the RI isoforms have a very low affinity for the AKAPs and are thus mainly soluble. Cyclic AMP acts

Module 2: | Table adenylyl cyclases
Regulatory properties and distribution of adenylyl cyclase.

Adenylyl cyclase (AC) isoform	Gα	Gβγ	$G\alpha_i$ or $G\alpha_o$	Modulation by Ca^{2+}, calmodulin (CaM), Ca^{2+}/CaM kinase II (CaMKII), protein kinase C (PKC), protein kinase A (PKA)	Tissue distribution
AC1	↑	↓	↓ $G\alpha_o$	↑ CaM and PKC ↓ CaMKII	Brain, adrenal medulla
AC2	↑	↑	-	↑ PKC	Brain, skeletal and cardiac muscle, lung
AC3	↑			↑ CaM and PKC ↓ CaMKII	Brain, olfactory epithelium
AC4	↑	↑		↑ PKC	Brain, heart, kidney, liver
AC5	↑	↓	↓	↓ Ca^{2+} and PKCα ↓ PKA	Brain, heart, kidney, liver, lung, adrenal
AC6	↑	↓	↓	↑ PKC ↓ Ca^{2+} and PKA	Ubiquitous
AC7	↑	↑		↑ PKC	Ubiquitous, high in brain
AC8	↑			↑ CaM	Brain, lung, heart, adrenal
AC9	↑				Brain, skeletal muscle
AC10	-	-	-	Activated by HCO_3^-	Testis

The membrane-bound adenylyl cyclases (AC1-AC9) are widely distributed. They are particularly rich in brain, but are also expressed in many other cell types. The soluble AC10 is restricted to the testis. The primary regulation of the AC1-AC9 isoforms is exerted through components of the heterotrimeric G proteins, which are dissociated upon activation of neurotransmitter and hormonal receptors into their α and βγ subunits. They are all activated by $G\alpha_s$, but only some of the isoforms are inhibited by $G\alpha_i$. The Gβγ subunit is able to activate some isoforms, but inhibits others. The isoforms also differ in the way they are modulated by components of other signalling pathways such as Ca^{2+} and PKC. Some of the others are inhibited by PKA, which thus sets up a negative-feedback loop whereby cyclic AMP can inhibit its own production. Modified from Table I in Whorton and Sunahara 2003. Reproduced from *Handbook of Cell Signaling*, Volume 2 (edited by R.A. Bradshaw and E.A. Dennis), Whorton, M.R. and Sunahara, R.K., Adenylyl cyclases, pp. 419-426. Copyright (2003), with permission from Elsevier.

by binding to the tandem cyclic AMP-binding domains to release active C subunits that then phosphorylate specific substrates. Since the RI subunits have a higher cyclic AMP-binding affinity, PKA I will be able to respond to the lower cyclic AMP concentrations found globally within the bulk cytoplasm.

Protein kinase A (PKA) II

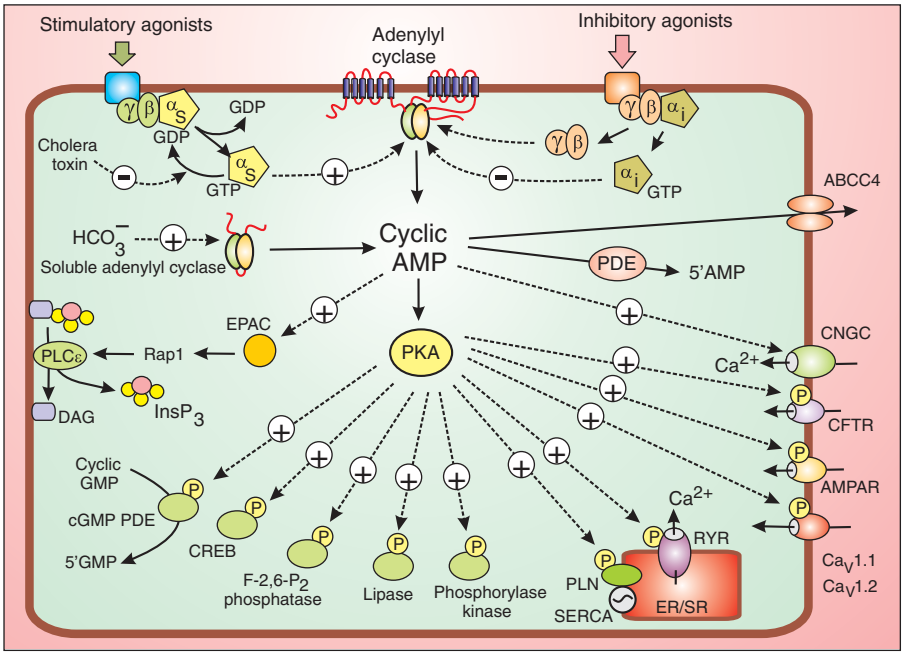
A characteristic feature of Type II protein kinase A (PKA) is that the regulatory dimer is made up of RII subunits. Since this RII subunit has a much higher affinity for the A-kinase-anchoring proteins (AKAPs), PKA II is usually docked to this scaffolding protein and thus has a much more precise localization to specific cellular targets.

The substrates phosphorylated by cyclic AMP (Module 2: Figure cyclic AMP signalling) fall into two main groups: the cyclic AMP substrates that regulate specific cellular processes and the cyclic AMP substrates that are components of other signalling systems.

Cyclic AMP substrates that regulate specific cellular processes:

- In neurons, cyclic AMP acts through PKA to phosphorylate Ser-845 on the AMPA receptor (Module 3: Figure AMPA receptor phosphorylation).
- Cyclic AMP acting through PKA stimulates the fructose-2,6-bisphosphatase component to lower the level of fructose 2,6-bisphosphate, which reduces glycolysis and promotes gluconeogenesis (Module 2: Figure AMPK control of metabolism).
- In insulin-secreting β-cells, the salt-inducible kinase 2 (SIK2) that phosphorylates transducer of regulated cyclic AMP response element-binding protein (TORC) is inhibited by a cyclic AMP/PKA-dependent phosphorylation (Module 7: Figure β-cell signalling).
- PKA phosphorylates the hormone-sensitive lipase (HLS) that initiates the hydrolysis of triacylglycerol to free fatty acids and glycerol in both white fat cells (Module 7: Figure lipolysis and lipogenesis) and in brown fat cells (Module 7: Figure brown fat cell).
- PKA phosphorylates the phosphorylase kinase that converts inactive phosphorylase *b* into active phosphorylase *a* in skeletal muscle (Module 7: Figure skeletal muscle E-C coupling) and in liver cells (Module 7: Figure glycogenolysis and gluconeogenesis).
- PKA activates the transcription factor cyclic AMP response element-binding protein (CREB) (Module 4: Figure CREB activation). This activation is a critical event in the induction of gluconeogenesis in liver cells (Module 7: Figure liver cell signalling).
- PKA inhibits the salt-inducible kinase 2 (SIK2) that normally acts to phosphorylate TORC2, thereby preventing it from entering the nucleus to facilitate the activity of CREB (Module 7: Figure liver cell signalling).
- PKA phosphorylates inhibitor 1 (I1), which assists the protein phosphorylation process by inactivating protein phosphatase 1 (PP1) .
- PKA contributes to the translocation and fusion of vesicles with the apical membrane during the onset of acid secretion by parietal cells (Module 7: Figure HCl secretion).
- PKA phosphorylates the regulatory (R) domain on the cystic fibrosis transmembrane conductance regulator (CFTR) to enable it to function as an anion channel (Module 3: Figure CFTR channel).
- In the small intestine, PKA phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR) channel (Module 3: Figure CFTR channel) that is responsible for activating fluid secretion (Module 7: Figure intestinal secretion). Uncontrolled activation of cyclic AMP formation by cholera toxin results in cholera.
- In kidney collecting ducts, cyclic AMP acts through PKA to phosphorylate Ser-256 on the C-terminal cytoplasmic tail of aquaporin 2 (AQP2), enabling this water

Module 2: | Figure cyclic AMP signalling



Organization and function of the cyclic AMP signalling pathway.
Cyclic AMP is formed both by membrane-bound adenylyl cyclase and by the bicarbonate-sensitive soluble adenylyl cyclase. The former is regulated by both stimulatory agonists that act through the α_S subunit or through inhibitory agonists that act through either the α_i or the $\beta\gamma$ subunits. The increase in cyclic AMP then acts through three different effector systems. It acts through the exchange protein activated by cyclic AMP (EPAC), which functions to activate Rap1. It can open cyclic nucleotide-gated channels (CNGCs). The main action of cyclic AMP is to activate protein kinase A (PKA) to phosphorylate a large number of downstream targets. Some of these drive specific processes such as gene transcription through phosphorylation of cyclic AMP response element-binding protein (CREB), and activation of ion channels [e.g. AMPA receptors and cystic fibrosis transmembrane conductance regulator (CFTR)] and various enzymes that control metabolism [e.g. fructose 2,6-bisphosphate (F-2,6-P_2) 2-phosphatase, lipase and phosphorylase kinase]. Other downstream targets are components of other signalling pathways such as the cyclic GMP phosphodiesterase (cGMP PDE), the phospholamban (PLN) that controls the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA), the ryanodine receptor (RYR) and the Ca^{2+} channels $Ca_V1.1$ and $Ca_V1.2$.

channel to fuse with the apical membrane to allow water to enter the cell (Module 7: Figure collecting duct function).

- In blood platelets, cyclic AMP activates the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), which is a member of the Ena/vasodilator-stimulated phosphoprotein (VASP) family resulting in a decrease in the actin-dependent processes associated with clotting (Module 11: Figure platelet activation).

Cyclic AMP substrates that are components of other signalling systems:

- Phosphorylation of the cyclic GMP phosphodiesterase PDE1A by PKA results in a decrease in its sensitivity to Ca^{2+} activation.
- Entry of Ca^{2+} through the L-type $Ca_V1.1$ channel (Module 3: Figure $Ca_V1.1$ L-type channel) and the L-type $Ca_V1.2$ channel (Module 3: Figure $Ca_V1.2$ L-type channel) is enhanced through PKA-dependent phosphorylation.
- PKA-dependent phosphorylation of dopamine- and cyclic AMP-regulated phosphoprotein of apparent molecular mass 32 kDa (DARPP-32) functions as a molecular switch to regulate the activity of protein phosphatase 1 (PP1).
- The ryanodine receptor 2 (RYR2) is modulated by phosphorylation through PKA, which is associated with the cytoplasmic head through an AKAP (Module 3: Figure ryanodine receptor structure).
- Sarco/endo-plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) increases its Ca^{2+}-pumping activity when the inhibitory effect of phospholamban (PLN) is removed following its phosphorylation by PKA on Ser-16 (Module 5: Figure phospholamban mode of action).

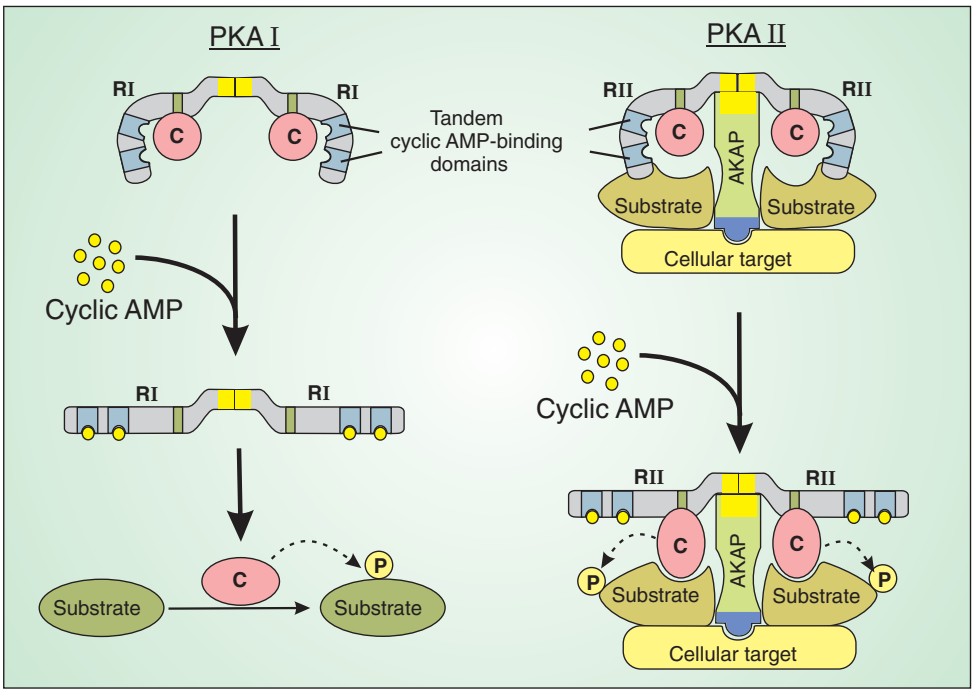
Salt-inducible kinase 2 (SIK2)

The salt-inducible kinase 2 (SIK2) functions to phosphorylate the transducer of regulated CREB (TORC2), thereby preventing it from entering the nucleus to facilitate the activity of the transcriptional factor cyclic AMP response element-binding protein (CREB) (Module 4: Figure CREB activation). It functions to regulate TORC2 in liver cells (Module 7: Figure liver cell signalling) and in insulin-secreting β-cells (Module 7: Figure β-cell signalling).

Exchange proteins activated by cyclic AMP (EPACs)

Other targets for the second messenger cyclic AMP are the exchange proteins activated by cAMP (EPACs). One of the

Module 2: | Figure protein kinase A (PKA)



The functional organization of protein kinase A (PKA).
There are two types of PKA, type I PKA (PKA I) and type II PKA (PKA II), which differ primarily in the type of R subunits that associate with the C subunits. There are four R subunit isoforms (RIα, RIβ, RIIα and RIIβ), which have somewhat different properties with regard to their affinity for cyclic AMP and their ability to associate with the A-kinase-anchoring proteins (AKAPs). It is these different R subunits that define the properties of the two types of PKA.

functions of EPACs is to activate Rap1 and Rap2B, which have many functions, many of which are related to controlling actin dynamics. In addition, the EPAC/Rap pathway can activate phospholipase Cε (PLCε) and this mechanism has been implicated in the control of autophagy (Module 11: Figure autophagy signalling mechanisms).

Cyclic AMP signalling functions

The cyclic AMP signalling pathway functions in the control of a wide range of cellular processes:

- Cyclic AMP suppresses spontaneous Ca^{2+} oscillations during oocyte maturation.
- Cyclic AMP has a potent anti-inflammatory action by inhibiting the activity of macrophages (Module 11: Figure macrophage signalling) and mast cells (Module 11: Figure mast cell inhibitory signalling).
- Melanocortin 4 receptors (MC4Rs) on second-order neurons use the cyclic AMP signalling pathway to induce the hypothalamic transcription factor Single-minded 1 (Sim1) to decrease food intake and weight loss (Module 7: Figure control of food intake).
- Cyclic AMP mediates the action of lipolytic hormones in white fat cells by stimulating a hormone-sensitive lipase (Module 7: Figure lipolysis and lipogenesis).
- Heat production by brown fat cells is controlled by noradrenaline acting through cyclic AMP (Module 7: Figure brown fat cell).
- The phosphorylation of dopamine- and cyclic AMP-regulated phosphoprotein of apparent molecular mass 32 kDa (DARPP-32) co-ordinates the activity of the dopamine and glutamate signalling pathways in medium spiny neurons (Module 10: Figure medium spiny neuron signalling).
- Adrenaline-induced glycogenolysis in skeletal muscle cells depends upon a cyclic AMP-dependent phosphorylation of phosphorylase kinase (Module 7: Figure skeletal muscle E-C coupling).
- Activation of the cyclic AMP-dependent transcription factor cyclic AMP response element-binding protein (CREB) contributes to the regulation of glucagon biosynthesis in glucagon-secreting α-cells (Module 7: Figure α-cell signalling).

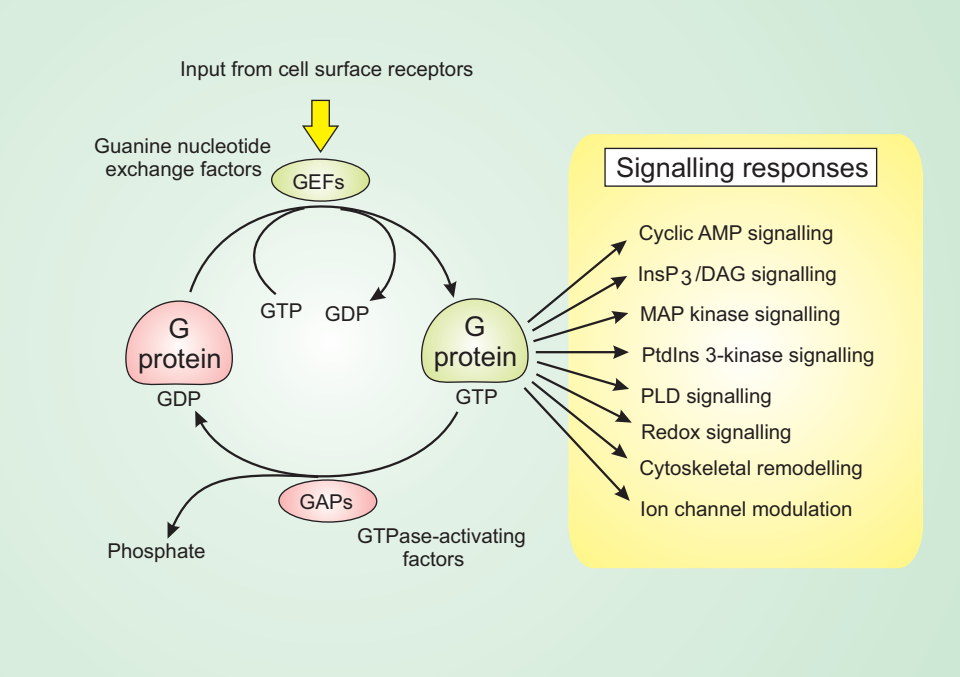
Cyclic AMP hydrolysis

There are two OFF reactions of the cyclic AMP signalling pathway, cyclic AMP efflux from the cell and cyclic AMP hydrolysis. The latter is carried out by a family of phosphodiesterase enzymes that hydrolyse cyclic AMP to AMP (Module 2: Figure cyclic AMP signalling).

Cyclic AMP efflux

There are two OFF reactions for the cyclic AMP signalling pathway, cyclic AMP hydrolysis and cyclic AMP efflux from the cell (Module 2: Figure cyclic AMP signalling). The latter is carried out by ABCC4, which is one of the ATP-binding cassette (ABC) transporters (Module 3: Table ABC transporters).

Module 2: | Figure G protein binary switching



Signal transduction through G protein binary switching.
All GTP-binding proteins (G proteins) function through a binary switching mechanism that is driven by the binding of GTP. The G protein is inactive when bound to GDP. When this GDP is exchanged for GTP, the G protein is activated and can stimulate a number of different signalling responses. The rapid switching to the active state is facilitated by the guanine nucleotide exchange factors (GEFs) that receive the information coming in from the receptors on the cell surface. By contrast, the GTPase-activating proteins (GAPs) accelerate the OFF reaction by enhancing the hydrolysis of GTP to GDP.

GTP-binding proteins

There are a large number of GTP-binding proteins, which usually are referred to as G proteins, which play a central role in cell signalling as molecular switches (Module 2: Figure G protein binary switching). These G proteins are also GTPases, and the switching is driven by both the binding of GTP and its hydrolysis to GDP. The G protein is inactive when bound to GDP, but when the GDP is exchanged for GTP, the G protein/GTP complex is active and transfers information down the signalling pathway until the endogenous GTPase activity hydrolyses GTP to GDP. The G proteins belong to two groups, the heterotrimeric G proteins and the monomeric G proteins, which have separate, but overlapping, functions.

Heterotrimeric G proteins

The heterotrimeric G proteins function as transducers for the G-protein-coupled receptors (GPCRs) that activate a number of cell signalling pathways (Module 1: Table G-protein coupled receptors). These G proteins are made up from 16 Gα, five Gβ and 11 Gγ genes (Module 2: Table heterotrimeric G proteins). These different subunits are characterized by having various lipid modifications that serve to insert them into the plasma membrane, where they are positioned to detect information coming in from the GPCRs and to relay it to various amplifiers. The Gα subunits are either palmitoylated or myristoylated near the N-terminus, whereas the Gβγ subunits are prenylated. The heterotrimeric G proteins are extremely versatile signalling elements, and both the Gα subunit and the Gβγ subunit are able to relay information to downstream components (Module 2: Figure heterotrimeric G protein signalling).

These heterotrimeric G proteins exist in two states. When the Gα subunit is bound to GDP, it forms a complex with Gβγ subunits to form the inactive heterotrimeric complex. When the GPCRs, which are sensitive to a wide range of stimuli (Module 1: Figure stimuli for cyclic AMP signalling), are activated, they function as a guanine nucleotide exchange factor (GEF) to replace the GDP with GTP. The binding of GTP not only activates the Gα subunit, but also liberates the Gβγ subunit, both of which can activate a range of signalling systems. This signal transduction process is switched off when the endogenous GTPase activity of the Gα subunit hydrolyses GTP to GDP and the Gα/GDP complex recombines with the Gβγ subunit to reform the inactive heterotrimeric complex. The normal rate of GTPase activity is very low (four to eight conversions per s), which means that the two subunits have a long time to find their targets. However, there are two mechanisms for speeding up the GTPase activity. Firstly, some of the targets can act to accelerate the GTPase activity. Secondly, a family of regulators of G protein signalling (RGS) proteins function as GTPase-activating proteins (GAPs) that accelerate the Gα subunit GTPase activity more than 1000-fold.

There are G-protein receptor kinases (GRKs) such as β-adrenergic receptor kinase 1 (βARK1), which phosphorylate active receptors to provide binding sites for

Module 2: | Table heterotrimeric G proteins
The heterotrimeric proteins are assembled from subunits taken from the G protein α (Gα), G protein β (Gβ) and G protein γ (Gγ) families.

Heterotrimeric G protein	Function
G protein α (Gα) subunits	
$G\alpha_s$	Stimulate adenylyl cyclase
$G\alpha_{olf}$	Stimulate adenylyl cyclase
$G\alpha_{i1}$	Inhibit adenylyl cyclase
$G\alpha_{i2}$	Inhibit adenylyl cyclase
$G\alpha_{i3}$	Inhibit adenylyl cyclase
$G\alpha_{o1}$	Inhibit adenylyl cyclase
$G\alpha_{o2}$	Inhibit adenylyl cyclase
$G\alpha_{t1}$	Stimulate cyclic GMP phosphodiesterase in rod photoreceptors
$G\alpha_{t2}$	Stimulate cyclic GMP phosphodiesterase in rod photoreceptors
Gz	Close K^+ channels. Inhibits exocytosis (see Module 10: Figure lactotroph regulation)
$G\alpha_{gust}$	Stimulate phospholipase Cβ (PLCβ) (see Module 10: Figure taste receptor cells and Module 7: Figure L cells)
$G\alpha_q$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_{11}$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_{14}$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_{15}$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_{16}$	Stimulate phospholipase Cβ (PLCβ)
$G\alpha_{12}$	Stimulate RhoGEFs to activate Rho (Module 2: Figure Rho signalling)
$G\alpha_{13}$	Stimulate RhoGEFs to activate Rho (Module 2: Figure Rho signalling)
G protein β (Gβ) subunits; β1-β5	These β subunits combine with γ subunits to form βγ dimers that have a number of control functions (for details see Module 2: Figure heterotrimeric G protein signalling)
G protein γ (Gγ) subunits; γ1-γ11	These γ subunits combine with β subunits to form βγ dimers that have a number of control functions (for details see Module 2: Figure heterotrimeric G protein signalling)

arrestin that prevent the heterotrimeric proteins from binding the receptor and this leads to receptor desensitization (Module 1: Figure homologous desensitization).

The active Gα/GTP and Gβγ subunits are able to relay information to a large number of signalling pathways, which are described in more detail for the different signalling pathways:

- The cyclic AMP signalling pathway (Module 2: Figure cyclic AMP signalling).
- The activation of phospholipase Cβ (PLCβ) in the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette (Module 2: Figure PLC structure and function).
- Modulation of the Ca_V2 family of N-type and P/Q-type channels (Module 3: Figure Ca_V2 channel family).
- Activation of the PtdIns 3-kinase signalling pathway (Module 2: Figure PtdIns 3-kinase signalling).
- Activation of phosphodiesterase 6 (PDE6) during phototransduction in photoreceptors (Module 10: Figure phototransduction).
- $G\alpha_{olf}$ functions in sperm motility and chemotaxis.
- $G\alpha_{gust}$, which is also known as gustudin, functions in taste cells and in the L cells that detect food components in the lumen of the intestine L cells (Module 7: Figure L cells).

Regulators of G protein signalling (RGS)

The regulators of G protein signalling (RGS) are a large family of approximately 30 proteins that function as GTPase-activating proteins (GAPs) for the heterotrimeric G proteins (Module 2: Figure heterotrimeric G protein signalling). The Gα subunit has a low intrinsic GTPase activity and this is greatly increased by the RGS proteins. RGS structure is defined by an RGS-box region that is responsible for binding to Gα/GTP. However, many of the RGS proteins contain a number of other protein–protein interaction domains, such as PDZ, phosphotyrosine-binding (PTB), pleckstrin homology (PH) and phox homology (PX) domains, indicating that, in addition to their GAP activity, they may have other functions. One of these might be the regulation of the G-protein-activated inwardly rectifying K^+ (GIRK) channel.

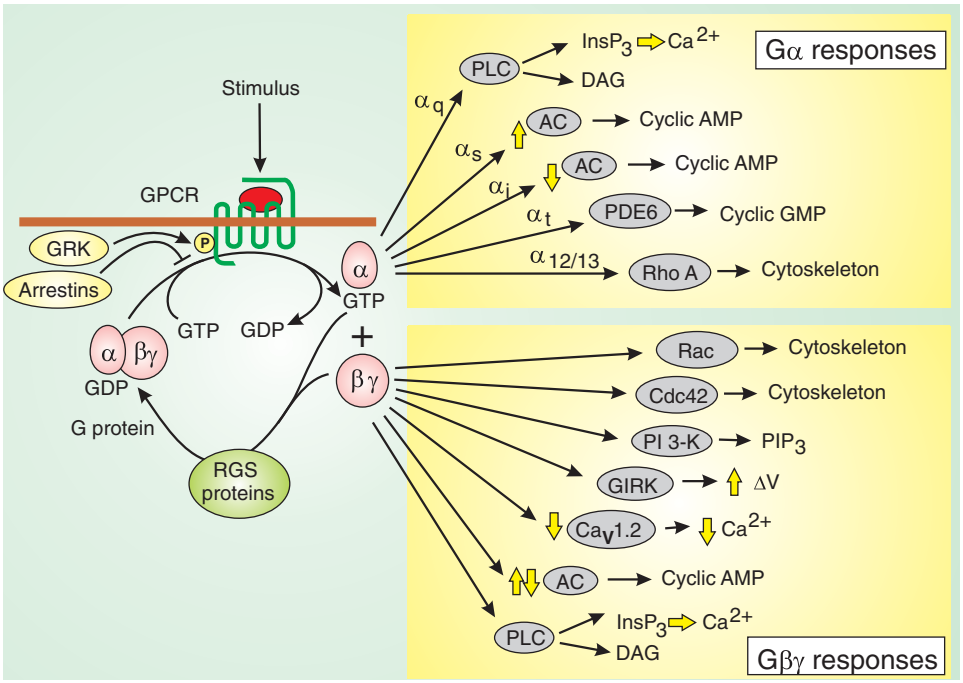
The function of RGS proteins has been clearly defined in phototransduction, where RGS9 acts to accelerate GTP hydrolysis by $G\alpha_t$ (step 6 in Module 10: Figure phototransduction).

Monomeric G proteins

The monomeric GTP-binding proteins (G proteins) belong to a large family of approximately 150 members. Ras was the founding member, and the family is often referred to as the Ras family of small G proteins. Within this family, it is possible to recognize five subfamilies: Ras, Rho, Rab, Ran and ADP-ribosylation factor (Arf) (Module 2: Table monomeric G protein toolkit). The Ran family plays a role in nuclear transport, whereas the large Rab family functions in membrane trafficking. The Ras and Rho family are primarily involved in cell signalling, where they function as binary switches to control a number of cell signalling systems. This binary switch is driven by the binding of GTP, which represents the ON reaction, and is followed by the hydrolysis of the GTP by the endogenous GTPase activity. Most attention has focused on a small number of these signal transducers, and the following will be described in detail to illustrate their role in cell signalling:

- Arf signalling mechanisms
- Cdc42 signalling mechanisms
- Rab signalling mechanisms
- Rac signalling mechanisms
- Ras signalling mechanisms
- Rap signalling mechanisms
- Rho signalling mechanisms

Module 2: | Figure heterotrimeric G protein signalling



Heterotrimeric G proteins function as transducers to activate many signalling pathways.
External stimuli that bind to G protein-coupled receptors (GPCRs) act as guanine nucleotide exchange factors (GEFs) for the heterotrimeric G proteins. When the GDP on the Gα subunit is replaced with GTP, the complex dissociates into Gα/GTP and Gβγ subunits that are then capable of activating or inhibiting a wide range of signalling systems. Most of the actions are stimulatory, but some are inhibitory, as illustrated by the yellow arrows. The regulators of G protein signalling (RGS) proteins function as GTPase-activating proteins (GAPs) to facilitate the GTPase activity of the Gα subunit, which is the OFF reaction that terminates signalling. G protein receptor kinase (GRK) phosphorylates active receptors and provides binding sites for arrestin that result in receptor desensitization by preventing the heterotrimeric G proteins from binding the receptor.

Ras signalling mechanisms

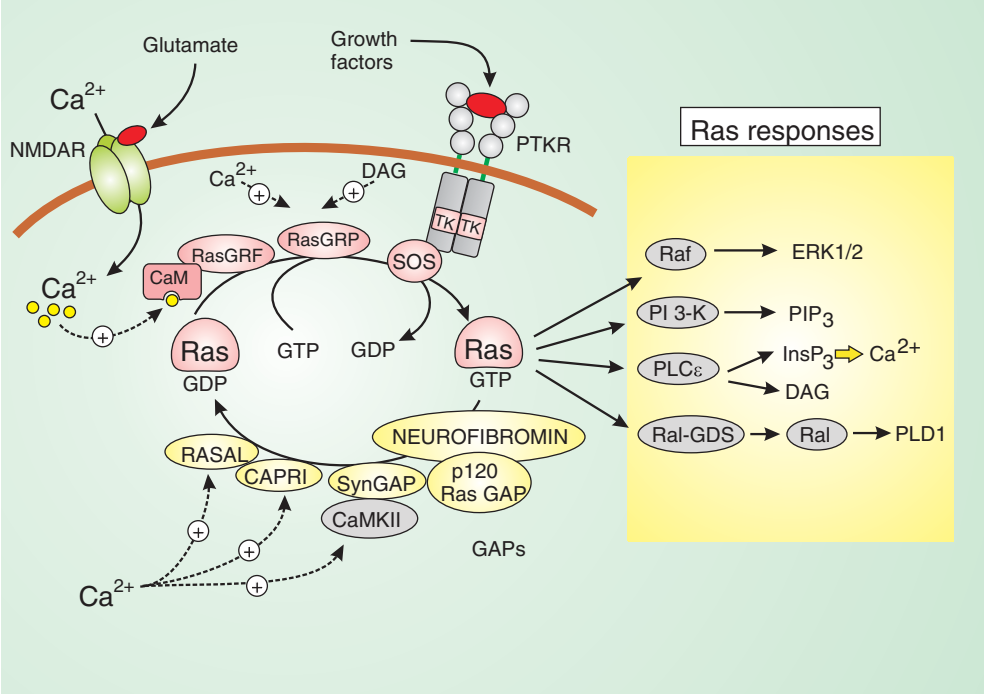
Ras is a small (21 kDa) GTPase that plays a central role as a signal transducer for a number of signalling systems. It was first identified as a transducer for the tyrosine kinase-linked receptors (Module 1: Figure stimuli for enzyme-linked receptors), where it functions to relay information to the mitogen-activated protein kinase (MAPK) signalling pathway. It is now known that activated Ras is able to relay information to a number of other signalling pathways. Like other G proteins (Module 2: Figure G protein binary switching), Ras functions as a binary switch that is activated by binding GTP. There are thus two critical aspects to Ras action: how is the switch controlled and how is information relayed out to different signalling pathways (Module 2: Figure Ras signalling)? With regard to the first question, the ON reaction of the binary switch is controlled by a number of Ras guanine nucleotide exchange factors (RasGEFs) such as Son-of-sevenless (SoS), Ras guanine nucleotide release-inducing factors (RasGRFs) and Ras guanine nucleotide releasing proteins (RasGRPs) (Module 2: Table monomeric G protein toolkit). This conversion of Ras·GDP into Ras·GTP is inhibited by the tumour suppressor protein merlin. Neurofibromatosis type 2 is caused by mutations in merlin.

There are a number of Ras GTPase-activating proteins (RasGAPs) that accelerate the OFF reaction by speeding up the hydrolysis of GTP to GDP by Ras. Examples of such RasGAPs include p120 RasGAP, neurofibromin, SynGAP, Ca^{2+}-promoted Ras inactivator (CAPRI) and Ras GTPase-activating-like (RASAL). The function of some of these RasGAPs such as CAPRI and RASAL are attracting considerable attention because they are Ca^{2+}-sensitive proteins that rapidly translocate to the membrane through their C2 domains whenever there is an increase in the level of Ca^{2+}. SynGAP, which is expressed mainly in brain, is also activated by Ca^{2+} following its phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). The role of Ca^{2+} in modulating the activity of both the RasGEFs and RasGAPs indicates an important feedback interaction between the Ca^{2+} and Ras signalling pathways.

The Ras signalling mechanism relays information to a number of signalling targets:

- It activates Raf to initiate the MAPK signalling pathway (Module 2: Figure ERK signalling).
- It activates phospholipase Cε (PLCε) (Module 2: Figure PLC structure and function).
- It activates the PtdIns 3-kinase signalling pathway.
- Ras can activate a family of RalGEFs such as Ral-GDP dissociation stimulator that then activates the Ral proteins (RalA and RalB) (Module 2: Figure Ras signalling). One of the functions of RalA is to stimulate the phospholipase D signalling pathway (Module 2: Figure PLD signalling).

Module 2: | Figure Ras signalling



Function of the monomeric Ras G protein in cell signal transduction.

Ras plays a role as a signal transducer to relay information from various external stimuli to a range of different Ras-dependent responses. When bound to GDP, Ras is inactive. Cell stimuli act through different guanine nucleotide exchange factors (GEFs) such as Son-of-sevenless (SoS), RasGRF and RasGRP to facilitate an exchange of GDP for GTP to create the activated Ras/GTP complex that can relay information through a number of signalling pathways. The action of Ras is terminated by a variety of GTPase-activating proteins (GAPs) that accelerate the ability of Ras to hydrolyse GTP back to GDP.

Son-of-sevenless (SoS)

Son-of-sevenless (SoS) is a classical Ras guanine nucleotide exchange factor (RasGEF). Its mode of action is evident in the way the platelet-derived growth factor receptor (PDGFR) is linked to the mitogen-activated protein kinase (MAPK) signalling pathway (Module 1: Figure PDGFR activation). SoS binds to the Src homology 3 (SH3)-containing adaptor growth factor receptor-bound protein 2 (Grb2), which is attached to the phosphotyrosine residues of the activated receptor. Once it is associated with the receptor, SoS comes into contact with Ras and can begin to facilitate the exchange of GDP for GTP, thus creating the active Ras/GTP complex that begins to stimulate the MAPK signalling pathway (for further details see Module 2: Figure ERK signalling).

Ras guanine nucleotide release-inducing factors (RasGRFs)

The Ras guanine nucleotide release-inducing factors (RasGRFs) and Ras guanine nucleotide releasing proteins (RasGRPs) are also important GEFs that can relay information to Ras. The RasGRFs, which are strongly expressed in brain, are particularly important for the activation of the MAPK signalling pathway in neurons, where they respond to activation of *N*-methyl-D-aspartate (NMDA) receptors. One of the functions of the latter is to gate Ca^{2+}, which then acts through calmodulin (CaM) to stimulate RasGRF. This is a highly localized signalling event, because the RasGRF is associated with the NMDA receptor and thus responds to the microdomain of Ca^{2+} near the mouth of the channel. This signalling mechanism functions in neuronal gene transcription (Module 10: Figure neuronal gene transcription).

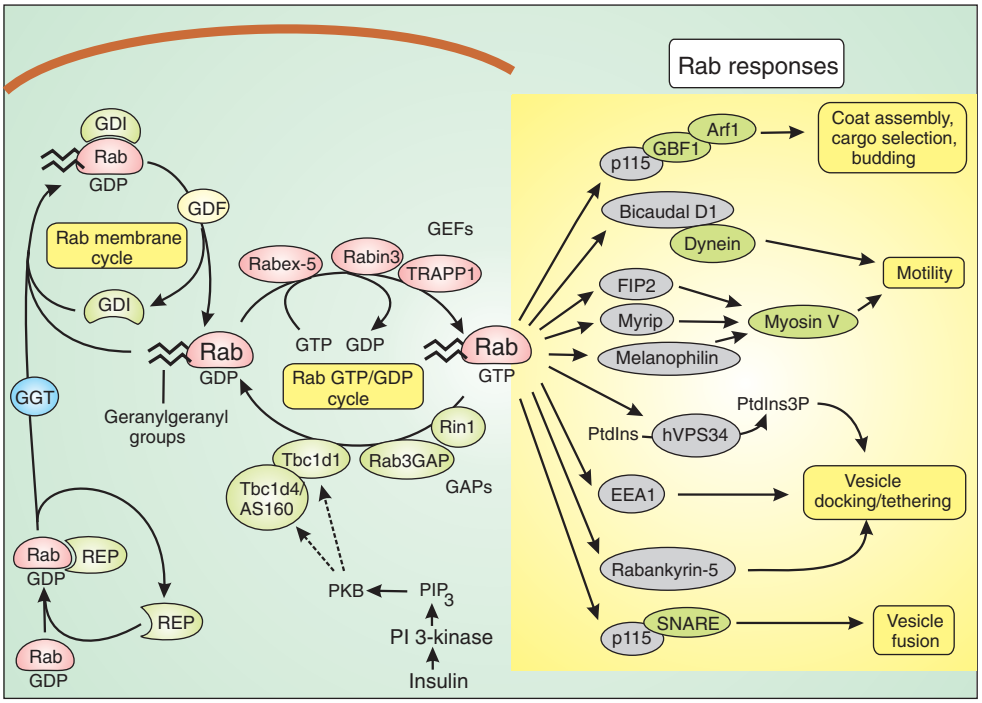
Ras guanine nucleotide releasing proteins (RasGRPs)

The Ras guanine nucleotide releasing proteins (RasGRPs) are mainly expressed in haematopoietic cells (Module 2: Table monomeric G protein toolkit). These GRPs are also known as CalDAG-GEFs because they are sensitive to diacylglycerol (DAG) and Ca^{2+}. They contain a C1 domain that binds DAG and they also have a pair of Ca^{2+}-binding EF-hands. The sensitivity to both DAG and Ca^{2+} suggests that these RasGRPs may couple phosphoinositide signalling to the activation of the various pathways that are linked to Ras (Module 2: Figure Ras signalling).

Rap signalling mechanisms

The Ras-related protein (Rap) family are typical small monomeric G-proteins that belong to the Ras superfamily (Module 2: Table monomeric G-protein toolkit). The Rap family has four members Rap1A, Rap1B, Rap2A and Rap2B. One of the guanine nucleotide-exchange factors (GEFs) is the cyclic AMP-dependent GEF EPAC that regulates the activation of Rap1 and Rap2B. This pathway can activate phospholipase Cε (PLCε) (Module 2: Figure cyclic AMP signalling). Such a mechanism has been implicated in the control of autophagy (Module 11: Figure autophagy).

Module 2: | Figure Rab signalling



Function of the Rab monomeric G-protein in cell signalling.
The monomeric Rab G-protein is regulated at a number of levels. Newly synthesized Rab bound to GDP is recognized by the Rab escort protein (REP), which presents it to the geranylgeranyl transferase (GGT). GGT adds geranylgeranyl groups to one or two of the C-terminal cysteine residues. The geranylgeranylated Rab is recognized by the Rab GDP dissociation inhibitor (GDI), which operates the Rab membrane cycle. The Rab.GDP is then targeted to specific membrane sites by interacting with the GDI displacement factor (GDF). Various guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs) operate the Rab GTP/GDP cycle responsible for the inter-conversions between inactive Rab.GDP and active Rab.GTP. The latter then acts on a variety of effectors to regulate many of the processes responsible for protein trafficking.

Rab signalling mechanism

The Ras-associated binding (Rab) proteins are typical small monomeric G-proteins that belong to the Ras superfamily. The primary function of the Rabs is to control multiple phases of membrane and protein trafficking such as vesicle formation, motility, tethering and fusion to the acceptor membrane and signalling to other organelles. There are more than 60 human Rabs (Module 2: Table monomeric G-protein toolkit). The enormous proliferation of this family reflects their central role in the regulation of so many vesicle trafficking processes.

The continuous cycling between the cytosol and different membrane-bound compartments is shepherded by a number of protein factors and also requires the prenylation of Rab. This post-translational lipid modification depends on the addition of isoprenes, which are intermediaries in cholesterol biosynthesis. Following its synthesis, Rab is bound to GDP and is soluble in the cytoplasm (Module 2: Figure Rab signalling). This Rab.GDP is then recognized by the chaperone protein Rab escort protein (REP), which transfers it to the Rab geranylgeranyl transferase (GGT) that adds either one or two geranylgeranyl groups to cysteine residue(s) located at the C-terminus of the Rab. Mutation of REP causes choroideraemia, a form of blindness resulting from degeneration of the retinal pigment epithelium.

The prenylated Rab enters the Rab membrane cycle by binding to the GDP dissociation inhibitor (GDI). The Rab.GDP/GDI complex is then targeted to specific membrane sites by interacting with the GDI displacement factor (GDF). Once Rab has performed its function it is extracted from the membrane by GDI and remains in the cytosol until it is required again to carry out the same or some other function. Some patients with X-linked non-specific mental retardation have mutations in one of the genes for GDI.

Rabs carry out their multiple tasks through the operation of the Rab GTP/GDP cycle regulated by the coordinated activity of guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Module 2: Figure Rab signalling). Examples of the RAB GEFs include Rabex-5, Rabin3 and TRAPP1. Examples of some of the Rab GAPs are Rab3GAP, Rin1, Tbc1d1 and Tbc1d4 (also known as AS160). Tbc1d1 and Tbc1d4/AS160 are the targets of insulin action resulting in insertion of GLUT4-containing vesicles in skeletal muscle (Module 7: Figure skeletal muscle E-C coupling). The Rab.GTP form has the dynamic conformation and mobility to interact with many different downstream effectors that drive the multiple Rab responses (Module 2: Figure Rab signalling).

An allele of Tbc1d4, which reduces the translocation of GLUT4 to the plasma membrane of skeletal muscle (Module 7: Figure skeletal muscle E-C coupling), is linked to a susceptibility to obesity.

Rab1

Rab1 functions in vesicle tethering and fusion especially during both ER-to-Golgi and Golgi-to-ER transport (see steps 1 and 2 in Module 4: Figure membrane and protein trafficking). During the COPII-mediated transport from the ER to the Golgi (Module 4: Figure COPII-coated vesicles), TRAPP1 is the guanine nucleotide-exchange factor (GEF) that converts inactive Rab1.GDP into active Rab1.GTP during vesicle tethering prior to fusion to the Golgi membrane. The transport from the Golgi back to the ER begins with the golgin protein p115 recruiting Rab1b, which then activates the Golgi-specific brefeldin A resistant factor 1 (GBF1), which is a guanine nucleotide-exchange factor (GEF) for the small monomeric G-protein ADP-ribosylation factor 1 (Arf1) that has an important role in controlling key events such as coat formation, actin polymerization and Golgi vesicle budding during COPI-mediated transport from the Golgi to the ER (Module 4: Figure COPI-coated vesicles).

Rab3

There are three Rab3 proteins (Rab3A, Rab3B, Rab3C and Rab3D) that function in regulated exocytosis. These Rab3 members tend to be restricted to cells types with high rates of exocytosis such as neurons and neuroendocrine cells (Rab3A and Rab3C). Rab3B is located on tight junctions and secretory granules in epithelial cells. The more widely expressed Rab3D is located on the secretory granules of non-neuronal cells such as fat cells, adipocytes, exocrine glands and certain haematopoietic cells. Rab3A and Rab3C tend to reduce exocytosis, whereas Rab3B and Rab3D have a more positive role.

The activation of these Rabs is regulated by a Rab3GAP which has two components: a catalytic RabGAP1 subunit and a non-catalytic RabGAP2 subunit. Mutations in RabGAP1 have been linked to Warburg Micro syndrome whereas a milder form called Martsolf syndrome is cause by mutations in RabGAP2.

Rab4

Rab 4, together with myosin V, functions in early endosome to plasma membrane trafficking (Module 4: Figure early endosome budding). Rab 4, together with myosin V, also functions in early endosome to *trans*-Golgi network (TGN) trafficking (Module 4: Figure early endosome budding).

Rab5

Rab5 plays a role in endosome vesicle fusion to early endosomes by targeting vesicles to the early endosome (Module 4: Figure endosome vesicle fusion). Such a role is manifest during phagosome maturation (Module 4: Figure phagosome maturation). Rab5 also has a role in activating hVPS34 to promote vesicle nucleation during autophagy (Module 11: Figure autophagy signalling mechanisms).

Rabex-5 is the guanine nucleotide-exchange factor (GEFs) that activates Rab5, which then interacts with the effector Rabaptin-5. Rab22 may play a role in recruiting Rabex-5 to the early endosome.

Rab6

During the process of COPII-mediated transport from ER to Golgi (Module 4: Figure COPII-coated vesicles), Rab6 binds to Bicaudal D and thus provides a mechanism to attach the dynein motor complex to the vesicle for its transfer between the ER and Golgi membranes (Module 4: Figure dynein)

Rab 7

The two Rab7 proteins (Rab7A and Rab7B), which are expressed ubiquitously, play a role in regulating vesicle trafficking between the endocytic and autophagic compartments. Rab7 is found on the acidic and degradative organelles, such as the late endosomes, lysosomes, multivesicular bodies, phagosomes and autophagolysosomes. These mechanisms regulate the degradation of endocytic cargos such as EGF receptors and neurotrophic factor receptors such as TrkA and internalized cholesterol. The role of RAB7 in lysosomal transport depends on the effector protein Rab7-interacting lysosomal protein (RILP). Rab7 may also play a role in the retrograde trafficking of the signalling endosomes that supply trophic support to peripheral neurons.

Charcot-Marie-Tooth disease type 2B is caused by mutations in Rab7A.

Rab8

Rab8A interacts with myosin Vb to carry out non-clathrin-dependent endocytosis. It also plays a role in the biogenesis of cilia by interacting with cenexin/ODF2.

A defect in the transcriptional regulation of Rab8A has been linked to Microvillus inclusion disease.

Rab11

There are three Rab11 proteins (Rab11A, Rab11B and Rab11C), which function in both endocytic recycling and cytokinesis. Rab11 plays an important role in the transport of synaptic vesicles where it is attached to myosin Vb through the Rab11-family interacting proteins (FIPs) (Module 4: Figure myosin motor). Such trafficking events leading to receptor insertion are key components in the relationship between Ca^{2+} and synaptic plasticity during the process of learning (Module 10: Figure Ca^{2+}-induced synaptic plasticity).

Rab11, together with its effector FIP2, also plays a role in the trafficking and insertion of aquaporin-2 (AQP2) (Module 7: collecting duct function) and also plays a role in controlling the trafficking and insertion of new vesicles to form the cleavage furrow during cytokinesis (Module 9: Figure cytokinesis).

Rab27

There are two Rab27 proteins (Rab27A and Rab27B), which function in intracellular transport of secretory granules and melanosomes (Module 4: Figure myosin motor). Rab27 exerts its actions through various effectors that fall into three families that have distinct functions. There are synaptotagmin-like proteins (Slps), Slp lacking C2 domains (Slac2s) and the Rab3-binding domain (RBD) family. They all have an N-terminal Rab27-binding domain known as the Slp-homology domain (SHD), which

binds to the GTP-bound form of Rab27. The Slp and RBD families also have two C-terminal C2 domains, which enables these effectors to bind to phospholipids at the plasma membrane and thus enables Rab27a to regulate the secretory machinery at the vesicle docking step. The Slac2-a/Melanophilin and Slac2-c/MyRIP complexes link Rab27a to molecular motors such as MyoVa (Module 4: Figure myosin motor). The active Rab27.GTP carries out a number of functions:

- One of the functions of RAB27A is to move melanosomes along actin filaments in retinal pigment epithelium cells and in melanocytes. A defect in the transport of melanocytes results in choroideraemia. In the case of melanogensis, Rab27A interacting with melanophilin that then uses myosin Va to move melanosomes to the cell periphery of the melanocytes (Module 7: Figure melanogenesis).
- Another function of Rab27A is to regulate the secretion of perforin- and granzyme-containing cytotoxic granules at the immunological synapse that forms when cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells interacts with their target cells, such as virus-infected cells and tumour cells. Interaction of the T cell receptor (TCR) with an antigen-presenting target cell induces the rapid polarization of the microtubule-organizing center (MTOC), which orchestrates secretion of the cytotoxic granules at the immunological synapse. The Rab27 effector hMunc13-4 contributes to the maturation and exocytosis of the perforin-containing granules. Several inherited conditions, which lead to functional impairment of this cytotoxic pathway result from defects in either Rab27A or hMunc13-4. Mutations in Rab27A have been linked to Griscelli syndrome type 2 (GS2), whereas mutations in hMunc13-4 cause familial hemophagocytic lymphohistiocytosis type 3 (FHL3).
- Insulin granules are tethered to the plasma membrane using the effector Slp4-a/granuphilin-a to interact with both Rab27A and the SNARE protein syntaxin-1a.
- In glucagon-secreting α-cells in the pancreas, glucagon granules are targeted to the plasma membrane by Slp2a.

Rab11-family interacting proteins (FIPs)

The Rab11-family interacting proteins (FIPs) serve as effectors for a number of Rabs and ADP-ribosylation factors (Arfs). There are five members, which all have a Rab11-binding domain (RBD) at the C-terminus and are divided into two classes:

Class I (have a phospholipid-binding C2 domain)

- Rip11, which is also known as pp75 or FIP5, functions in the transport of GLUT-4 vesicles to the cell surface when adipocytes are stimulated by insulin.
- FIP2, which is also known as optineurin (OPTN), functions to couple Rab 11 to the AMPA-containing synaptic vesicles (Module 4: Figure myosin motor). Such trafficking events leading to receptor insertion are key components in the relationship between Ca^{2+} and synaptic plasticity during the process of learning (Module 10: Figure Ca^{2+}-induced synaptic plasticity).
- Rab-coupling protein (RCP) is also known as FIP1C

ClassII (have typical Ca^{2+}-binding EF-hands)

- FIP3, which is also known as Arfophilin and Eferin, contributes to the role of Rab11 in trafficking and insertion of membrane vesicles during formation of the cleavage furrow responsible for cytokinesis (Module 9: Figure cytokinesis).
- FIP4, which is also known as Arfophilin-2, functions like FIP3 to regulate cytokinesis.

Mutations in FIP2/optineurin have been linked to some forms of glaucoma and amyotrophic lateral sclerosis (ALS).

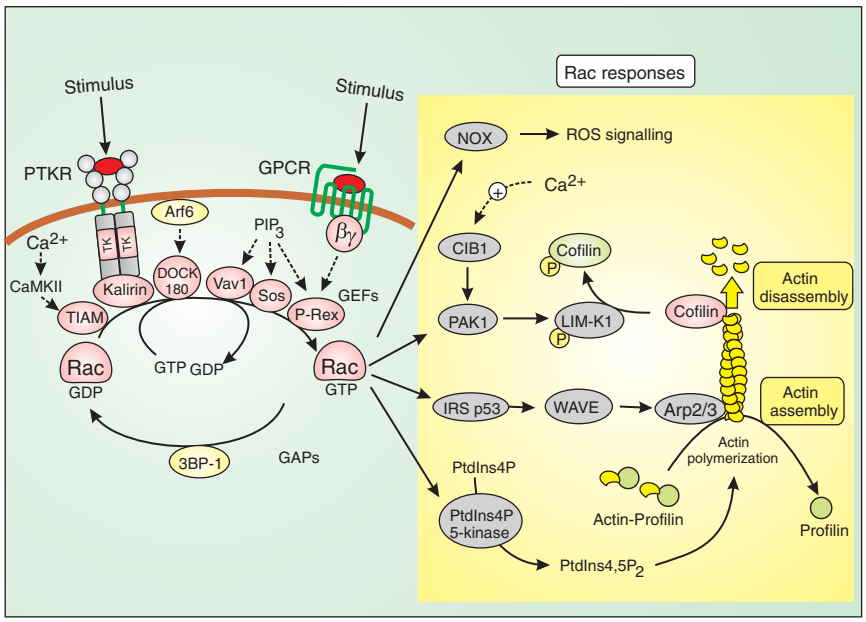
Rac signalling mechanisms

The Rac signalling pathway has an important role in activating a number of signalling pathways (Module 2: Figure Rac signalling). Like other G proteins, Rac functions as a binary switch. It is inactive when bound to GDP, but when this GDP is exchanged for GTP, the Rac/GTP complex becomes active. External stimuli can activate this switch using different guanine nucleotide exchange factors (GEFs) such as T cell lymphoma invasion and metastasis (Tiam), Kalirin, Vav, SoS and P-Rex. Arf6, which participates in the Arf signalling pathway, stimulates the Rac GEF called downstream of Crk-180 homologue (DOCK180). Kalirin functions in the Ephrin (Eph) receptor signalling pathway, where it links the EphB receptor to Rac and actin assembly (Module 1: Figure Eph receptor signalling). The lipid second messenger PtdIns3,4,5P_3, which is produced by the PtdIns 3-kinase signalling pathway, is particularly effective in stimulating many of these GEFs.

The activated Rac/GTP complex has a number of important actions:

A. Rac is responsible for mediating the action of the PtdIns 3-kinase signalling pathway in stimulating the NADPH oxidase to initiate the reactive oxygen species (ROS) signalling pathway (Module 2: Figure plasma membrane ROS formation).
B. It stimulates the JNK (c-Jun N-terminal kinase) signalling pathway (Module 2: Figure JNK signalling).
C. It promotes actin stability by stimulating p21-activated kinase (PAK) to phosphorylate LIM kinase, which phosphorylates cofilin to inhibit its ability to cut actin.
D. It contributes to focal adhesion integrin signalling (Module 6: Figure integrin signalling).
E. It promotes actin remodelling in several ways. It stimulates PtdIns4P 5-kinase to increase the formation of PtdIns4,5P_2 to control actin remodelling (Module 4: Figure actin remodelling). It also acts through insulin receptor substrate p53 (IRSp53), which activates Wiskott-Aldrich syndrome protein (WASP) to co-ordinate the activity of the actin-related protein 2/3 complex (Arp2/3 complex) that initiates actin polymerization (Module 4: Figure actin remodelling). Such a role is evident in neurons

Module 2: | Figure Rac signalling



Function of the Rac monomeric G protein in signal transduction.
Rac is inactive when bound to GDP, but switches into an active form when this GDP is exchanged for GTP. This GTP for GDP exchange is facilitated by a number of guanine nucleotide exchange factors (GEFs) such as Tiam, Kalirin, Vav, SoS and P-Rex, which are sensitive to various messengers such as Ca^{2+}, PtdIns3,4,5P_3 (PIP_3) or Gβγ subunits of heterotrimeric G proteins. The activated Rac/GTP then relays information out to different signalling pathways as described in the text. Further details concerning the role of Wiskott-Aldrich syndrome protein (WASP) verprolin homologous (WAVE) are shown in Module 4: Figure actin remodelling.

during spine morphogenesis (Module 10: Figure post-synaptic density) and during the Ca^{2+} and synaptic plasticity changes that occur during long-term potentiation (LTP) (Module 10: Figure Ca^{2+}-induced synaptic plasticity).

Rho signalling mechanisms

The Rho (Ras homologue) signalling pathway has an important role in regulating a number of systems, in particular the operation of the cytoskeleton. It regulates the function of actin in two main ways. Firstly, it controls some of the processes that function in actin assembly and it also operates one of the control mechanisms that regulate the myosin II–actin interaction responsible for contraction both in smooth muscle cells and non-muscle cells (Module 2: Figure Rho signalling). Like other G proteins, Rho functions as a binary switch. It is inactive when bound to GDP, but when this GDP is exchanged for GTP, the Rho/GTP complex becomes active. External stimuli can activate this switch using different Rho guanine nucleotide exchange factors (RhoGEFs). There are a large number of such RhoGEFs that are characterized by having a Dbl homology (DH) domain. The term Dbl comes from diffuse B cell lymphoma, which is the cell line where the first RhoGEF was identified. The family of Dbl-containing GEFs has now expanded to 69 members, some of which are shown in Module 2: Table monomeric G protein toolkit. These GEFs are not necessarily specific for Rho: some are rather promiscuous and will also function as Rac and Cdc42 GEFs. In the case of Rho, ephexin appears to be specific for coupling ephrin receptors to Rho (Module 2: Figure Rho signalling). Also leukaemia-associated RhoGEF (LARG), p115-RhoGEF and PDZ-RhoGEF are specific for coupling G-protein-coupled receptors (GPCRs) to Rho activation. Net-1, which appears to be sensitive to PtdIns3,4,5P_3 (PIP_3), is normally found in the nucleus, but translocates to the cytoplasm, where it functions to activate RhoA. Another important GEF is Ect2, which is also found in the nucleus during interphase, but then associates with microtubules during mitosis, where it stimulates the activation of Rho cytokinesis (Module 9: Figure cytokinesis).

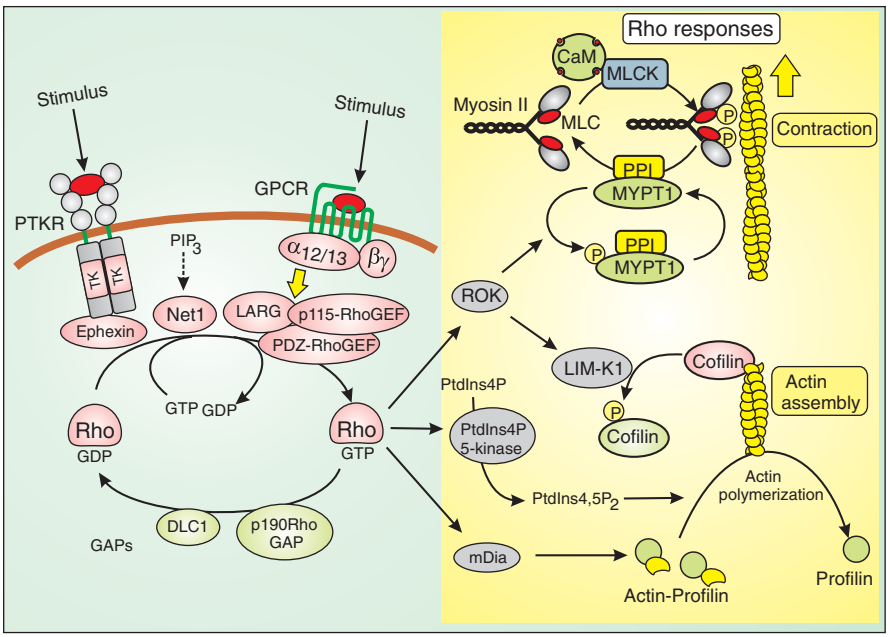
The activated Rho/GTP complex is able to stimulate a number of signalling processes, many of which are directed towards remodelling of actin and its contraction. Many of the actions of Rho are carried out through a Rho kinase (ROK). Diaphanous-related forming 1 (Dia1) is one of the downstream effectors that responds to Rho/GTP by interacting with profilin to increase actin polymerization.

Oligophrenin-1 (OPHN1) is a Rho Gap that functions to stabilize AMPA receptors at neuronal synapses and thus plays an important role in both synapse formation and synaptic plasticity. Mutations in the *OPHN1* gene have been linked to mental retardation.

The following are some examples of the function of Rho signalling:

- Activation of smooth muscle contraction (Module 7: Figure smooth muscle cell E-C coupling).
- Assembly of the actomyosin contractile ring and activation of the contraction during cytokinesis (Module 9: Figure cytokinesis).
- Endothelial cell contraction to open the permeability barrier (Module 7: Figure endothelial cell contraction).

Module 2: | Figure Rho signalling



Function of the Rho monomeric G protein in cell signal transduction.
Rho is a typical G protein that is activated when GDP is exchanged for GTP. This exchange is facilitated by a number of Rho guanine nucleotide exchange factors (RhoGEFs). For example, the ephexins mediate the action of protein tyrosine-linked receptors (PTKRs), such as the ephrin receptors, whereas the G protein-coupled receptors (GPCRs) use the $\alpha_{12/13}$ subunit of heterotrimeric G proteins to activate leukaemia-associated RhoGEF (LARG), p115-RhoGEF or PDZ-RhoGEF. The activated Rho/GTP complex then activates a number of signalling systems as outlined in the text.

- Assembly of the actin ring in osteoclast podosomes (Module 7: Figure osteoclast podosome).
- Eph receptor-induced growth cone collapse in developing neurons (Module 1: Figure Eph receptor signalling).
- Activation of uropod contraction during neutrophil chemotaxis (Module 11: Figure neutrophil chemotactic signalling).
- Physical interactions between endothelial cells and the extracellular matrix (ECM) activate p190RhoGAP that is responsible for the mechanosensitive control of VEGF receptor expression.

Cdc42 signalling mechanisms

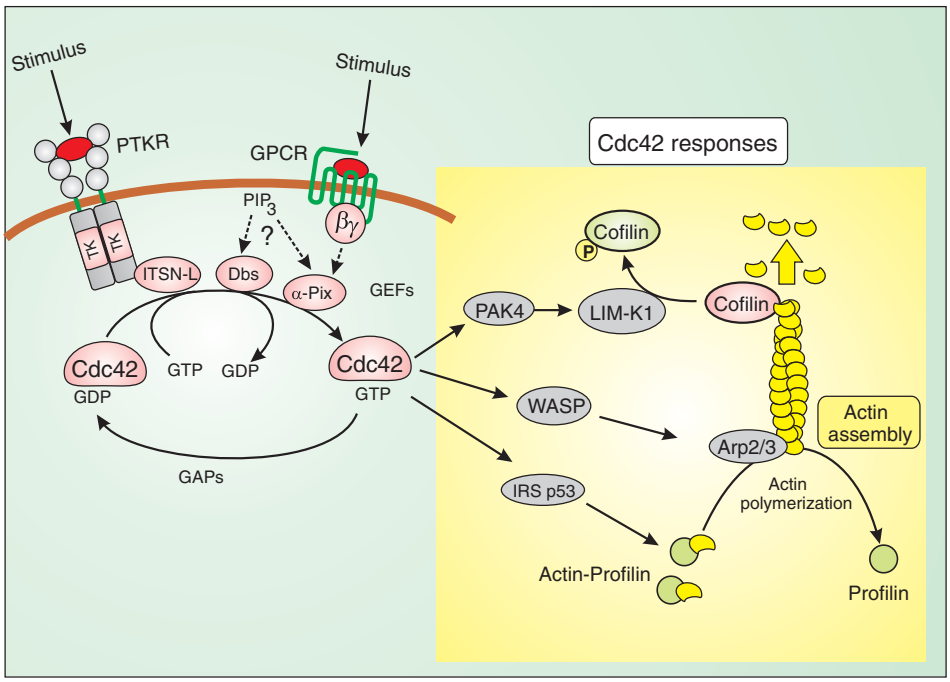
The Cdc42 signalling mechanism has an important role in controlling the actin cytoskeleton. It regulates some of the processes that function in actin assembly (Module 2: Figure Cdc42 signalling). Like other G proteins, Cdc42 functions as a binary switch. It is inactive when bound to GDP, but when this GDP is exchanged for GTP, the Cdc42/GTP complex becomes active. External stimuli activate this switch using different guanine nucleotide exchange factors (GEFs), such as intersectin-long (ITSN-L) and PAK-interacting exchange factor α (α-Pix). Just how these Cdc42 RhoGEFs are activated is still not properly understood. In the case of the Ephrin (Eph) receptor signalling pathway, intersectin is activated by binding to the EphA receptor (Module 1: Figure Eph receptor signalling). The activated Cdc42/GTP complex then stimulates various elements that regulate actin polymerization. It promotes actin stability by stimulating p21-activated kinase (PAK) to phosphorylate LIM kinase, which phosphorylates cofilin to inhibit its ability to cut actin. One of its main actions is to stimulate the Wiskott-Aldrich syndrome protein (WASP) that acts on the actin-related protein 2/3 complex (Arp2/3 complex) to initiate actin polymerization (Module 4: Figure actin remodelling). This action is facilitated by Cdc42 acting on profilin through insulin receptor substrate p53 (IRSp53) and Mena.

The Cdc42 signalling mechanism plays an important role in actin polymerization in synaptic spines as part of the events associated with Ca^{2+} and synaptic plasticity (Module 10: Figure Ca^{2+}-induced synaptic plasticity).

Arf signalling mechanisms

The ADP-ribosylation factors (Arfs) are best known for their role in membrane and protein trafficking. There are six mammalian Arfs that are divided into three classes (Module 2: Table monomeric G-protein toolkit). The Class I and Class II Arfs are found mainly at the Golgi and also on various endosomal membranes where they function in actin remodelling and formation of the coat protein complexes responsible for the vesicle budding that transfers cargo form one compartment to another. Such a role is evident in the case of COPI-mediated transport from Golgi to ER (Module 4: Figure COPI-coated vesicles). Class III has a single member Arf6, which functions at the plasma membrane where it can have marked effects on actin polymerization. One action of Arf6 is to induce the trafficking of Rac1 to the plasma membrane where it is activated by the Rac GEF called downstream of Crk-180 homologue (DOCK180) to increase membrane ruffling and focal adhesion actin attachment (Module 6: Figure integrin signalling). The DOCK180 acts as a complex in association with a protein called engulfment and

Module 2: | Figure Cdc42 signalling



Function of the Cdc42 monomeric G protein in signal transduction.
When bound to GDP, Cdc42 is inactive, but it is activated when the GDP is exchanged for GTP. This exchange is accelerated by guanine nucleotide exchange factors (GEFs), but how these are activated is still somewhat of a mystery. The primary action of the Cdc42/GTP complex is to stimulate actin assembly by inhibiting the action of cofilin, by promoting actin polymerization by acting on actin/profilin and Wiskott-Aldrich syndrome protein (WASP). Further details on the role of WASP are shown in Module 4: Figure actin remodelling.

cell motility (ELMO). Arf6 has also been implicated in the endocytosis of AMPA receptors during the process of Ca^{2+}-induced synaptic plasticity at synaptic endings (See step 4 in Module 10: Figure Ca^{2+}-induced synaptic plasticity). This function of Arf6 depends on the activity of BRAG2, which is an Arf GEF that binds to the Glu2 subunit of the AMPA receptor.

Like other monomeric GTP-binding proteins (G-proteins), Arf activity is regulated by a balance between Arf guanine nucleotide-exchange factors (Arf GEFs) and the Arf GTPase-activating proteins (Arf GAPs) (Module 2: Figure Arf signalling). Examples of Arf GEFs are Golgi-specific brefeldin A resistant factor 1 (GBF1) and Arf nucleotide-binding site opener (ARNO). These activation mechanisms are counteracted by the Arf GAPs that facilitate the hydrolysis of GTP to GDP thus converting the complex back into its inactive Arf.GDP state. There are 24 human genes coding for Arf GAPs, which are characterized by having an Arf GAP domain, that can be separated into two main groups: the Arf GAP1 type and the Arf GAP with ANK repeats and PH domain (AZAPs) (Module 2: Table monomeric G protein toolkit).

The Arf GAP1 group has three genes coding for Arf GAP1-3, two genes for stromal membrane-associated protein 1 and 2 (SMAP1 and SMAP2) and two genes coding for G-protein-coupled receptor kinase interactors 1 and 2 (Git1 and Git2). ArfGAP1 and ArfGAP2/3 inactivates Arf1.GTP resulting in the shedding of the COPI coat as the vesicles approach the endoplasmic reticulum (See step 7 in Module 4: Figure COPI-coated vesicles).

Another group of Arf GAPs with ANK repeats and PH domain (AZAPs) have 12 genes that fall into four groups and the 'Z' refers to the protein domain that characterizes each subgroup:

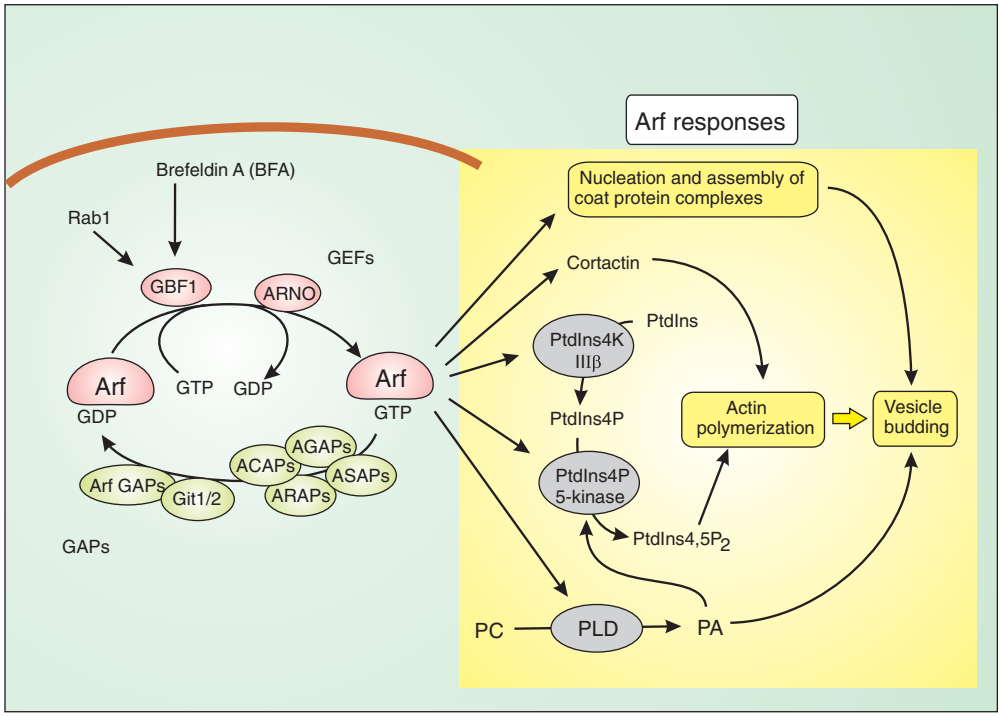
- Arf GAP with coiled-coil, ANK repeats and PH domain (ACAP)
- Arf GAP with GLD domain, ANK repeats and PH domain (AGAP)
- Arf GAP with Rho GAP, ANK repeats and PH domain (ARAP)
- Arf GAP with Src homology 3, ANK repeats and PH domain (ASAP)

The ASAP family has three genes (ASAP1–3). The ASAP3–Arf6 complex is of interest because it is sensitive to Ca^{2+} that binds to a specific region at the complex interface. This sensitivity to Ca^{2+} suggests that there may be cross-talk between the Arf and Ca^{2+} signalling pathways.

To carry out its role in vesicle trafficking, the Arfs have two main actions. First, they catalyse the nucleation and assembly of coat protein complexes during the process of vesicle budding. This role is particularly evident at the Golgi during the formation of COPI vesicles (Module 4: Figure COPI-coated vesicles). The Arfs also play a role in recruiting various Arf-associated golgins, such as GMAP-210, to the Golgi membrane.

The Arfs also function by activating the $PtdIns4,5P_2$ signalling cassette responsible for controlling multiple functions (Module 2: Figure $PtdIns4,5P_2$ signalling). The Arfs

Module 2: | Figure Arf signalling



Function of the Arf monomeric G-protein in signal transduction

When bound to GDP, Arf is inactive, but it is activated when the GDP is exchanged for GTP. This exchange is accelerated by guanine nucleotide-exchange factors (GEFs), but how these are activated is still somewhat of a mystery. In some cases, such as Golgi-specific brefeldin A resistant factor 1 (GBF1), activation depends on other G-proteins such as Rab1 (Module 4: Figure COPI-coated vesicles). The primary action of the Arfs is to stimulate actin polymerization and membrane remodelling during protein trafficking.

act by recruiting and stimulating both PtdIns 4 kinase IIIβ and the PtdIns4P 5-kinase (Module 2: Figure Arf signalling). All of the Arfs are responsible for phospholipase D (PLD) activation (Module 2: Figure PLD signalling). The subsequent formation of phosphatidic acid (PA), which is a bioactive lipid, has two actions: it acts synergistically to stimulate PtdIns4P 5-kinase and it alters membrane curvature during vesicle budding (Module 2: Figure Arf signalling).

Dedicator of cytokinesis 8 (DOCK8)

There is a family of eleven dedicator of cytokinesis (DOCK) proteins that are guanine nucleotide-exchange factors (GEFs) that appear to act primarily on the Rac signalling mechanism and the Cdc42 signalling mechanism. A typical example is DOCK8, which has an N-terminal DOCK Homology Region-1 (DHR-1) responsible for the guanine nucleotide-exchange reaction and a C-terminal DHR-2 domain that is thought to bind phospholipids. This family of DOCK proteins may act by remodelling actin to carry out a variety of cellular functions such as the control of cell migration, morphology, adhesion and proliferation. During the process of B-cell differentiation in the lymph node, DOCK8 is activated following its interaction with the integrin intercellular adhesion molecule 1 (ICAM1) (Module 8: Figure B cell maturation signalling).

Loss-of-function mutations in DOCK8 have been linked to variants of hyper-IgE syndromes (HIES) and mental retardation.

p21-activated kinase (PAK)

The p21-activated kinases (PAKs) are some of the major downstream targets of Rac and Rho (Module 2: Figure Rho-regulated kinases). They have been implicated in a large number of processes such as actin remodelling, cell cycle control, transcription and apoptosis. There are six isoforms (PAK1–PAK6) with slightly different functions. Rac acts through LIM kinase 1 (LIMK1) to phosphorylate cofilin (Module 2: Figure Rac signalling). Rho acts through PAK4 to phosphorylate the same LIMK1 (Module 2: Figure Cdc42 signalling). Another function of PAK1 is to phosphorylate myosin light chain kinase (MLCK), resulting in a decrease in the activity of the actin–myosin contractile system. PAK1 can also be activated independently of Rac through the calcium and integrin-binding protein 1 (CIB1) that acts by stimulating PAK1 (Module 2: Figure Rac signalling).

The fragile X mental retardation protein 1 RMRP1, which is mutated in fragile X syndrome (FXS), may act to inhibit the function of PAK particularly during actin remodelling in neuronal spines (Module 10: Figure Ca^{2+}-dependent synaptic plasticity).

Myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK)

Myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) has a number of functional domains and belongs to the same family as Rho kinase (ROK) (Module 2: Figure Rho-regulated kinases). MRCK, which is

Module 2: | Table monomeric G protein toolkit
Summary of the monomeric G proteins with their guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs).

Monomeric G protein, GEF or GAP	Comments
Monomeric G proteins (approximately 150 members)	
Ras family (36 members)	Contribute to the activation of multiple signalling pathways (Module 2: Figure Ras signalling)
H-Ras	
K-Ras	
N-Ras	
R-Ras	
RalA	
RalB	
Rap1	
Rap2	Rap2B has been implicated in the control of autophagy (Module 11: Figure autophagy)
RagA	The RagB/RagD heterotrimer interacts with TORC1 to control autophagy (Module 11: Figure autophagy)
RagB	
RagC	
RagD	
Rho family (22 members)	
Rho	Primary function is cytoskeletal remodelling (Module 2: Figure Rho signalling)
RhoA	
RhoD	
RhoE	
RhoF	
RhoH	
RhoV	
Mito Rho	
Rho BTB	
Rac	Cytoskeletal remodelling and ROS signalling (Module 2: Figure Rac signalling)
Rac1	
Rac2	
Rac3	
Cdc42	Primary function is cytoskeletal remodelling (Module 2: Figure Cdc42 signalling)
Rab family	A large family of GTPases that function in membrane trafficking
Rab1A	ER-to-Golgi trafficking. Rab1 interacts with the golgins (golgin-84, golgin-95, p115 and giantin)
Rab1B	
Rab2A	ER-to-Golgi trafficking. Rab2 interacts with the golgins (golgin-45)
Rab2B	
Rab3A	Control exocytosis by interacting with effectors such as Rabphilin, Rabin3, RIM1/2 and synapsin
Rab3B	
Rab3C	
Rab3D	
Rab4A	Endocytic recycling and functions in GLUT4 biogenesis, sorting and exocytosis
Rab4B	
Rab4C	
Rab4D	
Rab5A	Functions in early endosome fusion and phagosome maturation. Interacts with Rabaptin-5, Rabenosyn-5, EEA1 and Rabanykyrin-5 (see Module 4: Figure endosome vesicle fusion)
Rab5B	
Rab5C	
Rab5D	
Rab6A	Functions at the Golgi where it interacts with Giantin, Rabankyrin. Couples to dynein through Bicaudal D1 (see Module 4: Figure dynein)
Rab6B	
Rab6C	
Rab6D	
Rab7A	Operates in the late endosomal pathway during formation of lysosomes. Rab7A is mutated in Charcot-Marie-Tooth type 2B disease
Rab7B	
Rab8A	Functions in trafficking from Golgi to the plasma membrane. Interacts with optineurin and FIP-2. Rab8A binds to myosin Vb and functions in non-clathrin-dependent endocytosis
Rab8B	
Rab8C	
Rab9C	Functions in trafficking from late endosomes to the Golgi.
Rab9D	
Rab10	Functions in trafficking from Golgi to the plasma membrane. Interacts with Rim1.
Rab11A	Located in recycling endosomes. Functions in trafficking and internalization of aquaporin-2 (AQP2). Interacts with multiple effectors (FIPs, myosin Vb and PtdIns 4-kinaseβ)
Rab11B	
Rab12	Functions in trafficking from Golgi to the plasma membrane.
Rab13	Functions in biogenesis of tight junctions
Rab14	Functions in trafficking from Golgi to the early endosomes.
Rab15	Early endosome trafficking
Rab17	Operates in the apical recycling endosomes and functions in ciliogenesis
Rab18	Functions in formation of lipid droplets from the ER
Rab19A	Located in the Golgi
Rab19B	
Rab20	Trafficking of connexin-43
Rab21	Located on early endosomes and functions in cytokinesis
Rab22	Located on early endosomes
Rab23	Functions in formation of phagosomes
Rab24	Functions in autophagy
Rab25	Functions in integrin recycling
Rab26	Interacts with Rim1 in exocytosis
Rab27A	Intracellular transport of secretory granules and melanosomes (Module 4: Figure myosin motor)
Rab27B	

Module 2: | Table *continued*

Monomeric G protein, GEF or GAP	Comments
Rab28	
Rab29	
Rab30	Located on Golgi
Rab31	Located on early endosomes
Rab32	Located on early melanosmes and mitochondria
Rab33A	ER-to-Golgi trafficking
Rab33B	
Rab34	Functions in macropinocytosis
Rab35	Located on recycling endosomes and functions in cytokinesis and formation of the immunological synapse
Rab36	
Rab37	Located on secretory granules and functions in mast cell degranulation
Rab38	Functions in formation of melanosomes
Rab39	Located on the Golgi
Rab40A	
Rab40B	
Rab40C	
Rab41	
Rab42	
Rab43	ER-to-Golgi trafficking
Rab44	
Arf family	There are six ADP-ribosylation factors that function in actin remodelling and membrane trafficking (Module 2: Figure PLD signalling)
Class I	Operate mainly at the Golgi
Arf1	
Arf2	
Arf3	
Class II	Operate mainly at the Golgi
Arf4	
Afr5	
Class III	
Arf6	Operates mainly at the plasma membrane. It functions in endocytosis of AMPA receptors at synaptic endings (Module 10: Figure Ca^{2+}-induced synaptic plasticity)
Arf-like (ARL)	A small GTPase that interacts with golgins
Ran	A small GTPase that functions in nucleocytoplasmic transport
Guanine nucleotide-exchange factors (GEFs)	
RasGEFs	
SoS1	Son-of-sevenless-1
SoS2	Son-of-sevenless-2
RasGRF1	Expressed in brain, particularly hippocampus
RasGRF2	
RasGRF3	Expressed in brain
RasGRP1	Expressed in T cells
RasGRP2	Expressed in neutrophils and platelets
RasGRP3	Expressed in B cells
RasGRP4	Expressed in mast cells
Rho family GEFs	
The Dbl family	The 69 family members have a Dbl homology domain (DH)
Dbl	Diffuse B cell lymphoma
Dbs	Dbl's big sister
Ect2	Epithelial-cell transforming gene-2 that functions in cytokinesis (Module 9: Figure cytokinesis)
Ephexin	Functions in growth cone collapse mediated by EphA receptors (Module 1: Eph receptor signalling)
DOCK180	Downstream of Crk-180 homologue is a Rac GEF (Module 2: Figure Arf signalling), which functions in focal adhesion integrin signalling (Module 6: Figure integrin signalling)
Fgd1	Facial genital dysplasia
ITSN-L	Intersectin-long controls Cdc42 activity during EphB receptor signalling (Module 1: Eph receptor signalling)
Kalirin	Functions to activate Rac during the action of Eph receptors on dendritic spine morphogenesis (Module 1: Eph receptor signalling)
LARG	Leukaemia-associated RhoGEF
NET1	Neuroepithelioma transforming gene 1
p115-RhoGEF	Links G protein-coupled receptors to Rho
PDZ-RhoGEF	Links G protein-coupled receptors to Rho
P-Rex1	PtdIns3,4,5P_3-dependent Rac exchanger 1
α-Pix	PAK-interacting exchange factor α
β-Pix	PAK-interacting exchange factor β
Tiam1	T cell lymphoma invasion and metastasis-1
Tiam2	T cell lymphoma invasion and metastasis-2
Trio	
Vav1	
Vav2	
Vav3	
RAB GEFs	See Module 2: Figure Rab signalling
Rabex-5	A GEF for Rab 5 (Module 4: Figure endosome vesicle fusion)
Rabin3	
TRAPP1	A GEF for Rab1 (Module 4: Figure COPII-coated vesicles)

Module 2: | Table *continued*

Monomeric G protein, GEF or GAP	Comments
Arf GEFs	
Cytohesin 1	
Cytohesin 4	
GBF1	Golgi-specific brefeldin A resistant factor 1 functions in COPI-mediated protein transport from the Golgi to the ER (Module 4: Figure COPI-coated vesicles)
ARNO/Geα2	Arf nucleotide-binding site opener (ARNO)
BRAG2	This Arf6 GEF binds to AMPA receptors to promote endocytosis
GTPase-activating proteins (GAPs)	
Ras GAPs	
p120RasGAP	
Neurofibromin	This tumour suppressor is lost in the inherited disorder neurofibromatosis type 1
SynGAP	
GAP1m	
GAP1IP$_4$BP	Ins1,3,4,5P$_4$ binds to this protein, causing it to dissociate from the plasma membrane
	Ca^{2+}-promoted Ras inactivator
CAPRI	Ras GTPase-activating-like
RASAL	
DAB2IP	
Miro	Mitochondrial Rho-GTPase functions in mitochondrial motility (Module 5: Figure mitochondrial motility)
Rac GAPs	
3BP-1	3BP-1 inactivates Rac.GTP by enhancing GTP hydrolysis (Module 2: Figure Rac signalling)
Rho GAPS	
DLC1 (p122RhoGAP in mice)	Deleted in liver cancer 1 is altered in many tumours. It regulates actin formation in adhesion complexes (Module 6: Figure integrin signalling)
p190-RhoGAP	Inhibits Rho activity during neutrophil chemotaxis (Module 11: Figure neutrophil chemotactic signalling)
Oligophrenin-1 (OPHN1)	Mutations in OPHN1 have been linked to mental retardation
Rab GAPs	See Module 2: Figure Rab signalling
Rab3GAP	
Rin1	
Tbc1d1	These Rab GAPs function in the insertion of the GLUT4 transporter (Module7: Figure skeletal muscle E-C coupling)
Tbcd14/AS160	
Arf GAPS	Function to inactivate Arf.GTP (Module 2: Figure Arf signalling)
Arf GAP1 group	
ArfGAP1	Function in removing the coat from COPI-coated vesicles (Module 4: Figure COPI-coated vesicles)
ArfGAP2	
ArfGAP3	
SMAP1	Stromal membrane-associated protein
SMAP2	
Git1	G-protein-coupled receptor kinase interactors
Git2	
AZAP group	Arf GAP with ANK repeats and PH domain group of Arf GAPs
ACAP1	Arf GAP with coiled-coil, ANK repeats and PH domain (ACAP)
ASCP2	
ASCP3	
ASAP1	Arf GAP with Src homology 3, ANK repeats and PH domain (ASAP)
ASAP2	
ASAP3	
AGAP1	Arf GAP with GLD domain, ANK repeats and PH domain (AGAP)
AGAP2	
AGAP3	
ARAP1	Arf GAP with Rho GAP, ANK repeats and PH domain (ARAP)
ARAP2	
ARAP3	

Since some of the families are very large, only representative members have been included. Data for the large Rab family was based on that from Supplementary Table S1 from Stenmark (2009).

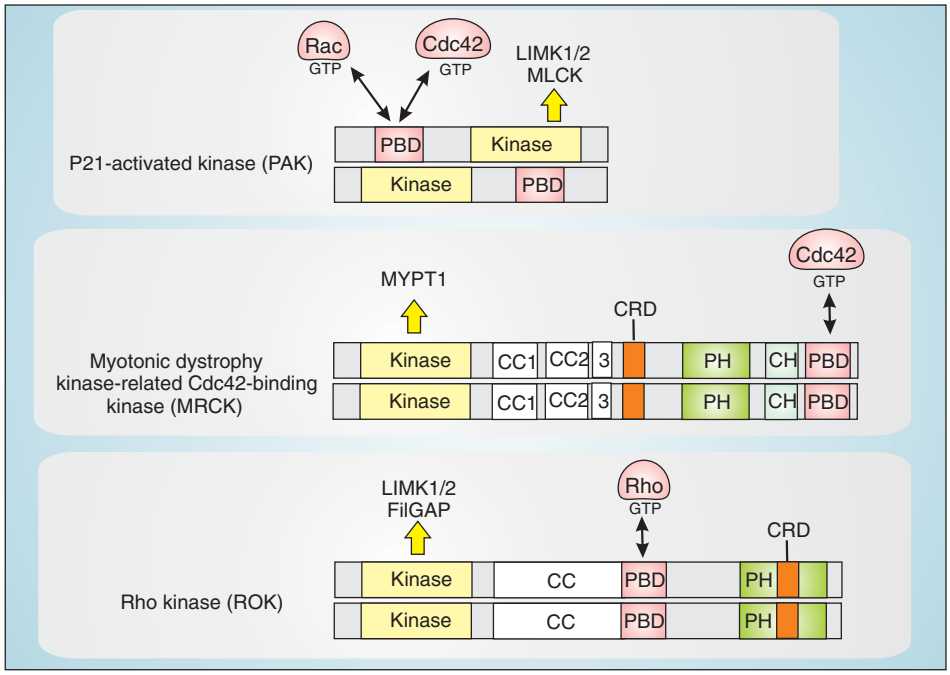
activated by Cdc42·GTP, may play a role in actin formation during the growth of neurites. One of the downstream targets of MRCK is the MYPT1 subunit (Module 5: Table PP1 regulatory and inhibitory subunits and proteins) that binds protein phosphatase 1δ (PP1δ) and which dephosphorylates the myosin light chain (MLC).

Rho kinase (ROK)

Rho kinase (ROK) is a serine/threonine kinase that phosphorylates key regulators of actin and myosin function (Module 2: Figure Rho-regulated kinases). ROK acts to stabilize actin by phosphorylating LIM, which then phosphorylates cofilin to prevent it from severing actin. Rho also acts on diaphanous-related formin protein (Dia), which belongs to a formin-related protein family that interacts with profilin to promote actin polymerization.

The other major function of ROK is to control contraction by activating myosin II in smooth muscle cells and in non-muscle cells. The activity of myosin II is regulated by the phosphorylation status of the myosin light chain (MLC) that is associated with the myosin head. When MLC is dephosphorylated, myosin is inactive, but when it is phosphorylated by the Ca^{2+}-sensitive myosin light

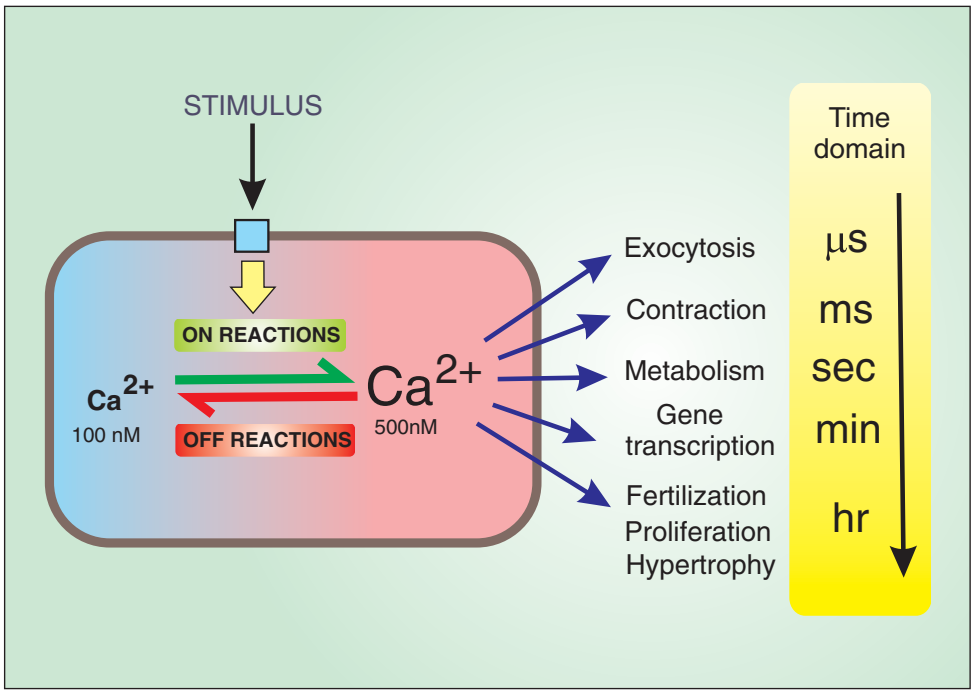
Module 2: | Figure Rho-regulated kinases



Structure of Rho GTPase-regulated kinases.

The Rho family exert many of their actions by stimulating protein kinases such as PAK, MRCK and ROK. These kinases exist as homodimers with PAK being arranged in a head-to-tail manner, whereas MRCK and ROK have a parallel alignment. The GTP-bound form of these p21 monomeric G proteins bind to a p21-binding domain (PBD). CC, coiled-coil domain: CH, citron homology domain; CRD, cysteine-rich domain; FilGap, filamin GTPase-activating protein; MLCK, myosin light chain kinase; PH, pleckstrin homology domain.

Module 2: | Figure basic Ca^{2+} signalling mechanism



The basic mechanism of Ca^{2+} signalling.

The concentration of Ca^{2+} in cells at rest is approximately 100 nM, but this increases to 500 nM or more following a stimulus that activates the Ca^{2+} ON reactions. When the stimulus is removed, the Ca^{2+} OFF reactions return the concentration of Ca^{2+} to its resting level. Ca^{2+} is a universal signal capable of activating many different cellular processes operating over a very wide time domain.

chain kinase (MLCK), myosin can begin to interact with actin to induce contraction. ROK can influence the phosphorylation of MLC by inhibiting the protein phosphatase 1 by phosphorylating the scaffolding protein myosin phosphatase targeting subunit 1 (MYPT1) (Module 5: Table PP1 regulatory, targeting and inhibitory subunits).

ROK may also function to inhibit endocytosis by phosphorylating endophilin.

Diaphanous-related formin 1 (Dia1)
There are two diaphanous-related formin proteins (Dia1 and Dia2) that belong to a formin-related family. They contain three formin homology domains that are used to bind to various effectors. Dia1 is activated by the Rho signalling mechanism and functions to control actin polymarization by binding to profilin (Module 2: Figure Rho signalling). Dia1 has also been implicated in the control of polycystin 2.

Ca^{2+} signalling

Ca^{2+} signalling is one of the major signalling systems in cells (Module 2: Figure cell signalling pathways). It functions to regulate many different cellular processes throughout their life history. It triggers new life at the time of fertilization. It controls many processes during development, and once cells have differentiated, it governs the activity of most cellular processes, effectively determining how we metabolize, secrete, move and think. There also is a darker side to its action, because larger than normal elevations can cause cell death, either in the controlled manner of programmed cell death (apoptosis) or in the more catastrophic necrotic changes that occur during processes such as stroke or cardiac ischaemia.

The basic mechanism of Ca^{2+} signalling is relatively simple in that it depends upon an increase in the intracellular concentration of this ion. The Ca^{2+} concentration is low when cells are at rest, but when an appropriate stimulus arrives, there is a sudden elevation, which is responsible for a change in cellular activity. However, there are multiple variations of this relatively simple theme. The versatility of Ca^{2+} signalling is achieved by having an extensive Ca^{2+} toolkit from which a large number of Ca^{2+} signalling signalsomes are assembled. This large toolkit contains many different components that can be mixed and matched to create many different Ca^{2+} signalling modules. There are Ca^{2+} entry channels, which control the entry of Ca^{2+} from the outside. There are Ca^{2+} release channels, which control the release of Ca^{2+} from internal stores. The Ca^{2+} buffers ensure that the concentration of Ca^{2+} remains within its operation range and does not rise to levels that can induce cell death. Once Ca^{2+} has carried out its signalling function, there are Ca^{2+} pumps and exchangers that remove it from the cytoplasm by either extruding it from the cell or returning it to the internal stores. Ca^{2+} signalling functions are carried out by various Ca^{2+} sensors and Ca^{2+} effectors that are responsible for translating Ca^{2+} signals into a change in cellular activity.

Basic mechanism of Ca^{2+} signalling

Cells at rest maintain a low intracellular concentration of Ca^{2+} (approximately 100 nM), but this increases rapidly into the micromolar range when cells are stimulated (Module 2: Figure basic Ca^{2+} signalling mechanism). This increase in intracellular Ca^{2+} can operate over a very wide time domain (e.g. microseconds to hours) to regulate many different cellular processes. This very wide temporal range of Ca^{2+} signalling is an intrinsic property of the Ca^{2+} signalling modules.

An important feature of Ca^{2+} signalling is its dynamic nature, as exemplified by the fact that Ca^{2+} signals invariably appear as a brief transient. The rising phase of each transient is produced by the ON reactions, whereas the falling phase depends on the OFF reactions (Module 2: Figure Ca^{2+} signalling dynamics). At any moment, the level of Ca^{2+} is determined by the balance between the Ca^{2+} ON reactions that increase Ca^{2+} and the Ca^{2+} OFF reactions that remove it from the cytosol. An important aspect of the ON and OFF reactions is their spatial location. An example of this spatial organization is the ER/mitochondrial Ca^{2+} shuttle (Module 5: Figure ER/mitochondrial shuttle), where events at the ER are closely linked to those in the mitochondria. Another important spatial aspect is that the ON reactions are often closely associated with the effector systems that respond to Ca^{2+}. For example, voltage-operated channels (VOCs) in presynaptic endings are associated with the synaptic vesicles, thus producing a highly localized puff of Ca^{2+} to trigger exocytosis (Module 4: Figure Ca^{2+}-induced membrane fusion). Similarly, the type 2 ryanodine receptors (RYR2s) of cardiac cells are lined up close to the contractile filaments to ensure that Ca^{2+} will rapidly stimulate contraction.

In cases where cells need to be stimulated over a long time, these transients are repeated at set intervals to set up Ca^{2+} oscillations. These oscillations are part of the spatiotemporal aspects of Ca^{2+} signalling.

Ca^{2+} signalling signalsome

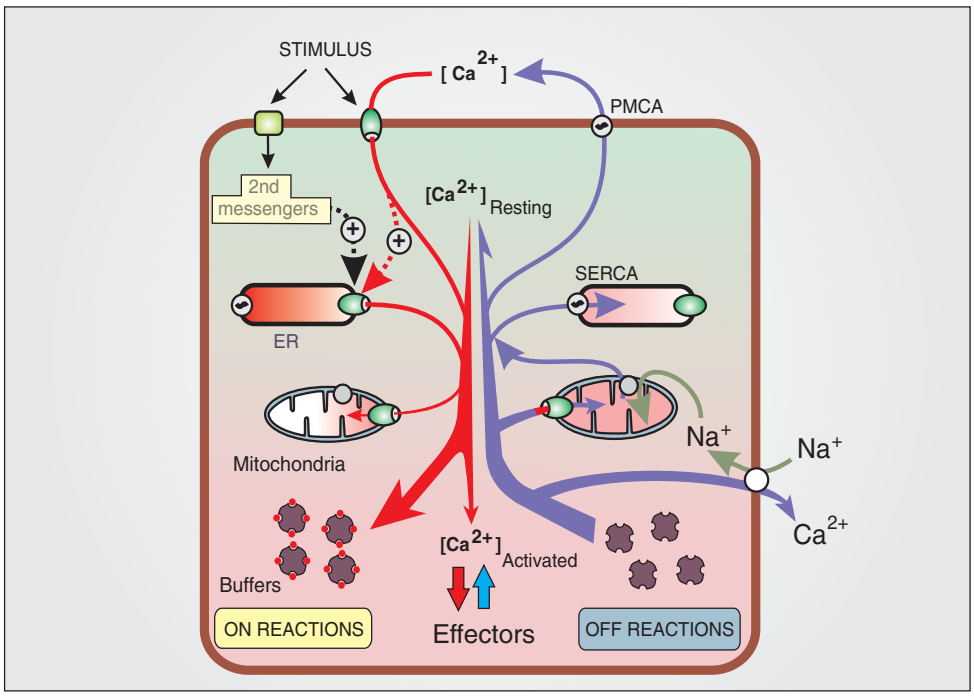
Cells have access to a large Ca^{2+} signalling toolkit (Module 2: Table Ca^{2+} signalling toolkit). Many of the toolkit components have similar functions (Module 2: Figure Ca^{2+} signalling toolkit), which represents a generic Ca^{2+} signalling system. In reality, however, each cell type has a clearly defined subset of toolkit components that will be referred to as a Ca^{2+} signalling signalsome. These cell-specific signalsomes are put in place during development when a process of signalsome expression enables each differentiating cell to select out those signalling components it will require to control its particular functions (Module 8: Figure signalsome expression). There are an enormous number of cell-specific Ca^{2+} signalsomes (Module 2: Figure cell-specific Ca^{2+} signalsomes). The important point is that each signalsome generates a cell-specific Ca^{2+} signal with characteristic spatial and temporal properties.

A signalsome is defined here as the collection of signalling components that make up each cell-specific signalling system. The Ca^{2+} signalsomes of different cell types often display recurring themes in the form of Ca^{2+} signalling modules (Module 2: Figure Ca^{2+} modules).

Ca^{2+} signalling modules

The Ca^{2+} signalling system in specific cell types is often not a single entity, but is made up of distinct modules, which are mixed and matched to produce the cell-specific systems found in different cell types. Some of the main modules used by cells are summarized in Module 2: Figure Ca^{2+} modules:

Module 2: | Figure Ca^{2+} signalling dynamics



The dynamics of Ca^{2+} signalling.

The dynamics of Ca^{2+} signalling are governed by an interplay between the ON and OFF reactions that control the fluxes of Ca^{2+} across both the plasma membrane and the internal organelles such as the endoplasmic reticulum (ER) and mitochondria. External stimuli activate the ON reactions, which introduce Ca^{2+} into the cytoplasm either through channels in the plasma membrane or from internal stores such as the ER. Most cells make use of both sources, but there are examples of cells using either external or internal sources to control specific processes. Most of the Ca^{2+} that enters the cytoplasm is adsorbed on to buffers, while a much smaller proportion activates the effectors to stimulate cellular processes. The OFF reactions remove Ca^{2+} from the cytoplasm using a combination of mitochondria and different pumping mechanisms. When cells are at rest, these OFF reactions keep the concentration low, but these are temporarily overwhelmed when external stimuli activate the ON reactions. Sequential activation of the ON and OFF reactions gives rise to the Ca^{2+} transients (Module 2: Figure Ca^{2+} transient mechanisms), which are such a characteristic feature of Ca^{2+} signalling systems. PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco/endo-plasmic reticulum Ca^{2+}-ATPase.

1. Agonists such as the neurotransmitters glutamate and ATP act directly on receptor-operated channels (ROCs) in the plasma membrane to allow external Ca^{2+} to enter the cell.
2. Second messengers such as diacylglycerol (DAG), cyclic AMP, cyclic GMP and arachidonic acid acting from the cytoplasmic side open second messenger-operated channels (SMOCs) in the plasma membrane.
3. Membrane depolarization (ΔV) activates voltage-operated channels (VOCs) in the plasma membrane to allow a rapid influx of external Ca^{2+}.
4. Membrane depolarization (ΔV) activates a specific VOC isoform, the $Ca_V1.1$ L-type channel, that activates the ryanodine receptor 1 (RYR1) in skeletal muscle through a direct conformational-coupling mechanism.
5. Membrane depolarization (ΔV) activates voltage-operated channels (VOCs) in the plasma membrane to allow a rapid influx of external Ca^{2+} (see Module 3) to provide a Ca^{2+} trigger that then activates the ryanodine receptor 2 (RYR2) to release Ca^{2+} stored in the sarcoplasmic reticulum (SR) through a process of Ca^{2+}-induced Ca^{2+} release (CICR). This mechanism is found in cardiac muscle and neurons.
6. Agonists acting on cell-surface receptors generate inositol 1,4,5-trisphosphate ($InsP_3$) (Module 2: Figure $InsP_3$ and DAG formation), which then diffuses into the cell to activate the $InsP_3$ receptor ($InsP_3R$) to release Ca^{2+} from the ER.

Ca^{2+} ON reactions

In response to external stimuli, channels in the plasma membrane or ER are opened, and Ca^{2+} flows into the cytoplasm to bring about the elevation of cytosolic Ca^{2+} responsible for cell activation (Module 2: Figure Ca^{2+} signalling dynamics). During these ON reactions, the cell employs a variety of both Ca^{2+} entry channels and Ca^{2+} release channels to create Ca^{2+} signals with markedly different spatial and temporal properties.

The entry of Ca^{2+} across the plasma membrane is carried out by many different channels whose names indicate how they are opened:

- Voltage-operated channels (VOCs)
- Agonist-operated channels (AOCs)
- Receptor-operated channels (ROCs)
- Second messenger-operated channels (SMOCs)
- Store-operated channels (SOCs)

Release of Ca^{2+} from internal stores is carried out by different types of channels and control mechanisms:

- Ryanodine receptors (RYRs)
- $InsP_3$ receptors ($InsP_3Rs$)

Module 2: | Figure cell-specific Ca^{2+} signalsomes

	SKELETAL MUSCLE CELL	CARDIAC ATRIAL CELL	Ca1 NEURON	T CELL
Receptors		ET-1R/α1R AngIIR	mGluR1 M1	TCR
PLC		PLCβ	PLCβ	PLCγ1
Entry channels	$Ca_V1.1$	$Ca_V1.2$	$Ca_V1.2$/ $Ca_V2.1$ $Ca_V2.2$/ NMDAR	Orai1
Release channels	RYR1	RYR2 $InsP_3R2$	RYR2 $InsP_3R2$	$InsP_3R1$
PMCAs	PMCA1a, 1c,1d	PMCA1c, 1d,2a	PMCA1a, 2a 3a	PMCA4b
SERCAs	SERCA1a, 1b	SERCA2a	SECA2b, 3	SERCA2b, 3
Na^+/Ca^{2+} exchanger	NCX	NCX1	NCX1, 3	–
Buffers	Parvalbumin		Parvalbumin Calbindin 28K	
Sensors	Troponin c Calmodulin	Troponin c Calmodulin	Calmodulin	Calmodulin

Some examples of cell-specific Ca^{2+} signalling signalsomes.

The four cell types represented here generate Ca^{2+} signals with very different spatial and temporal properties. For example, the skeletal muscle signalsome selects out those components specialized to deliver rapid pulses of Ca^{2+} to activate contraction, whereas the T cell signalsome has different components that generate the much slower repetitive pulses of Ca^{2+} necessary to stimulate cell proliferation.

- NAADP control of Ca^{2+} release

One of the major problems in Ca^{2+} signalling has been to determine how stimuli arriving at the cell surface gain access to these internal stores. Two main mechanisms have been identified:

1. Conformational coupling through a protein–protein interaction. This is a very fast mechanism that depends upon a sensor in the plasma membrane interacting directly with an internal release channel. The receptor on the cell surface is the $Ca_V1.1$ L-type channel (a voltage sensor), which is coupled to the type 1 ryanodine receptor 1 (RYR1) (Ca^{2+} module 4 in Module 2: Figure Ca^{2+} modules). Information is transferred through a process of conformational coupling. This mechanism is restricted to skeletal muscle (Module 7: Figure skeletal muscle E-C coupling) and perhaps also to some neurons.
2. Generation of diffusible second messengers. Activation of receptors or channels on the cell surface generate second messengers that then diffuse into the cell to activate release channels. One of the most significant Ca^{2+}-mobilizing messengers is Ca^{2+} itself, which is a potent activator of the two main internal release channels, the ryanodine receptors (RYRs) and the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$). This Ca^{2+}-induced Ca^{2+} release (CICR) mechanism has the unique property of being autocatalytic and plays a central role in generating those Ca^{2+} signals that appear as regenerative Ca^{2+} waves (Module 2: Figure Ca^{2+}-induced Ca^{2+} release).

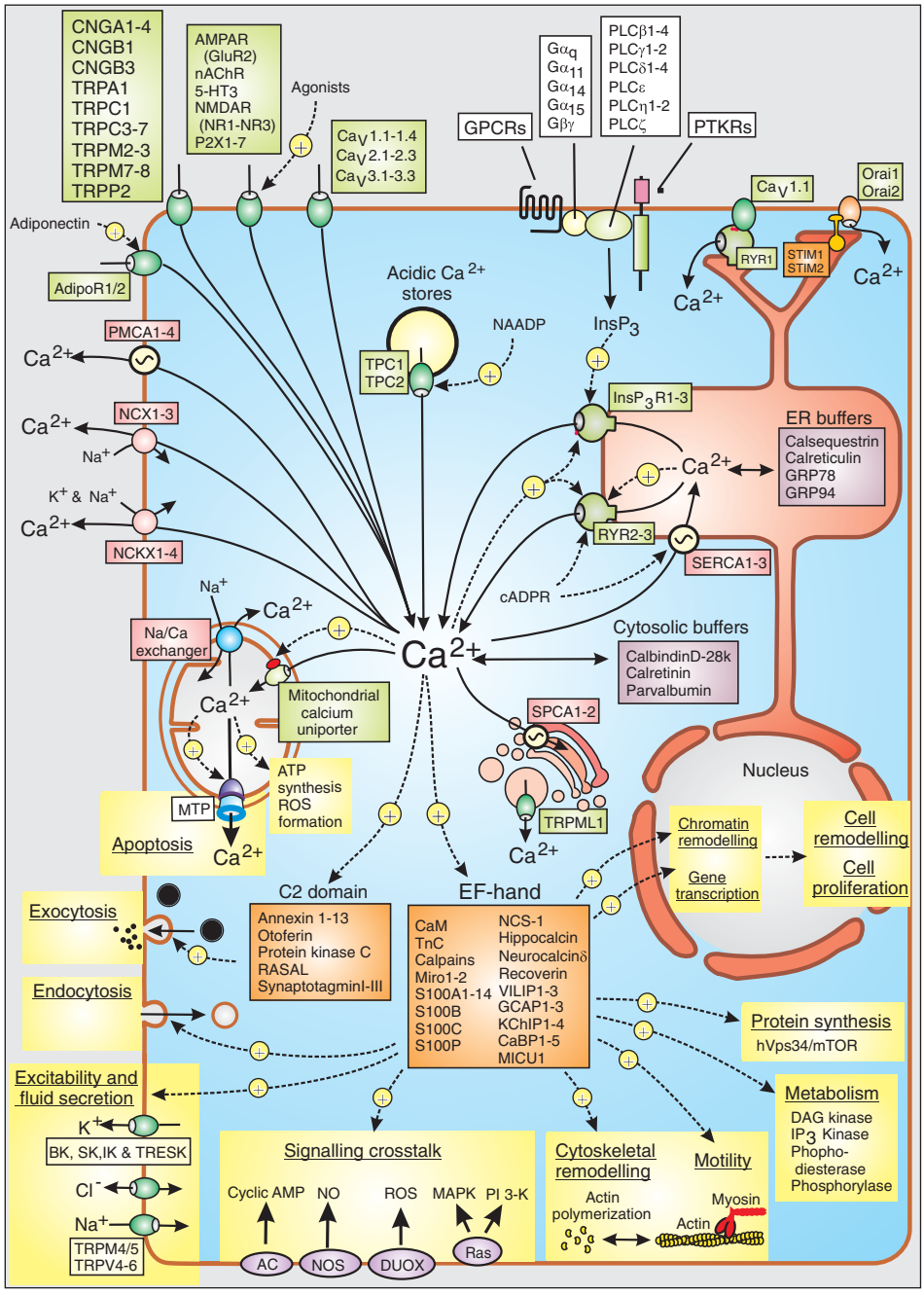
Another classical process for releasing internal Ca^{2+} is the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette (Ca^{2+} module 6 in Module 2: Figure Ca^{2+} modules). Other Ca^{2+}-mobilizing messengers have been described such as sphingosine 1-phosphate (S1P), cyclic ADP ribose (cADPR) and nicotinic acid–adenine dinucleotide phosphate (NAADP).

The channels responsible for these Ca^{2+} ON reactions usually have powerful inactivation mechanisms that rapidly curtail the entry or release processes to prevent the cell being swamped with Ca^{2+}, which can result in cell stress and apoptosis. Once the ON reactions have been curtailed, the Ca^{2+} OFF reactions rapidly take over to return the activated level of Ca^{2+} back to its resting level.

Ca^{2+}-induced Ca^{2+} release (CICR)

A process of Ca^{2+}-induced Ca^{2+} release (CICR) plays a central role in the way Ca^{2+} signals are generated. This positive-feedback mechanism whereby Ca^{2+} triggers its own release has two important functions in cells. It enables Ca^{2+} entering across the plasma membrane to function as a messenger to release Ca^{2+} from the internal store (Module 2: Figure Ca^{2+}-induced Ca^{2+} release). This function of CICR was first described in cardiac cells, where the $Ca_V1.2$ L-type channel provides an influx of trigger Ca^{2+} that then diffuses into the cell to activate the ryanodine receptor 2 (RYR2) (Ca^{2+} module 5 in Module 2: Figure Ca^{2+} modules). A similar interaction is particularly evident for neuronal Ca^{2+} entry and release channels.

Module 2: | Figure Ca^{2+} signalling toolkit



Summary of the major components that contribute to the Ca^{2+} signalling signalsome.

There is an extensive Ca^{2+} signalling toolkit. The green boxes illustrate the membrane Ca^{2+} channels, whereas the red boxes are the pumps and exchangers that move Ca^{2+} either out of the cell or back into the endoplasmic reticulum (ER). The purple boxes represent the buffers located in the cytoplasm or in the endoplasmic reticulum (ER). To carry out its signalling function, Ca^{2+} binds to sensors with C2 or EF-hand domains that then employ a range of effectors to stimulate cellular processes shown in the yellow boxes. These different components are mixed and matched to construct various Ca^{2+} signalling modules (Module 2: Figure Ca^{2+} modules) that are then assembled to produce cell-specific signalsomes.

The other main function of CICR is to set up intracellular Ca^{2+} waves where an elevated level of Ca^{2+} in one region of the cell (the initiation site) propagates throughout the rest of the cytoplasm as a regenerative Ca^{2+} wave. Waves can progress by recruiting either RYRs or inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) (Module 2: Figure Ca^{2+}-induced Ca^{2+} release). These Ca^{2+} waves are made up of elementary Ca^{2+} events such as the sparks and puffs produced by the RYRs and $InsP_3Rs$ respectively. It is these unitary events that are used to generate the regenerative waves that make up global Ca^{2+} signals.

Ca^{2+} OFF reactions

Cells use a variety of mechanisms to remove Ca^{2+} from the cytoplasm (Module 2: Figure Ca^{2+} signalling dynamics). The introduction of Ca^{2+} into the cell during the

Module 2: | Table Ca^{2+} signalling toolkit
The Ca^{2+} signalling toolkit.

Component	Comments
RECEPTORS AND TRANSDUCERS	
G protein-coupled receptors (GPCRs)	See Module 1: Table G-protein-coupled receptors for a list of those GPCRs that act by stimulating phospholipase Cβ
Tyrosine-kinase-linked receptors	See Module 1: Figure tyrosine kinase-linked receptors. (The receptors shown opposite can activate phospholipase Cγ)
Platelet-derived growth factor receptor (PDGFR)	See Module 1: Figure PDGFR activation
PDGFRα	
PDGFRβ	
Epidermal growth factor receptor (EGFR)	
ERBB1-ERBB4	
Vascular endothelial growth factor receptor (VEGFR)	See Module 9: Figure VEGF-induced proliferation
VEGFR1-VEGFR3	
G Proteins	
$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, $G\alpha_{16}$, $G\beta\gamma$	These G protein subunits activate phospholipase C (Module 2: Figure heterotrimeric G protein signalling)
Guanine nucleotide exchange factors	
RasGRF1	A Ca^{2+}-activated GEF expressed in brain (Module 2: Figure Ras signalling)
Regulators of G protein signalling (RGS)	
RGS1, RGS2, RGS4, RGS16	
Phospholipase C (PLC)	
PLCβ1-4, PLCγ1-2, PLCδ1-4, PLCε, PLCζ	PLC hydrolyses PtdIns4,5P_2 to form InsP_3 and DAG (Module 2: Figure PLC structure and function)
ADP-ribosyl cyclase	Functions to generate cADPR and NAADP (Module 2: Figure cADPR/NAADP function)
CD38	
CHANNELS	
Plasma membrane channels	
Voltage-operated channels (VOCs)	See Module 3: Table VOC classification for further details
$Ca_V1.1$ (L-type)	
$Ca_V1.2$ (L-type)	
$Ca_V1.3$ (L-type)	
$Ca_V1.4$ (L-type)	
$Ca_V2.1$ (P/Q-type)	
$Ca_V2.2$ (N-type)	
$Ca_V2.3$ (R-type)	
$Ca_V3.1$ (T-type)	
$Ca_V3.2$ (T-type)	
$Ca_V3.3$ (T-type)	
Ca^{2+}-sensitive ion channels	
Ca^{2+}-activated K^+ channels	See Module 3: Table properties of Ca^{2+}-sensitive K^+ channels for further information
SK (small conductance Ca^{2+}-sensitive channel)	
IK (intermediate conductance Ca^{2+}-sensitive channel)	
BK (large conductance Ca^{2+}-sensitive channel)	
Ca^{2+}-activated chloride channel	
HCLCA1 (human chloride channel, Ca^{2+}-activated)	
Receptor-operated channels (ROCs)	See Module 3: Table receptor-operated channel toolkit for further details)
Nicotinic acetylcholine receptors	
$5\text{-}HT_3$	5-Hydroxytryptamine receptor
AMPA receptors	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor
NMDA receptors	*N*-methyl-D-aspartate receptor
P2X receptors	
Second messenger-operated channels (SMOCs)	Module 3: Figure Ca^{2+} entry mechanisms
Arachidonate-regulated Ca^{2+} channel (I_{ARC})	
Cyclic nucleotide-gated channels (CNGs)	Module 3: Figure cyclic nucleotide-gated channels
CNGA1-CNGA4, CNGB1, CNGB3	
Transient receptor potential (TRP) ion channel family	Module 3: Figure TRP channel phylogeny
TRPC1-TRPC7	Canonical
TRPV1-TRPV6	Vanilloid
TRPM1-TRPM8	Melastatin
Polycystins	Module 3: Figure polycystin domain structure
PC1	
PC2	
Endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) Ca^{2+} release channels	
$InsP_3$ receptors ($InsP_3Rs$)	Module 3: Figure $InsP_3R$ structure
$InsP_3R1$	
$InsP_3R2$	
$InsP_3R3$	
Ryanodine receptors (RYRs)	Module 3: Figure ryanodine receptor structure
RYR1	
RYR2	
RYR3	

Module 2: | Table continued

Component	Comments
Channel regulators	
Triadin	
Junctin	
Sorcin	EF-hand protein that can bind to annexin 7
FKBP12	FK506-binding protein 12 kDa
FKBP12.6	FK506-binding protein 12.6 kDa
Phospholamban	Module 5: Figure phospholamban mode of action
MICU1	Mitochondrial calcium uptake 1 regulates the mitochondrial uniporter
Ca^{2+} BUFFERS	
Cytosolic buffers	
Calbindin D-28k	
Calretinin	
Parvalbumin	
Endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) buffers and chaperones	
Calnexin	
Calreticulin	
Calsequestrin	
GRP78	Glucose-regulatory protein of 78 kDa. Also known as BiP
GRP94	Glucose-regulatory protein of 94 kDa. Also known as endoplasmin
Ca^{2+} SENSORS	
EF-hand Ca^{2+}-binding proteins	
Calmodulin (CaM)	A ubiquitous Ca^{2+} sensor
Calcineurin B (CaNB)	A sensor that resembles CaM and controls the function of calcineurin (Module 4: Figure calcineurin)
Troponin C (TnC)	The Ca^{2+} sensor in striated muscle
Miro	Mitochondrial Rho-GTPase functions in mitochondrial motility (Module 5: Figure mitochondrial motility)
DAG kinase α	A Ca^{2+}-sensitive enzyme that phosphorylates diacylglycerol (DAG) (Module 2: Figure $InsP_3$/DAG recycling)
Stromal interaction molecule (STIM)	Senses Ca^{2+} in the ER lumen (Module 3: Figure STIM-induced Ca^{2+} entry)
Apoptosis-linked gene 2 (ALG-2)	A penta-EF-hand protein that may control ER-to-Golgi trafficking (Module 4: Figure COPII-coated vesicles)
S100 proteins	
S100 protein clustered on chromosome 1	S100A1-S100A14 are all clustered on 1q21 (Module 4: Figure S100 phylogenetic tree)
S100A1	
S100A2	
S100A3	
S100A4	
S100A5	
S100A6	
S100A7	
S100A8	
S100A9	
S100A10	
S100A11	
S100A12	
S100A13	
S100A14	
S100 protein clustered on other chromosomes	
S100B	
S100C	
S100P	
Neuronal Ca^{2+} sensor proteins (NCS)	A family of 14 EF-hand Ca^{2+}-binding proteins
NCS-1	Brain and retina
Hippocalcin	Restricted to hippocampal neurons
Neurocalcin δ	Brain and retina
Recoverin	Retina
Visinin-like proteins (VILIPs)	
VILIP-1	Brain and retina
VILIP-2	Retina
VILIP-3	Retina and cerebellar Purkinje cells
Guanylyl cyclase-activating proteins (GCAPs)	Module 10: Figure phototransduction
GCAP1	Retina
GCAP2	Retina
GCAP3	Retina
Kv-channel-interacting proteins (KChIPs)	
KChIP1	Brain
KChIP2	Brain
KChIP3/downstream regulatory element modulator (DREAM)/calsenilin	This protein has three names which are currently in use
KChIP4	Brain
Ca^{2+}-binding proteins (CaBPs)	
Caldendrin	Brain and retina
L-CaBP1	A long splice variant of caldendrin

Module 2: | Table continued

Component	Comments
S-CaBP1	A short splice variant of caldendrin
CaBP2	Retina
CaBP3	Retina
CaBP4	Brain and retina
CaBP5	Retina
Calneuron-1	The calneurons act to regulate the trafficking function of PtdIns 4-KIIIα at the Golgi
Calneuron-2	
C2 domain Ca^{2+}-binding proteins	
Synaptotagmins	The Ca^{2+} sensors that control exocytosis (Module 4: Figure Ca^{2+}-induced membrane fusion)
Synaptotagmin I	
Synaptotagmin II	
Synaptotagmin III	
Otoferin	The Ca^{2+} sensors that functions in hair cell transmitter release
Annexins	Ca^{2+}-dependent phospholipid-binding proteins (Module 4: Figure annexin structure)
Annexin A1	
Annexin A2	
Annexin A3	
Annexin A4	
Annexin A5	
Annexin A6	
Annexin A7	
Annexin A8	
Annexin A9	
Annexin A10	
Annexin A11	
Annexin A13	
Ca^{2+}-SENSITIVE ENZYMES AND PROCESSES	
Ca^{2+}-regulated enzymes	
Ca^{2+}-dependent protein kinases (CaMKs)	
CaMKI	
CaMKII	
CaMKIII	
CaMKIV	
Myosin light chain kinase (MLCK)	
Phosphorylase kinase	
Ins1,4,5P_3 3-kinase	
Proline-rich tyrosine kinase 2 (Pyk2)	A Ca^{2+}-sensitive tyrosine kinase that functions in osteoclast podosomes (Module 7: Figure osteoclast podosome)
Lipid kinase	
hVps34	See Module 9: Figure target of rapamycin signalling
PKC-α	
PKC-βI	
PKC-βII	
PKC-γ	
Phosphodiesterases	
Cyclic GMP phosphodiesterase (PDE)	Some of the phosphodiesterases are sensitive to Ca^{2+} see Module 5: Table PDE family properties
PDE1A	
PDE1B	
PDE1C	
Adenylyl cyclases (ACs)	See Module 2: Table adenylyl cyclases for details of these Ca^{2+}-sensitive enzymes
AC-1	
AC-III	
AC-VIII	
AC-V	
AC-VI	
Dual oxidases (DUOX1-2)	See Module 2: Figure redox signalling components
Nitric oxide synthase (NOS)	Module 2: Figure NO and cyclic GMP signalling
Endothelial NOS (eNOS)	
Neural NOS (nNOS)	
Ca^{2+}-activated proteases	
Calpain I	
Calpain II	
Transcription factors	
Nuclear factor of activated T cells (NFAT)	Module 4: Figure NFAT activation
NFATc1	
NFATc2	
NFATc3	
NFATc4	
Cyclic AMP response element-binding protein (CREB)	Module 4: Figure CREB activation
Downstream regulatory element modulator (DREAM)	
CREB-binding protein (CBP)	

Module 2: | Table continued

Component	Comments
Ca^{2+} PUMPS AND EXCHANGERS	
Na^+/Ca^{2+} exchanger (NCX)	See Module 5: Table Ca^{2+} pumping toolkit for further details
NCX1	
NCX2	
NCX3	
$Na^+/Ca^{2+}/K^+$ exchanger (NCKX)	
NCKX1	
NCKX2	
NCKX3	
NCKX4	
Mitochondrial channels and exchangers	Module 5: Figure mitochondrial Ca^{2+} signalling
Permeability transition pore (PMT)	
Na^+/Ca^{2+} exchanger	
MCU	Mitochondrial Ca^{2+} uniporter
Plasma membrane Ca^{2+}-ATPases (PMCAs)	See Module 5: Table Ca^{2+} pumping toolkit for further details
PMCA1	
PMCA2	
PMCA3	
PMCA4	
Sarco/endo-plasmic reticulum Ca^{2+}-ATPases (SERCAs)	See Module 5: Table Ca^{2+} pumping toolkit for further details
SERCA1	
SERCA2	
SERCA3	
Secretory-pathway Ca^{2+}-ATPase (SPCA) pumps	
SPCA1	Ubiquitous; located in the Golgi
SPCA2	

The Ca^{2+} signalling system has a very large toolkit of signalling components. The ways in which the major components are organized are summarized in Module 2: Figure Ca^{2+} signalling toolkit. Components from this toolkit can be mixed and matched to create a diverse array of cell-specific Ca^{2+} signalsomes (Module 2: Figure cell-specific Ca^{2+} signalsomes) that are capable of delivering Ca^{2+} signals with very different spatial and temporal properties.

Ca^{2+} ON reactions usually occurs for a relatively brief period during which there is a rapid increase in the intracellular concentration of Ca^{2+}. In fact, the increase in free Ca^{2+} that can be measured in the cytoplasm using aequorin or fluorescent indicators is a very small proportion of the total amount of Ca^{2+} that enters during the ON reactions. Much of this Ca^{2+} is rapidly bound by the cytosolic buffers or is taken up by the mitochondria. As the Ca^{2+} concentration returns to its resting level, Ca^{2+} leaves the buffers and the mitochondria and is returned to the ER or is pumped out of the cell resulting in a brief Ca^{2+} transient.

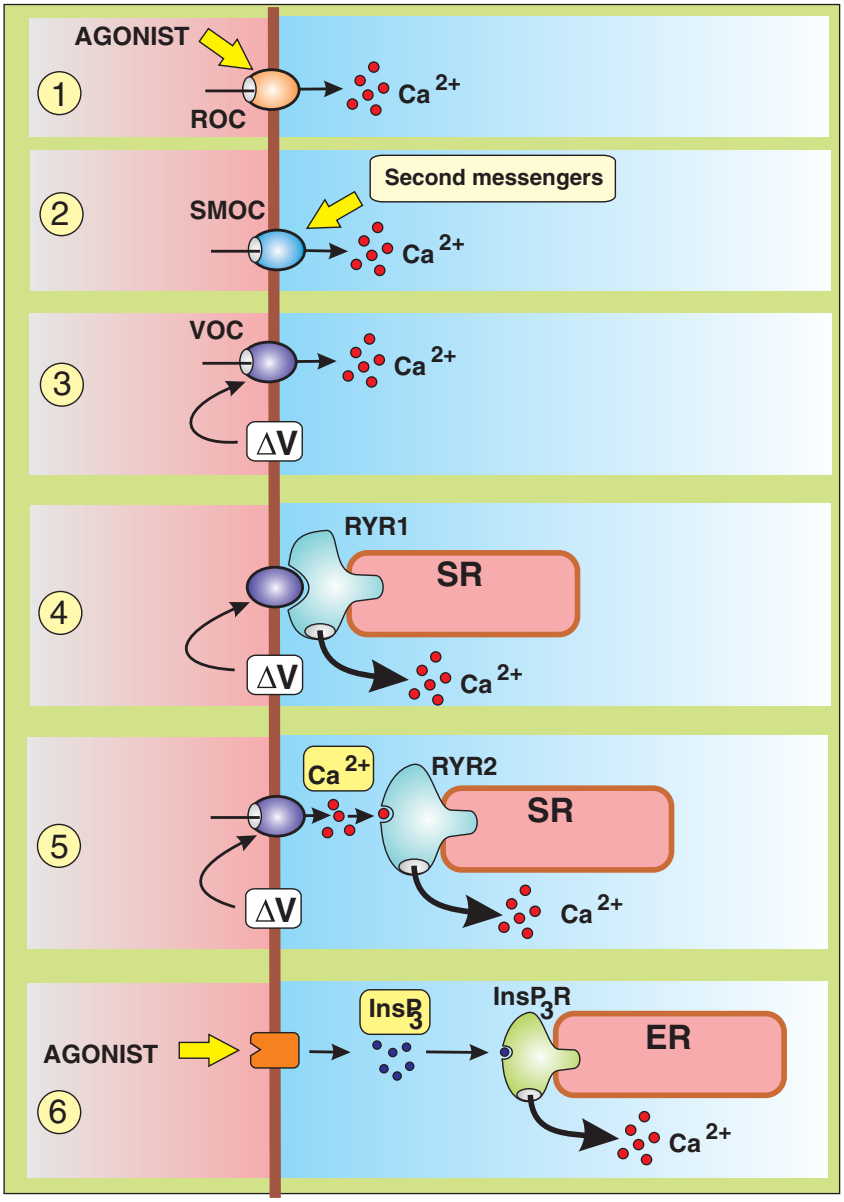
The recovery process thus depends upon a complex interplay between cytosolic Ca^{2+} buffers, mitochondria and Ca^{2+} pumps and exchangers on the internal stores and on the plasma membrane (Module 2: Figure Ca^{2+} signalling dynamics). These Ca^{2+} OFF reactions operate at different stages during the recovery phase of a typical Ca^{2+} spike. The buffers and mitochondria operate early, and the Ca^{2+} pumps and exchangers are responsible for restoring the status quo by pumping Ca^{2+} out of the cell or back into the ER. These pumps and exchangers operate at different times during the recovery process. The Na^+/Ca^{2+} exchangers have low affinities for Ca^{2+}, but have very high capacities and this enables them to function at the beginning of the recovery process to rapidly remove large quantities of Ca^{2+}. On the other hand, the plasma membrane Ca^{2+}-ATPase (PMCA) and sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps have lower capacities, but their higher affinities mean that they can complete the recovery process and can continue to pump at lower Ca^{2+} levels, thus enabling them to maintain the internal stores and the resting level (Module 5: Figure Ca^{2+} uptake and extrusion).

Some of these OFF reactions interact with each other during the recovery period, and this is particularly evident in the case of the ER/mitochondrial Ca^{2+} shuttle (Module 5: Figure ER/mitochondrial shuttle). When Ca^{2+} is released from the ER, Ca^{2+} is rapidly taken up by the mitochondria and this is then released slowly back to the ER once Ca^{2+} has returned to the resting level.

In addition to its role of returning the level of Ca^{2+} to its resting level following a stimulus, the Ca^{2+} OFF reactions are in constant operation to maintain the resting level of Ca^{2+} within a fairly narrow range. This calciostat is a dynamic system in that the OFF reactions are operating continuously to reverse the constant basal rate of Ca^{2+} entry through various 'Ca^{2+} leak pathways' that remain poorly defined. There is increasing evidence that presenilins may function as such a leak channel and this has been incorporated into the calcium hypothesis of Alzheimer's disease (see step 10 in Module 12: Figure amyloid processing and Ca^{2+} signalling). The latter can result in an elevation of cytosolic Ca^{2+} if the OFF reactions are inhibited. For example, agents such as thapsigargin, which inhibit the SERCA pumps on the ER, will result in an increase in Ca^{2+} concentration. Indeed, the rate at which the Ca^{2+} concentration rises following pump inhibition provides a measure of the activity of these leak pathways.

In pathological situations, the lack of oxygen reduces the supply of energy, thus compromising the function of the OFF reactions, resulting in the rise of Ca^{2+} that is so damaging during stroke or cardiac ischaemia. Under

Module 2: | Figure Ca^{2+} modules



Ca^{2+} signalling modules.

Some of the main Ca^{2+} signalling modules that cells employ to generate Ca^{2+} signals are depicted. Ca^{2+} is either derived from the external medium or released from internal stores such as the sarcoplasmic reticulum (SR) in muscle or the endoplasmic reticulum (ER) in other cell types. The ion channels responsible for gating Ca^{2+} in these different modules are explained in the text.

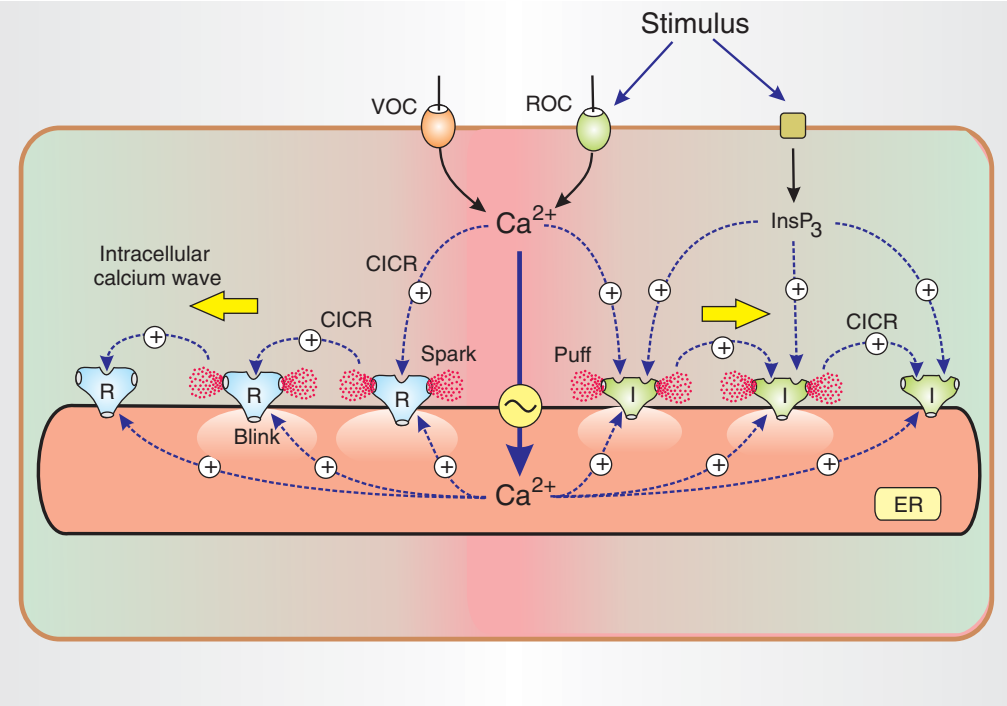
normal circumstances, however, the fully energized OFF reactions can rapidly reduce the pulse of Ca^{2+} introduced by the ON mechanisms, thus generating a brief Ca^{2+} transient (Module 2: Figure Ca^{2+} transient mechanisms). Such brief pulses of Ca^{2+} are a characteristic feature of the Ca^{2+} signalling pathway and contribute to the spatiotemporal aspects of Ca^{2+} signalling.

Ca^{2+} buffers

Cells express a large number of Ca^{2+}-binding proteins, which fall into two main groups: Ca^{2+} sensors and Ca^{2+} buffers. The Ca^{2+} sensors respond to changes in intracellular Ca^{2+} by activating some downstream effector process. In a sense, all proteins capable of binding Ca^{2+} will act as a buffer, and this applies to the sensors. However, the concentration of these sensors is usually rather low, so they have little buffering capacity. The role of Ca^{2+} buffering is carried out by the other major group of Ca^{2+}-binding proteins. Cells have a number of buffers (Module 2: Table Ca^{2+} signalling toolkit) that are capable of binding to Ca^{2+} both in the cytoplasm and within the lumen of the endoplasmic reticulum (ER). The sole function of these Ca^{2+} buffers is to bind Ca^{2+}, and this has an important function in shaping both the spatial and temporal properties of Ca^{2+} signals.

The major cytosolic buffers in cells are parvalbumin (PV), calbindin D-28k (CB) and calretinin (CR). The major buffers that operate within the lumen of the ER

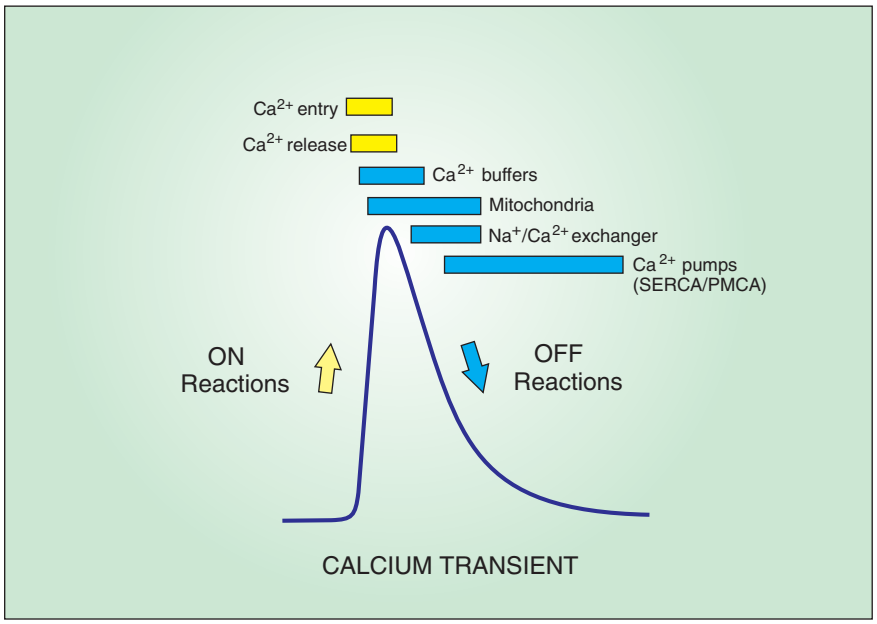
Module 2: | Figure Ca^{2+}-induced Ca^{2+} release



The role of Ca^{2+}-induced Ca^{2+} release (CICR) in mobilizing Ca^{2+} from internal stores.

The Ca^{2+}-sensitive channels are the ryanodine receptors (R) and the $InsP_3$ receptors (I) located on the endoplasmic reticulum (ER). CICR has two important functions. Firstly, it provides a mechanism for transferring information from the plasma membrane to these internal release channels. The primary mechanism is based on the voltage-operated channels (VOCs) that open in response to membrane depolarization to allow a small amount of Ca^{2+} to enter, which then diffuses into the cell to activate either R or I. The other important function of CICR is to link together these intracellular channels so that the Ca^{2+} being released from one channel diffuses across to neighbouring channels that are excited to release further Ca^{2+}, thereby setting up regenerative waves (yellow arrows).

Module 2: | Figure Ca^{2+} transient mechanisms



The sequence of ON and OFF reactions during the generation of a typical Ca^{2+} transient.

The rising phase of the Ca^{2+} spike results from the activation of Ca^{2+} entry and release mechanisms (yellow bars), which are then terminated by inactivation processes. Once the ON reactions have been inactivated, a series of OFF reactions operate in a sequential manner to restore Ca^{2+} to its resting level (blue bars). During the rising phase of the Ca^{2+} transient, large amounts of Ca^{2+} are rapidly bound to the Ca^{2+} buffers (calbindin D-28k and parvalbumin) and are taken up by the mitochondria. The mitochondria and cytosolic buffers help to shape the Ca^{2+} signal by reducing the impact of the ON reactions. In effect, they enable the cell to generate very fast transients without running the risk of being overwhelmed by Ca^{2+}.

are calsequestrin (CSQ) in the sarcoplasmic reticulum of muscle cells and calreticulin (CRT) in the ER of non-muscle cells. The latter is unusual in that it functions both as a cytosolic and a luminal buffer. The role of calsequestrin is discussed elsewhere and here we concentrate on the function of the cytosolic buffers. The latter have subtly different Ca^{2+}-binding properties and are expressed in cells in differing combinations and concentrations to create Ca^{2+} signals that are tailored to carry out different functions. For example, neurons such as Purkinje cells express large amounts of PV and CB. As a consequence, Purkinje cells have a large endogenous Ca^{2+} buffering capacity, e.g. their buffers bind approximately 2000 Ca^{2+} ions for each free ion. Lower capacities of 50–100:1 are found in other cells. Motor neurons have a very low buffering capacity and consequently have large Ca^{2+} signals in both the soma and dendrites during normal physiological responses, and this makes them particularly susceptible to neurodegeneration. Buffer concentration is one of the important parameters in determining buffer capacity. The other key parameters include affinity for Ca^{2+} and other metal ions, the kinetics of Ca^{2+} binding, release and mobility.

Alterations in Ca^{2+} buffers have been linked to schizophrenia.

Parvalbumin (PV)

Parvalbumin (PV) is a slow-onset buffer. It has relatively low on and off rates, which means that it cannot respond to the rapid onset of most Ca^{2+} signals. However, PV can soak up Ca^{2+} once the signal has appeared and thus influences the rate at which Ca^{2+} recovers. It is strongly expressed in skeletal muscle, where it plays an important role in facilitating the rate of relaxation. In $PV^{-/-}$ mice, there is a slowing in the recovery of the Ca^{2+} transient. Some of the effects of removing PV are compensated for by an increase in the volume of mitochondria that has a similar ability to PV of removing free Ca^{2+} from the cytoplasm during the recovery phase.

Reductions in the level of PV have been recorded in schizophrenia.

Calbindin D-28k (CB)

Calbindin D-28k (CB) is one of the major cytosolic buffers, particularly in neurons. It is a fast buffer that can have a major effect on both the spatial and temporal properties of Ca^{2+} transients. CB thus plays a major role in restricting the size of the elementary Ca^{2+} events that form around Ca^{2+} channels. CB is thus of central importance for the ability of neurons to create the highly localized Ca^{2+} events that occur in spines. The existence of these buffers has enabled neurons to increase the numbers of their synaptic connections, and this neuronal miniaturization greatly enhances the signal processing capacity of the brain. Some support for such a notion emerged from the finding of a remarkable compensatory mechanism whereby the volume of the spines increases markedly in neurons when these buffers are knocked out. In effect, the lack of these buffers reduces the capacity of neurons to miniaturize the Ca^{2+} component of their signalling systems.

The ability of CB to buffer internal Ca^{2+} may play an important role in facilitating Ca^{2+} reabsorption by the kidney tubule (Module 7: Figure kidney Ca^{2+} reabsorption). In the case of Ca^{2+} reabsorption by the intestine, the flux of Ca^{2+} is facilitated by calbindin D-9k (Module 7: Figure intestinal Ca^{2+} reabsorption).

An important aspect of the calcium hypothesis of Alzheimer's disease is that there is a decrease in the expression of CB, which increases the sensitivity of neurons to the enhanced Ca^{2+} signals that arise during the onset of Alzheimer's disease (Module 12: Figure amyloid cascade hypothesis).

Calretinin (CR)

Calretinin (CR) is an EF-hand Ca^{2+}-binding protein that is closely related to calbindin D-28k (CB). It has six EF-hand domains all of which can bind Ca^{2+} except for domain VI. CR is usually located in the cytoplasm, but can also be found localized to specific sites in the cell where it may function as a buffer to regulate microdomains of Ca^{2+}. This Ca^{2+} buffer is an excellent biochemical marker for certain GABAergic inhibitory interneurons such as the Cajal–Retzius neurons and double bouquet neurons in the dorsolateral prefrontal cortex (DLPFC) (Module 10: Figure dorsolateral prefrontal cortex).

Calreticulin (CRT)

Calreticulin (CRT) is a low-affinity Ca^{2+}-binding protein that is located within the lumen of the endoplasmic reticulum (ER). It has also been detected in the nucleus and cytoplasm and it may also be secreted into the extracellular environment during periods of cell stress. Its primary location, however, is within the lumen of the ER, where it functions both as a chaperone protein and as the major ER Ca^{2+} buffer. CRT has three main domains; there is a globular N-domain of unknown function followed by a proline-rich P-domain, which has sequences unique to CRT and its homologous proteins calnexin and calmegin. Finally, there is a highly acidic C-terminal domain, which has a large number of low-affinity Ca^{2+}-binding sites capable of binding 20–30 mol of Ca^{2+}/mol of protein. This C-terminal region terminates in the KDEL sequence responsible for retaining CRT in the lumen of the ER.

The chaperone function of CRT is carried out in conjunction with a related chaperone calnexin. A calnexin/calreticulin cycle ensures the correct folding and subunit assembly of glycoproteins and is thus essential for protein trafficking and secretion. The proper folding and assembly of proteins is very dependent on a constant level of Ca^{2+} within the ER lumen and the chaperones. The ability of CRT to bind large amounts of Ca^{2+} enables it to function as an ER Ca^{2+} buffer to help maintain this constancy of ER Ca^{2+}. In addition to functioning as a passive buffer, CRT may play a more direct role in maintaining homoeostatic control of their working environment by modulating the activity of the Ca^{2+} channels and pumps. When the Ca^{2+} level within the lumen gets too

high, CRT and calnexin inhibit the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump and, when Ca^{2+} is released, this inhibition is relieved and the SERCA pumps can restore Ca^{2+} to its normal level. In addition to its role as an ER buffer, CRT may play an active role in ensuring that the Ca^{2+} concentration within the ER lumen is maintained at the optimal level for protein folding to occur.

Ca^{2+} signalling function

The function of Ca^{2+} as an intracellular second messenger is carried out by a combination of Ca^{2+} sensors and Ca^{2+} effectors (Module 2: Figure Ca^{2+} signalling toolkit). The major sensors are the EF-hand proteins troponin C (TnC), calmodulin (CaM), neuronal Ca^{2+} sensor proteins (NCS) and the S100 proteins. However, there are a number of other sensors (Module 2: Table Ca^{2+} signalling toolkit).

These sensors are then responsible for relaying information through a range of effectors:

- Ca^{2+}-sensitive K^+ channels
- Ca^{2+}-sensitive Cl^- channels (CLCAs)
- Ca^{2+}/calmodulin-dependent protein kinases (CaMKs)
- Calcineurin
- Phosphorylase kinase
- Myosin light chain kinase (MLCK)
- Ca^{2+}-promoted Ras inactivator (CAPRI) (Module 2: Figure Ras signalling)

Spatiotemporal aspects of Ca^{2+} signalling

The use of Ca^{2+} as a universal signal for cell regulation is somewhat paradoxical because this ion can be very toxic to cells if its level remains high for a prolonged period. Such toxicity is avoided by presenting Ca^{2+} signals in a pulsatile manner (Module 2: Figure temporal aspects). In addition to this temporal aspect, the Ca^{2+} signal is also highly organized in space. The elementary and global aspects of Ca^{2+} signalling greatly increase the versatility of the Ca^{2+} signalling system in that it can act either locally or globally. For example, muscle contraction is activated by a global elevation in Ca^{2+}, whereas the release of neurotransmitters results from a minute punctate pulse of Ca^{2+} delivered directly to the docked vesicle by a Ca^{2+} sensor tightly associated with exocytotic machinery (Module 4: Figure Ca^{2+}-induced membrane fusion). In between these two extremes, there are many variations in the way the Ca^{2+} signal is presented to cells. Perhaps the most dramatic are the Ca^{2+} waves that initiate at fixed localities and then process through the cytoplasm in a regenerative manner through the process of CICR (Module 2: Figure Ca^{2+}-induced Ca^{2+} release).

These spatiotemporal aspects greatly enhance the versatility of Ca^{2+} signalling, thus providing the flexibility to regulate so many cellular processes.

Cyclic ADP-ribose (cADPR) signalling

Cyclic ADP-ribose (cADPR) is one of the messengers associated with the NAD^+ signalling pathways. cADPR has attracted considerable attention as a putative messenger to regulate the Ca^{2+} signalling pathway. cADPR generation and metabolism is unusual in that it is carried out by the same enzyme that possesses both synthase and hydrolase activity. The cADPR control of Ca^{2+} release is somewhat controversial, as its precise mode of action is still unclear. A cADPR working hypothesis has been put forward to provide a framework to understand some of the current information on how this messenger appears to operate. A relationship between cADPR and cell regulation has been established in a number of different cell types.

cADPR working hypothesis

There is sufficient evidence to take seriously the possibility that cADPR functions to regulate Ca^{2+} signalling. What seems to be in question is exactly how it functions. This working hypothesis has two main components:

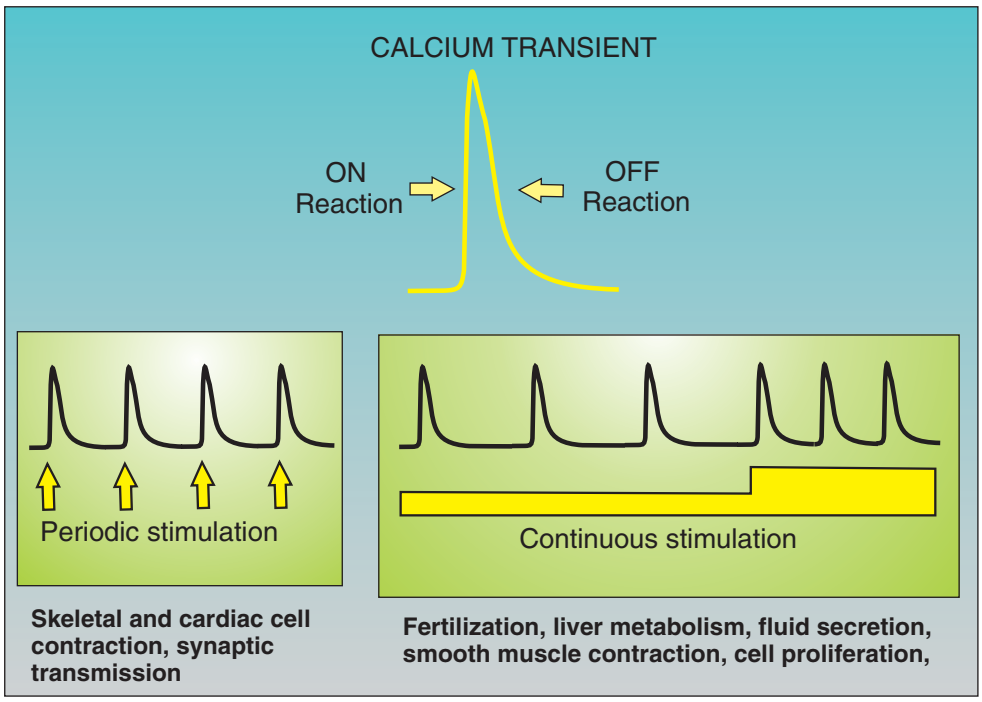
- Firstly, the generation of cADPR is closely coupled to cellular metabolism, as described in cADPR generation and metabolism (Module 2: Figure cADPR/NAADP function). The idea is that cADPR may function as a metabolic messenger responsible for relaying information about the state of metabolism to various systems in the cell, especially those that require a heavy expenditure of energy. An example would be Ca^{2+} signalling and the downstream elements regulated by Ca^{2+}. When energy is abundant, an increase in cADPR will set the stage for Ca^{2+} signalling to occur.
- Secondly, the cADPR control of Ca^{2+} release may occur indirectly through an activation of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump to increase the uptake of Ca^{2+} into the endoplasmic reticulum/sarcoplasmic reticulum. This increased loading of the internal store will serve to sensitize release channels such as the RYRs, thus leading to an increase in Ca^{2+} signalling. This messenger should thus be considered as a modulator rather than a mediator of Ca^{2+} signalling.

cADPR generation and metabolism

The generation and metabolism of cADPR are described together because both processes are carried out by the same enzyme: the ADP-ribosyl cyclase (Module 2: Figure cADPR metabolism). The hydrolysis of cADPR produces ADPR, which has been implicated as a messenger regulating melastatin-related transient receptor potential 2 (TRPM2), which is a Ca^{2+} channel in the plasma membrane (Module 2: Figure cADPR/NAADP function). This is a highly versatile enzyme. In addition to synthesizing and metabolizing cADPR, it is also responsible for synthesizing NAADP. In mammals, this bifunctional enzyme appears to be the lymphocyte antigen CD38, which is expressed widely and is located both in the plasma membrane and at internal sites. With regard to the former location, its enzymatic region is located on the outside. This is unusual because its substrate and its site of action are on the inside of the cell. It has been suggested that the enzyme might use NAD^+ derived from dying cells at sites of infections to generate cADPR, which is then transported into the cell.

The intracellular enzyme is more likely to be the one that functions in most cells. Just how the cyclase is

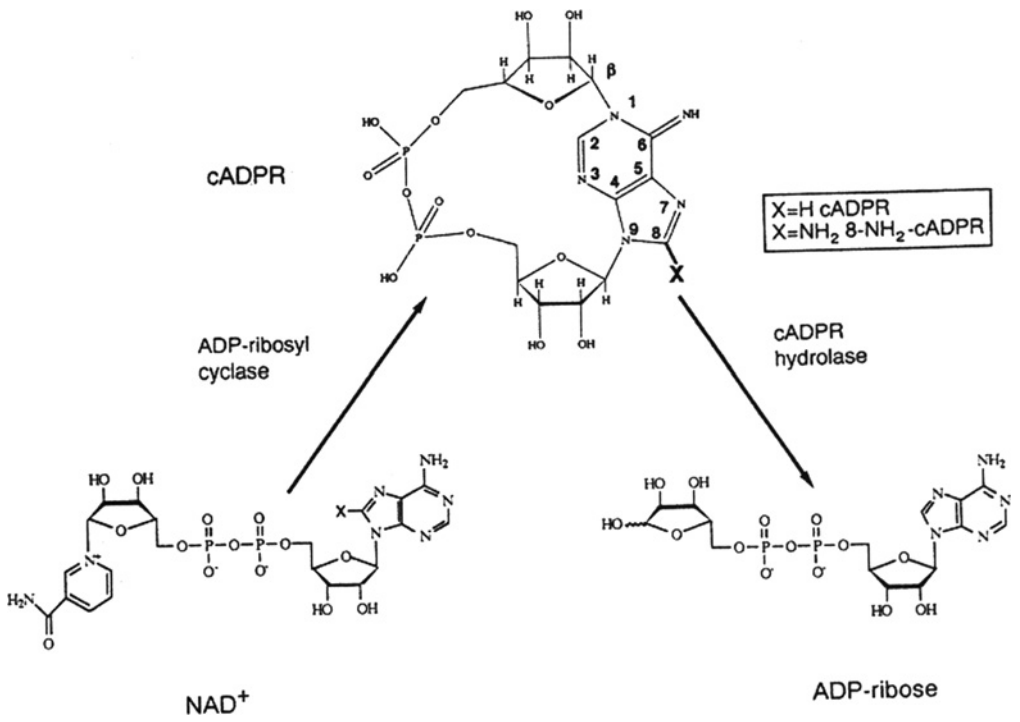
Module 2: | Figure temporal aspects



Temporal aspects of Ca^{2+} signalling.

In almost every example where Ca^{2+} is used as a signal, it is presented as a brief transient, which is the digital signal used to set up complex temporal patterns of Ca^{2+} signalling. In some cells, these pulses are produced on demand in that they are generated in response to periodic stimulation (blue arrows) as occurs in muscle contraction where a brief burst of Ca^{2+} activates the contractile machinery, which then recovers when the Ca^{2+} signal is removed. Likewise, the release of neurotransmitters from nerve terminals is triggered by a brief localized pulse of Ca^{2+}. In many other tissues, which receive a continuous stimulation over a prolonged period (blue bar), the Ca^{2+} signal is again presented as brief spikes that are produced rhythmically to give highly regular Ca^{2+} oscillations whose frequencies vary with the level of cell stimulation.

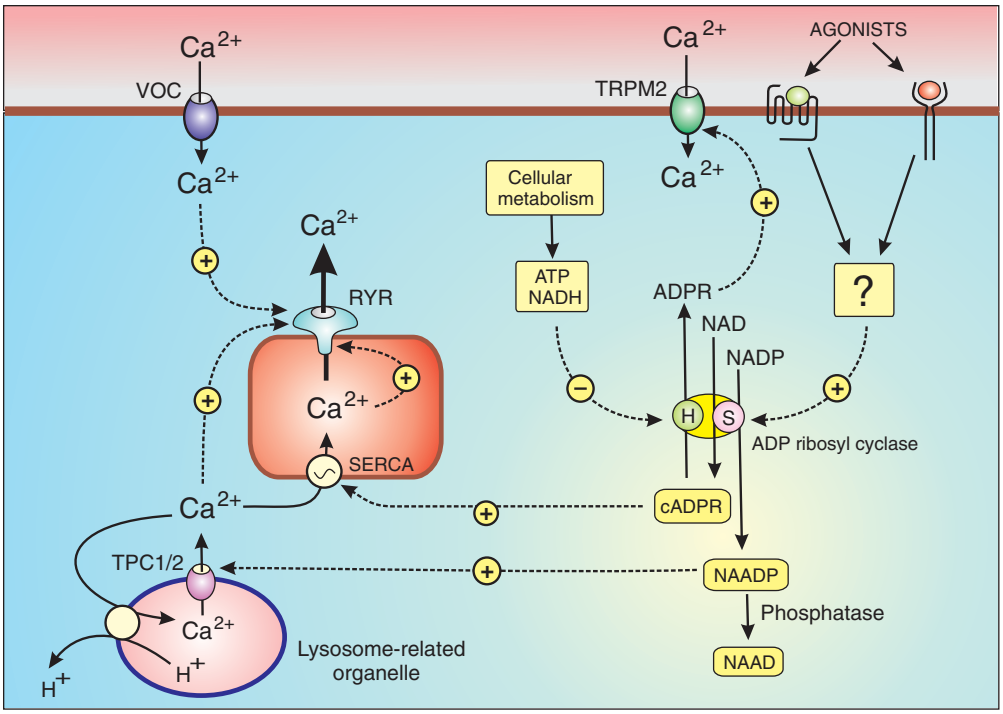
Module 2: | Figure cADPR metabolism



The generation and metabolism of cyclic ADP-ribose (cADPR).

The same enzyme ADP-ribosyl cyclase is responsible for both forming and degrading cADPR. The precursor NAD^+ is converted into cADPR by the cyclase activity, whereas the hydrolase component inactivates cADPR by hydrolysing it to ADP-ribose (ADPR). Reproduced from Trends Cell Biol., Vol. 4, Galione, A. and White, A., Ca^{2+} release induced by cyclic ADP-ribose, pp. 431-436. Copyright (1994), with permission from Elsevier; see Galione and White (1994).

Module 2: | Figure cADPR/NAADP function



Synthesis and mode of action of cADPR and NAADP.
The enzyme ADP-ribosyl cyclase is a bifunctional enzyme that has a synthase (S) component that synthesizes cADPR and NAADP from the precursors NAD^+ and NADP respectively, but it also has a hydrolase (H) activity that converts cADPR into ADPR. This hydrolase is sensitive to metabolism because it is inhibited by either ATP or NADH. The cADPR may act by stimulating the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump to increase the uptake of Ca^{2+} into the endoplasmic reticulum. NAADP acts on a channel to release Ca^{2+} from a lysosome-related organelle.

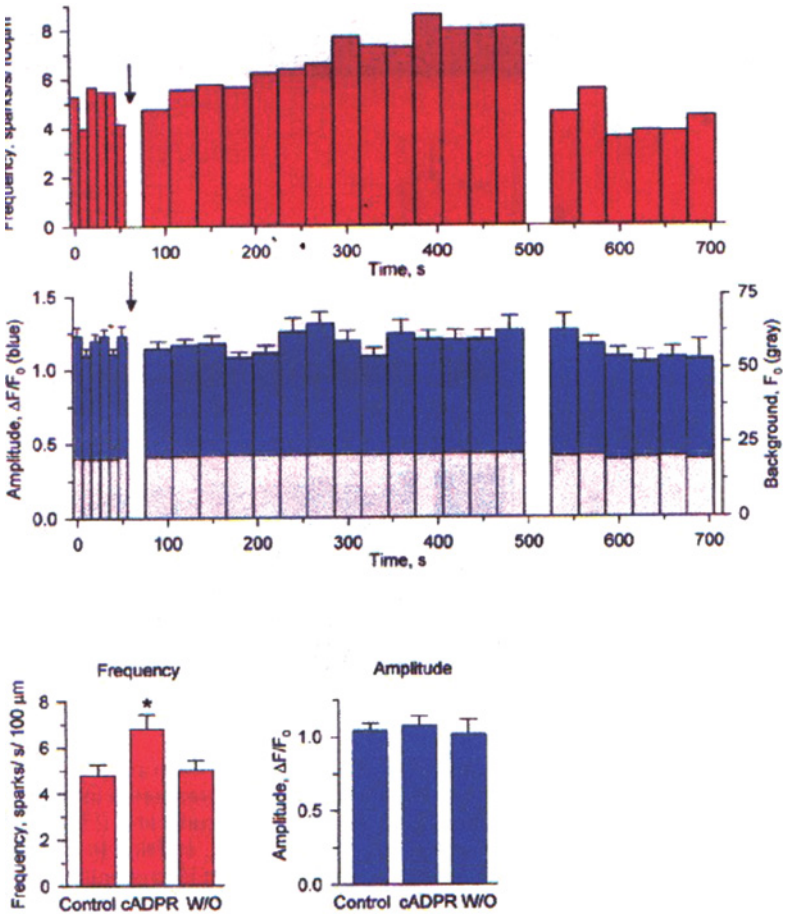
activated is still unclear. One suggestion is that the formation of cADPR and NAADP is sensitive to cellular metabolism (Module 2: Figure cADPR/NAADP function). In other words, cADPR and NAADP might be metabolic messengers that are capable of relaying information about the state of cellular metabolism to the Ca^{2+} signalling pathways. Such a notion is supported by the fact that cADPR metabolism by the hydrolase is inhibited by either ATP or NADH. Another suggestion is that it might be activated by agonists acting through cell-surface receptors, but the coupling mechanism remains to be established (Module 2: Figure cADPR/NAADP function). This absence of a coupling mechanism might be explained by the cADPR working hypothesis if the external agonist enhanced cADPR formation indirectly by first increasing cellular metabolism. Such a mechanism could explain the ability of β-adrenergic agents to increase cADPR levels in heart. Likewise, the glucose-dependent increase in cADPR in β-cells can be directly linked to the metabolism of glucose, with the resulting increase in ATP acting to reduce the hydrolase activity (Module 2: Figure cADPR/NAADP function).

In $CD38^{-/-}$ mice, there is a decrease in the amount of oxytocin (OT) released from hypothalamic neurons. Such mice show defects in maternal nurturing and in social behaviour.

cADPR control of Ca^{2+} release

One of the major uncertainties about cADPR is its mode of action in controlling the release of Ca^{2+}. There have been suggestions that it is a Ca^{2+}-mobilizing second messenger that acts by stimulating the ryanodine receptors (RYRs) to release Ca^{2+}. However, direct evidence for this assertion is not particularly convincing. Early single channel recordings seemed to provide such evidence by showing that cADPR could open RYRs in lipid membranes, but these observations were challenged on the basis that the cADPR was acting through the ATP-binding site. When cADPR is injected into cells, it usually fails to release Ca^{2+}, but after a period of time, it can begin to enhance the sensitivity of the RYRs. An example of such an effect is shown in heart cells, where there is a gradual increase in spark frequency following addition of cADPR (Module 2: Figure cADPR action in heart cells). This observation on cardiac cells forms the basis of the second part of the cADPR working hypothesis, which argues that cADPR acts indirectly as a modulator of Ca^{2+} release (Module 2: Figure cADPR/NAADP function) by stimulating the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump to increase the load of Ca^{2+} within the lumen of the store. This increase in luminal Ca^{2+} then sensitizes the RYRs so that they either begin to open spontaneously to give Ca^{2+} sparks (Module 2: Figure cADPR action in heart cells) or begin to

Module 2: | Figure cADPR action in heart cells



Effect of cADPR on spark frequency in heart cells.
When permeabilized rat ventricular myocytes were treated with 5 μM cADPR, there was a gradual increase in spark frequency that recovered to the resting level when it was washed off (wo). There was no change in spark amplitude. This increase in spark frequency was shown to depend upon sensitization of the RYRs due to an increase in the luminal load of Ca^{2+} resulting from an increase in the activity of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump. Reproduced from Lukyanenko, V., Györke, I., Wiesner, T.F. and Györke, S. (2001) Potentiation of Ca^{2+} release by cADP-ribose in the heart is mediated by enhanced SR Ca^{2+} uptake into the sarcoplasmic reticulum. Circ. Res. 89:614-622, with permission from Lippincott Williams & Wilkins (http://lww.com); see Lukyanenko et al. 2001.

produce larger Ca^{2+} transients when activated by a pulse of trigger Ca^{2+} as occurs in neurons (Module 2: Figure cADPR action in neurons).

cADPR and cell regulation

A role for cADPR has been implicated in a number of different cell types:

- Insulin-secreting β-cells (Module 7: Figure β-cell signalling)
- Hypothalamic neurons: in $CD38^{-/-}$ mice, there is a decrease in the amount of oxytocin (OT) released from hypothalamic neurons. Such mice show defects in maternal nurturing and in social behaviour.

Nicotinic acid–adenine dinucleotide phosphate (NAADP) signalling

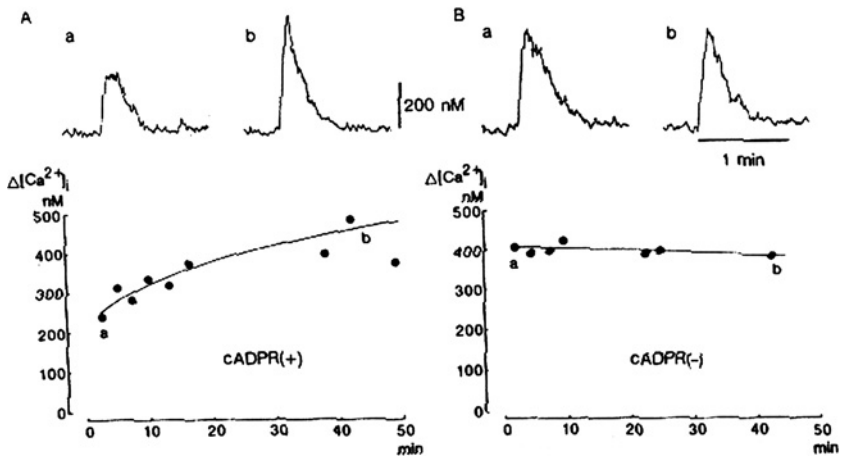
NAADP is one of the messengers associated with the NAD signalling pathways. NAADP has attracted considerable attention as a Ca^{2+}-mobilizing second messenger. NAADP generation and metabolism is unusual in that it is closely allied to that of cADPR in that the same enzyme synthesizes both messengers. The NAADP control of Ca^{2+} release depends upon this messenger acting on a store that is different from that controlled by the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) or the ryanodine receptors (RYRs). A relationship between NAADP and cell regulation seems to depend upon its interaction with the other Ca^{2+}-mobilizing messenger systems.

NAADP generation and metabolism

NAADP (Module 2: Figure NAADP structure) is another member of the NAD signalling pathways. Its synthesis from NADP is closely linked to that of cADPR in that they both share the same enzyme: the ADP-ribosyl cyclase (Module 2: Figure cADPR/NAADP function). This enzyme uses NAD^+ to make cADPR through a cyclization reaction, or it can use NADP as a substrate to produce NAADP through a base-exchange reaction during which the nicotinamide group is exchanged for nicotinic acid.

Unlike cADPR, which is hydrolysed by the same enzyme, NAADP is degraded to NAAD by phosphatases such as alkaline phosphatase.

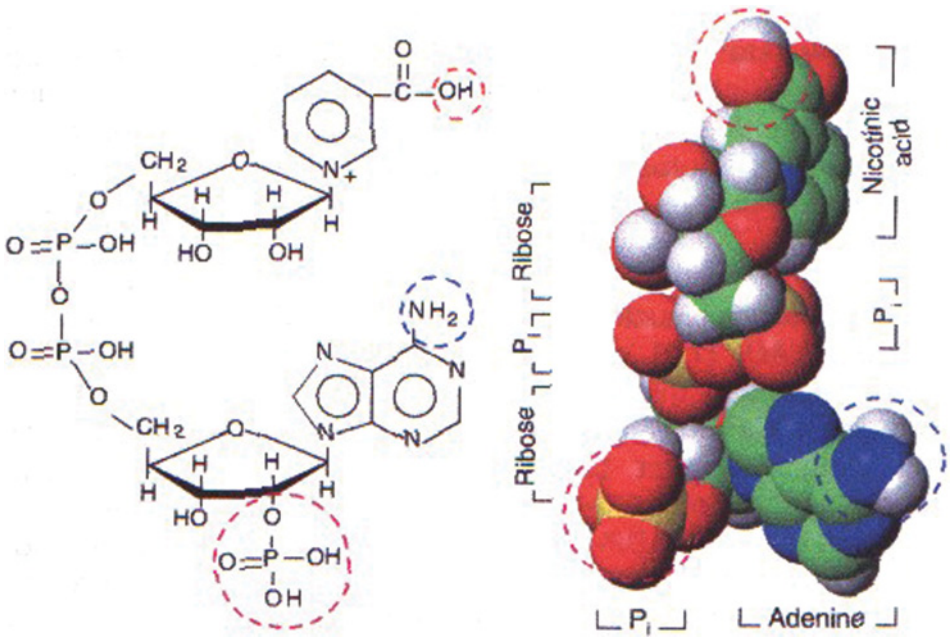
Module 2: | Figure cADPR action in neurons



cADPR potentiates Ca^{2+} transients induced by action potentials in sympathetic neurons.

When sympathetic neurons are stimulated for 10 ms at 20 Hz, there is a distinct transient made up of Ca^{2+} entering through voltage-operated channels (VOCs) together with release of Ca^{2+} from internal stores through a process of Ca^{2+}-induced Ca^{2+} release (CICR). The latter component is quite variable and depends upon the sensitivity of the ryanodine receptors (RYRs). When the neurons are perfused with cyclic ADP-ribose (cADPR), there is a large increase in the amplitude of the transient (compare responses a and b in panel A). Note how the increase developed slowly over the recording period. There was no augmentation when the neurons were perfused with control solution (panel B). Reproduced from Neuron, Vol. 12, Hua, S.-Y., Tokimasa, T., Takasawa, S., Furuya, S., Nohmi, M., Okamoto, H. and Kuba, K., Cyclic ADP-ribose modulates Ca^{2+} release channels for activation by physiological Ca^{2+} entry in bullfrog sympathetic neurons, pp. 1073–1709. Copyright (1994), with permission from Elsevier; see Hua et al. 1994.

Module 2: | Figure NAADP structure



The structure of NAADP.

The space-filling model on the right illustrates the molecular organization of the different components of NAADP. Reproduced from Curr. Biol., Vol. 13, Lee, H.C., Ca^{2+} signalling: NAADP ascends as a new messenger, pp. R186-R188. Copyright (2003), with permission from Elsevier; see Lee 2003.

NAADP control of Ca^{2+} release

NAADP functions as a Ca^{2+}-mobilizing messenger to release Ca^{2+} from an internal store (Module 2: Figure cADPR/NAADP function). This NAADP-sensitive store, which is distinct from the endoplasmic reticulum/sarcoplasmic reticulum store that is regulated by the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) and the ryanodine receptors (RYRs), is still being defined. On the basis of studies carried out in sea urchin eggs, this novel store appears to reside in a lysosome-related organelle. The receptors for NAADP appear to be a family of two-pore channels (TPC), with TPC1 being located on the endosomal membranes, whereas TPC2 is on lysosomal membranes.

NAADP and cell regulation

The amount of Ca^{2+} released by NAADP is relatively small and is unlikely to have a direct role in Ca^{2+} signalling. However, there are suggestions that it may act indirectly to trigger the release of Ca^{2+} by the other release channels [ryanodine receptors (RYRs) or the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) (Module 2: Figure cADPR/NAADP function)].

NAADP has been implicated in the control of insulin release by β-cells (Module 7: Figure β-cell signalling) and in the control of secretion by pancreatic acinar cells (Module 7: Figure control of pancreatic secretion).

ADP-ribosyl cyclase

ADP-ribosyl cyclase is the enzyme responsible for synthesizing the Ca^{2+} mobilizing messengers cADPR and NAADP. During cADPR generation and metabolism it uses NAD^+ to make cADPR through a cyclization reaction (Module 2: Figure cADPR/NAADP function). The same enzyme is also responsible for the hydrolysis of cADPR to ADPR. This enzyme can also use NADP as a substrate to produce NAADP through a base-exchange reaction during which the nicotinamide group is exchanged for nicotinic acid.

In mammals, this bifunctional enzyme appears to be the lymphocyte antigen CD38, which is expressed widely and is located both in the plasma membrane and at internal sites.

Phosphoinositide signalling

Signalling through the phosphoinositide lipids is complex because there are a number of signalling cassettes associated with both the synthesis and hydrolysis of the phosphoinositides. Phosphoinositide metabolism can be divided into two main parts. Firstly, there is inositol lipid metabolism, which describes the way in which the parent molecule PtdIns is metabolized to form a number of lipid intermediates, some of which are key elements in different signalling cassettes. Secondly, there is inositol phosphate metabolism, which is a complex pathway responsible for metabolizing soluble inositol phosphates. Some of the inositol phosphates generated by this metabolism have been implicated as messengers operating within the multipurpose inositol polyphosphate signalling pathway.

$PtdIns4,5P_2$ is of particular interest because it is the precursor used to generate the second messengers inositol 1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG) that function in the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette and the diacylglycerol (DAG)/protein kinase C (PKC) signalling cassette respectively. $PtdIns4,5P_2$ is also a precursor that is metabolized to $PtdIns3,4P_2$ and $PtdIns3,4,5P_3$, which function as second messengers to activate protein kinases in the PtdIns 3-kinase signalling pathway. In addition to being a precursor for these various signalling pathways, $PtdIns4,5P_2$ can also function as a messenger for the $PtdIns4,5P_2$ signalling cassette, where localized synthesis or hydrolysis of this lipid functions to regulate a number of cellular systems such as the actin cytoskeleton, membrane trafficking, exocytosis, ion channels and exchangers.

Alterations in the activity of this phosphoinositide lipid signalling pathway have been implicated in various diseases such as manic-depressive illness, Lowe's oculocerebrorenal (OCRL) syndrome and Cowden's disease.

Phosphoinositide metabolism

Phosphoinositide metabolism can be separated into two main components (Module 2: Figure phosphoinositide metabolism):

- Inositol lipid metabolism concerns the pathways responsible for converting PtdIns into a variety of phosphoinositide lipid signalling molecules.
- Inositol phosphate metabolism is responsible for creating a large array of inositol phosphates, some of which function in the multipurpose inositol polyphosphate signalling pathway. This metabolic pathway also forms the inositol that is used to resynthesize the lipid precursor PtdIns.

These two pathways are connected by the processes of lipid hydrolysis and lipid synthesis. The hydrolysis occurs when external signals activate phospholipase C (PLC) to hydrolyse $PtdIns4,5P_2$ to form the second messengers inositol 1,4,5-trisphosphate ($Ins1,4,5P_3$) and diacylglycerol (DAG). The $Ins1,4,5P_3$ is one of the major inputs into the inositol phosphate metabolic pathway that produces the inositol required for lipid synthesis.

This complex phosphoinositide metabolic network participates in a number of highly versatile signalling cassettes (Module 2: Figure phosphoinositide signalling systems):

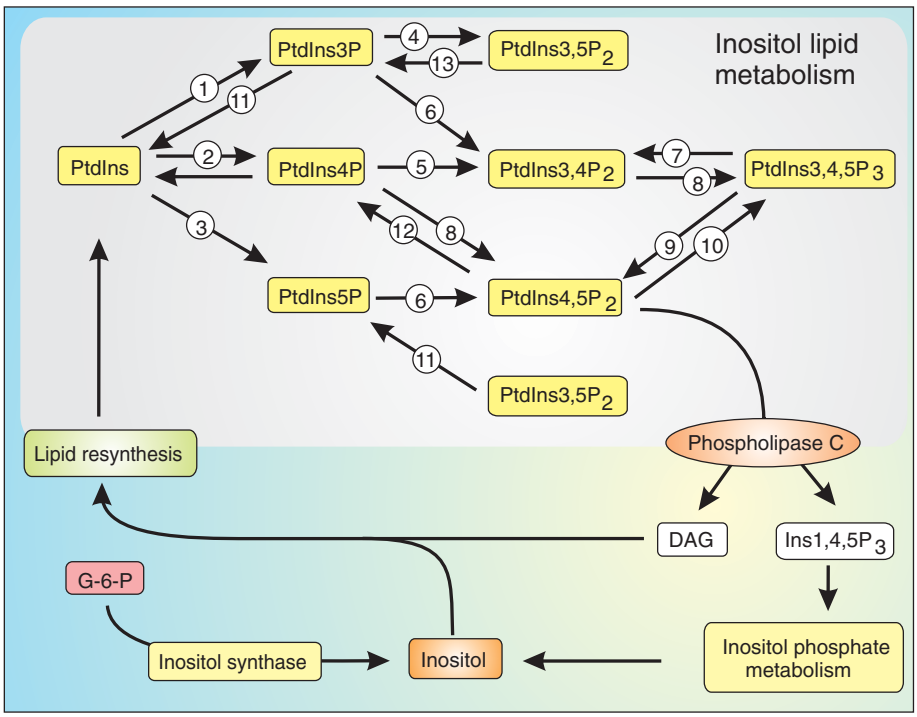
- Inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette
- Diacylglycerol (DAG)/protein kinase C (PKC) signalling cassette
- PtdIns 3-kinase signalling
- $PtdIns4,5P_2$ signalling cassette
- Multipurpose inositol polyphosphate signalling pathway

Inositol lipid metabolism

The different phosphoinositide signalling pathways are derived from the parent molecule phosphatidylinositol (PtdIns) (Module 2: Figure PtdIns structure). Unlike the other phospholipids found in cellular membranes, PtdIns is unique in that the free hydroxy groups at the 3-, 4- and 5-positions on the inositol ring can be phosphorylated further to create a family of phosphoinositides (Module 2: Figure phosphoinositide metabolism). One of the more important lipids involved in phosphoinositide signalling is $PtdIns4,5P_2$, which is a nodal point for a number of signalling systems (Module 2: Figure phosphoinositide signalling systems).

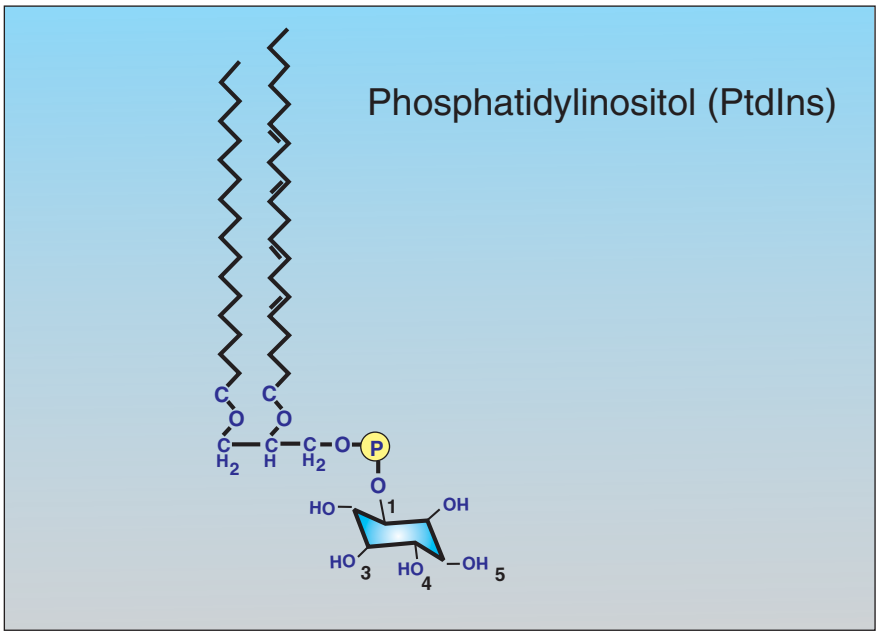
The metabolism of PtdIns is carried out by a collection of inositol lipid kinases and inositol lipid phosphatases. The numbers below refer to the different reactions shown in Module 2: Figure phosphoinositide metabolism:

Module 2: | Figure phosphoinositide metabolism



Summary of phosphoinositide metabolism.
The metabolism of phosphoinositides is separated into inositol lipid metabolism and inositol phosphate metabolism. In the former case, the parent molecule phosphatidylinositol (PtdIns) (Module 2: Figure PtdIns structure) undergoes a series of phosphorylation reactions to create various polyphosphoinositides many of which have messenger functions. For example, PtdIns4,5P_2 functions both as a messenger and as a precursor for the formation of other phosphoinositide lipid signalling molecules such as PtdIns3,4,5P_3. In addition, it can be hydrolysed to form the second messengers inositol 1,4,5-trisphosphate (Ins1,4,5P_3) and diacylglycerol (DAG). The Ins1,4,5P_3 is the major input into a complex pathway of inositol phosphate metabolism (Module 2: Figure inositol phosphate metabolism). One of the outputs of this metabolism is inositol that is used for the resynthesis of PtdIns. Cells also have a 1-L-*myo*-inositol-1-phosphate synthase that can convert glucose 6-phosphate (G-6-P) into inositol.

Module 2: | Figure PtdIns structure



Molecular structure of PtdIns.
The backbone of the molecule consists of a glycerol moiety. Two of the carbons carry fatty acids that insert into the hydrophobic domain of the membrane, whereas the remaining carbon is attached to a phosphate group that is linked to an inositol ring that projects out into the cytosol. The hydroxy groups on the 3-, 4- and 5-positions can be phosphorylated further (see Module 2: Figure phosphoinositide metabolism)

Module 2: | Table inositol lipid kinases and phosphatases
Inositol lipid kinases and phosphatases

	Substrate	Product	Comments
Inositol lipid kinases			
Class III PtdIns 3-kinase	PtdIns	PtdIns3P	Also known as hVps34 (Step 1 in Module 2: Figure phosphoinositide metabolism). Functions in protein trafficking (Module 2: Figure localized inositol lipid signalling)
Phosphatidylinositol 4-kinase (PtdIns 4-K)			Step 2 in Module 2: Figure phosphoinositide metabolism.
PtdIns 4-KIIα	PtdIns	PtdIns4P	
PtdIns 4-KIIβ	PtdIns	PtdIns4P	
PtdIns 4-KIIIα		PtdIns4P	
PtdIns 4-KIIIβ		PtdIns4P	
Phosphatidylinositol 5-kinase (PtdIns 5-K)	PtdIns	PtdIns5P	Step 3 in Module 2: Figure phosphoinositide metabolism.
Class II PtdIns 3-kinase	PtdIns	PtdIns3P	Step 5 in Module 2: Figure phosphoinositide metabolism.
	PtdIns4P	PtdIns3,4P_2	
Phosphatidylinositol4P 5-kinase (PtdIns4P 5-K) Type I			Step 8 in Module 2: Figure phosphoinositide metabolism
PtdIns4P 5-KIα	PtdIns4P	PtdIns4,5P_2	.
PtdIns4P 5-KIβ	PtdIns4P	PtdIns4,5P_2	
PtdIns4P 5-KIγ	PtdIns4P	PtdIns4,5P_2	
PhosphatidylinositolP kinase (PtdInsP 4-K) Type II	PtdIns3P	PtdIns3,4P_2	Step 6 in Module 2: Figure phosphoinositide metabolism.
	PtdIns5P	PtdIns4,5P_2	
PhosphatidylinositolP 5-kinase (PIKfyve) Type III	PtdIns3P	PtdIns3,5P_2	Step 4 in Module 2: Figure phosphoinositide metabolism. Functions in protein trafficking (Module 2: Figure localized inositol lipid signalling). Mutations of this kinase are associated with Francois-Neetens mouchetee fleck corneal dystrophy (CFD)
Class I PtdIns 3-kinases			Step 10 in Module 2: Figure phosphoinositide metabolism.
Class IA PtdIns 3-kinase			
p110α (catalytic subunit)	PtdIns4,5P_2	PtdIns3,4,5P_3	The structure of these lipid kinases is shown in Module 2: Figure PI3-K family
p110β (catalytic subunit)	PtdIns4,5P_2	PtdIns3,4,5P_3	
p110δ (catalytic subunit)	PtdIns4,5P_2	PtdIns3,4,5P_3	
p85α (regulatory subunit)			
p85β(regulatory subunit)			
p55α(regulatory subunit)			
p50(regulatory subunit)			
p55γ(regulatory subunit)			
Class IB PtdIns 3-kinase			
p110γ	PtdIns4,5P_2	PtdIns3,4,5P_3	
p84 (regulatory subunit)			
p101 (regulatory subunit)			
Class II PtdIns 3-kinase			
PtdIns 3KC2α	PtdIns4,5P_2	PtdIns3,4,5P_3	
PtdIns 3KC2β	PtdIns4,5P_2	PtdIns3,4,5P_3	
PtdIns 3KC2γ	PtdIns4,5P_2	PtdIns3,4,5P_3	
Inositol lipid phosphatases			
Src homology 2 (SH2) domain-containing inositol phosphatase (SHIP)			Step 7 in Module 2: Figure phosphoinositide metabolism.
SHIP1	PtdIns3,4,5P_3	PtdIns3,4,P_2	
SHIP2	PtdIns3,4,5P_3	PtdIns3,4,P_2	
Phosphatase and tensin homologue deleted on chromosome 10 (PTEN)	PtdIns3,4P_2	PtdIns4P	Step 9 in Module 2: Figure phosphoinositide metabolism.
	PtdIns3,4,5P_3	PtdIns4,5P_2	This lipid phosphatase is mutated in many human cancers
Myotubularins (MTMR1-MTMR13)			Step 11 in Module 2: Figure phosphoinositide metabolism.
MTM1	PtdIns3	PtdIns	Mutations of MTM1 are responsible for X-linked recessive myotubular myopathy
MTM2	PtdIns3	PtdIns	MTMR2 is mutated in Charcot-Marie-Tooth disease 4B

Module 2: | Table *continued*

	Substrate	Product	Comments
Synaptojanin 1 (SJ1)	PtdIns4,5P_2	PtdIns4P	Step 8 in Module 2: Figure phosphoinositide metabolism.
Synaptojanin 1 (SJ1)	PtdIns4,5P_2	PtdIns4P	Removes protein coats following scission of endocytic vesicles (Module 4: Figure scission of endocytic vesicles)
Oculocerebrorenal syndrome of Lowe (OCRL)	PtdIns4,5P_2	PtdIns4P	Mutations of OCRL cause Lowe's syndrome
	PtdIns3,4,5P_3	PtdIns3,4P_2	
Sac3	PtdIns3,5P_2	PtdIns3P	Mammalian homologue of yeast Fig4 is part of the PAS complex (Module 2: PIKfyve activation)

1. The Class III PtdIns 3-kinase (PtdIns 3-K) adds a phosphate to the 3-position of PtdIns to form PtdIns3P.
2. PtdIns 4-kinase (PtdIns 4-K) adds a phosphate to the 4-position of PtdIns to form PtdIns4P. It can also be formed by removal of the 3-phosphate from PtdIns3,4P_2 by phosphatase and tensin homologue deleted on chromosome 10 (PTEN).
3. The PIKfyve kinase is thought to be responsible for adding a phosphate to the 5-position of PtdIns to form PtdIns5P.
4. The PIKfyve also adds a phosphate to the 5-position of PtdIns3P to form PtdIns3,5P_2. The formation of PtdIns3,5P_2, which changes in cells following osmotic stress, is a key component of the PtdIns3,5P_2 signalling cassette (Module 2: Figure PIKfyve activation).
5. Types I and II PtdIns 3-kinase (PtdIns 3-K) add a phosphate to the 3-position of PtdIns4P to form PtdIns3,4P_2. PtdIns3,4P_2 has been suggested to function as a messenger operating within the plasma membrane, where it serves to recruit and activate protein kinases such as protein kinase B (PKB). PtdIns3,4P_2 can also be formed by other enzymes (see Steps 6 and 7).
6. PtdInsP kinase II adds a phosphate to the 4-position of PtdIns3P to form PtdIns3,4P_2. The same enzyme can add a phosphate to the 4-position of PtdIns5P to form PtdIns4,5P_2.
7. Inositol polyphosphate 5-phosphatase such as Src homology 2 (SH2) domain-containing inositol phosphatase (SHIP) removes a phosphate from the 5-position of PtdIns 3,4,5P_3 to form PtdIns3,4P_2.
8. A PtdIns4P 5-kinase (PtdIns4P 5-K) adds a phosphate to the 5-position of PtdIns4P to form PtdIns4,5P_2. The latter can also be formed by Steps 6 and 9. PtdIns4P5-K can also phosphorylate PtdIns3,4P_2 to form PtdIns3,4,5P_3.
9. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) removes a phosphate from the 3-position of PtdIns3,4,5P_3.
10. A Class I PtdIns 3-kinase adds a phosphate to the 3-position of PtdIns4,5P_2 to form PtdIns3,4,5P_3. PtdIns3,4,5P_3, which functions as one of the second messengers in the PtdIns 3-kinase signalling pathway, can also be formed by the addition of a phosphate to the 5-position of PtdIns3,4P_2 by the PtdIns4P 5-kinase (PtdIns4P 5-K) (i.e. the same enzyme used for Step 8).
11. The myotubularins remove the 3-phosphate from PtdIns3P and also from PtdIns3,5P_2.
12. The 5-phosphate on PtdIns4,5P_2 is removed by various lipid phosphatases such as oculocerebrorenal syndrome of Lowe (OCRL) and the synaptojanins.
13. The 5-phosphate on PtdIns3,5P_2 is removed by the Sac domain phosphatase Sac3, which is an orthologue of yeast Fig 4.

Inositol lipid kinases

There are a number of inositol lipid kinases that function to phosphorylate hydroxyls on the inositol headgroup of phosphatidylinositol.

PtdIns 3-kinase (PtdIns 3-K)

The PtdIns 3-kinases (PtdIns 3-Ks) are a family of enzymes that have been classified into three classes (Module 2: Figure PI 3-K family).

Class I PtdIns 3-kinases

The primary function of the Class I PtdIns 3-kinases is to phosphorylate PtdIns4,5P_2 to form the lipid second messenger PtdIns3,4,5P_3 (Module 2: Figure PtdIns 3-kinase signalling). These Class I enzymes are divided into two groups: five regulatory subunits and three catalytic subunits (Module 2: Figure PI 3-K family).

The Class IA enzymes are heterodimers that are formed from a regulatory subunit combining with a catalytic subunit. There are five regulatory subunits (p85α, p85β, p55α, p50α and p55γ), which are typical adaptor proteins. The p85 regulatory subunit contains an Src homology 3 (SH3) domain, a breakpoint-cluster-region homology (BH) domain, two proline-rich regions (P) on either side of the BH domain and two Src homology (SH2) domains that are separated by a p110-binding domain, enabling them to interact with a p85-binding domain located on the three catalytic subunits (p110α, p110β and p110δ). The different binding domains on the regulatory subunits enhance the versatility of the Class IA enzyme by enabling the catalytic subunit to be activated by interacting with a variety of signalling molecules:

- The Src homology (SH2) domains of the regulatory subunit enable the enzyme to associate with phosphotyrosine residues on the cytoplasmic domains of receptors such as the platelet-derived growth factor receptor (PDGFR) (Module 1: Figure PDGFR activation) or the CD19 B cell co-receptor (Module 9: Figure B cell activation).
- The Src homology 3 (SH3) domain can bind to proline-rich regions of Shc, Cbl and dynamin.
- The proline-rich regions can bind to the SH3 domains of Abl, Src, Lck, Lyn, Fyn and growth factor receptor bound protein 2 (Grb2).
- The p85β inhibitory subunit is a target for miR-126, which plays an important role in the regulation of angiogenesis.

The Class IB enzyme has a different activation mechanism in that it is stimulated by heterotrimeric G proteins (Module 2: Figure PtdIns 3-kinase signalling). It has a p110γ catalytic subunit and two regulatory subunits (p101 and p84) (Module 2: Figure PI 3-K family). These regulatory subunits may mediate the translocation of the p110γ catalytic subunit to the membrane by binding to the $G_{\beta\gamma}$ subunit.

The Class I enzymes interact strongly with the oncogene Ras (Module 2: Figure PtdIns 3-kinase signalling) and this can have important consequences for both upstream and downstream events, depending on the cellular context. With regard to the former, the binding of Ras can enhance enzyme activation and the generation of lipid messengers. On the other hand, the association with Ras might mediate some of the downstream effects of the PtdIns 3-kinases.

Class I PtdIns 3-kinases are strongly inhibited by the fungal metabolite wortmannin (an irreversible inhibitor) and by LY294002 (a reversible inhibitor).

Class II PtdIns 3-kinase

The Class II PtdIns 3-kinase has a more restricted substrate range in that it phosphorylates just PtdIns and PtdIns4P. There are no regulatory subunits, and the three catalytic subunits (PI3KC2α, PI3KC2β and PI3KC2γ) are characterized by having a C-terminal phox homology (PX) domain and a C2 domain (Module 2: Figure PI 3-K family).

Class III PtdIns 3-kinase

The Class III PtdIns 3-kinase has a restricted substrate range in that it phosphorylates just PtdIns to form PtdIns3P. The prototype of this class is vacuolar protein sorting 34 (Vps34) protein found in *Saccharomyces cerevisiae* of which there is a human homologue (hVps34). The activity of hVps34 is regulated by Ca^{2+} and this contributes to the regulation of the target of rapamycin (TOR), which functions in cell growth control (see steps 4 and 5 in Module 9: Figure target of rapamycin signalling). There is a Ca^{2+}-dependent CaM-binding domain located between residues 318 and 334 in the accessory domain (Module 2: Figure PI3-K family).

The hVps34 plays an important role in intracellular trafficking such as the early endosome to plasma membrane trafficking (Module 4: Figure early endosome budding), early endosome protein sorting and intraluminal vesicle formation (Module 4: Figure intraluminal endosomal vesicle formation) and endosome vesicle fusion to early endosomes (Module 4: Figure endosome vesicle fusion). In the case of autophagy, hVps34 plays a central role in driving the early induction, nucleation and elongation steps responsible for forming the autophagic vacuole (Module 11: Figure autophagy signalling mechanisms).

PtdIns 4-kinase (PtdIns 4-K)

PtdIns 4-kinase catalyses phosphorylation of PtdIns on the 4-position of the inositol ring to produce PtdIns4P (Step 2 in Module 2: Figure phosphoinositide metabolism). Enzyme activity is found in most cellular membranes (plasma membrane, endoplasmic reticulum, Golgi, nuclear membrane and secretory vesicles). Cells have two classes of PtdIns 4-K: Type II and Type III.

The Type II PtdIns 4-K, which contains wortmannin-insensitive α- and β-isoforms, may be responsible for generating the lipid substrates in the membrane that are used for signalling. The enzymatic activity of PtdIns 4-kinase is greatly enhanced in various cancer cells.

The Type III PtdIns 4-K, which has α- and β-isoforms, is sensitive to the drug wortmannin. The PtdIns 4-KIIIα found in the endoplasmic reticulum and the PtdIns 4-KIIIβ found in the cytosol and Golgi are responsible for producing PtdIns4P, which is one of the localized phosphoinositide lipid signalling molecules used for vesicle trafficking (Module 2: Figure localized inositol lipid signalling). The PtdIns 4-KIIIβ at the Golgi is controlled by the Arf signalling pathway (Module 2: Figure Arf signalling). It is the Type III PtdIns 4-K isoform that is activated by the neuronal Ca^{2+} sensor-1 protein (NCS-1) to enhance exocytosis. Originally, it was considered that PtdIns4P functioned solely as a precursor for the formation of PtdIns4,5P_2, but there is increasing evidence for a role as part of a PtdIns4P signalling cassette.

PtdIns phosphate kinases

There is a family of PtdIns phosphate kinases that are divided into three subfamilies (Types I–III) that are localized to different compartments and function to phosphorylate different phosphoinositides.

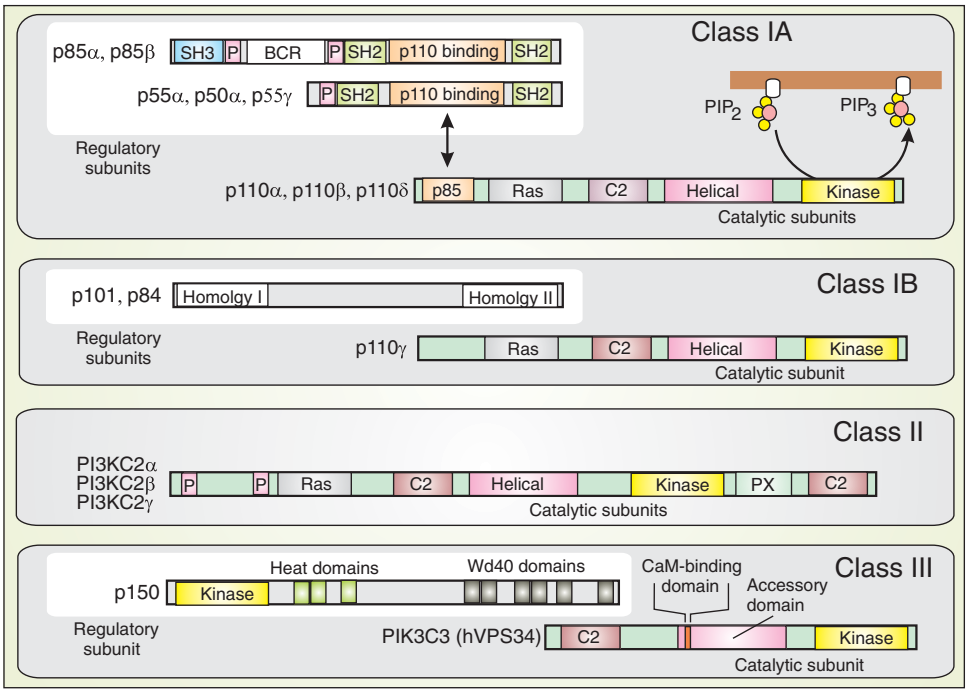
PtdIns4P 5-kinase (PtdIns4P 5-K)

This versatile family of Type I PtdIns phosphate kinases (also abbreviated as PIPKI) is composed of three isoforms (α, β and γ) that are located in different cellular regions such as the plasma membrane, focal adhesions, Golgi and nucleus. Their primary role is to phosphorylate PtdIns4P to PtdIns4,5P_2 (Step 8 in Module 2: Figure phosphoinositide metabolism).

PtdIns4P 5-kinase Iα

This enzyme functions at multiple locations in the cell. It is the major enzyme responsible for forming PtdIns4,5P_2 at the plasma membrane. It is also found within the nucleus at nuclear speckles that are sites of mRNA processing.

Module 2: | Figure PI 3-K family



The regulatory and catalytic subunits of the PtdIns 3-kinase family.
The family of PtdIns 3-kinase is divided into three classes. The kinase domain (shown in yellow) phosphorylates PtdIns4,5P_2 (PIP_2) to form the lipid messenger PtdIns3,4,5P_3 (PIP_3). The domain structures of the regulatory and catalytic subunits are described in the text. Information for this figure was taken from Hawkins et al. 2006.

PtdIns4P 5-kinase Iβ

This isoform is found on membranes surrounding the nucleus.

PtdIns4P 5-kinase Iγ

The PtdIns4,5P_2 function in focal adhesions depends upon a spliced form of this enzyme, which is targeted to focal adhesions by interacting with talin (Module 6: Figure integrin signalling). Talin contains a FERM domain, which is used to bring PtdIns4P 5-kinase Iγ into the adhesion complex. Similarly, this isoform plays a major role the control of endocytosis (Module 4: Figure endocytosis).

This Type I enzyme can be regulated by two separate mechanisms. Firstly, its activity is stimulated by phosphatidic acid (PA) that is formed either by the phospholipase D (PLD) signalling pathway where phosphatidylcholine (PC) is hydrolysed by phospholipase D (Module 2: Figure PLD signalling) or by the phosphorylation of diacylglycerol (DAG) by diacylglycerol kinase (DAG) kinase (Module 2: Figure $InsP_3$/DAG recycling). Secondly, enzyme activity can be enhanced by small monomeric G proteins such as Rho (Module 2: Figure Rho signalling). During its activation, PtdIns4P 5-kinase Iα translocates to the plasma membrane through an activation mechanism that is dependent upon Rac and Rho. This G protein regulation results in the localized synthesis of PtdIns4,5P_2, which then acts as a second messenger for the PtdIns4,5P_2 signalling cassette. For example, it can influence a variety of proteins to regulate processes such as actin remodelling, exocytosis, the PLD signalling pathway (Module 2: Figure PLD signalling) and the permeability of ion channels.

The PtdIns4P 5-Kα has an additional role in that it can phosphorylate PtdIns3,4P_2 to form the lipid second messenger PtdIns3,4,5P_3 (Step 8 in Module 2: Figure phosphoinositide metabolism).

PtdInsP kinase II

This enzyme, which is found in the cytosol, endoplasmic reticulum and nucleus, but not in the plasma membrane, is a PtdInsP 4-kinase capable of phosphorylating both PtdIns3P and PtdIns5P to produce PtdIns3,4P_2 and PtdIns4,5P_2 respectively (Steps 6 in Module 2: Figure phosphoinositide metabolism).

PtdInsP kinase III (PIKfyve)

This type III PtdInsP kinase, which is known as PhosphoInositide Kinase for five position containing a Fyve finger (PIKfyve), phosphorylates PtdIns3P to PtdIns3,5P_2 (Step 4 in Module 2: Figure phosphoinositide metabolism). Despite being classified as a PtdInsP kinase, this kinase may also phosphorylate PtdIns on the 5-position to form PtdIns5P (see Step 3). This PIKfyve kinase has a FYVE domain to target it to endomembranes where it functions in the PtdIns3,5P_2 signalling cassette (Module 2: Figure PIKfyve activation).

Mutations in PIKfyve have been linked to François-Neetens Mouchetée fleck corneal dystrophy (CFD).

Inositol lipid phosphatases

There are a large number of inositol lipid phosphatases that contribute to inositol lipid metabolism (Module 2: Figure phosphoinositide metabolism). The situation is complicated in that some of these phosphatases have a broad range of substrates and can act on both inositol lipids and inositol phosphates. The following are the phosphatases that act predominantly on inositol lipids:

- Phosphatase and tensin homologue deleted on chromosome 10 (PTEN)
- 5-Phosphatase II
- Inositol polyphosphate 5E-phosphatase (INPP5E)
- Myotubularins
- Oculocerebrorenal syndrome of Lowe (OCRL)
- Proline-rich inositol polyphosphate 5-phosphatase (PIPP)
- Skeletal muscle and kidney enriched inositol phosphatase (SKIP)
- Synaptojanins

5-Phosphatase II

5-Phosphatase II, which is also known as inositol polyphosphate 5B-phosphatase (INPP5B), is a 75 kDa protein that preferentially hydrolyses PtdIns4,5P_2 and PtdIns3,4,5P_3, but can also act on the inositol phosphates Ins1,4,5P_3 and Ins1,3,4,5P_4. This 5-phosphatase II closely resembles oculocerebrorenal syndrome of Lowe (OCRL) both in structure and function. Like OCRL, it has a central 5-phosphatase catalytic domain followed by an ASH domain and a RhoGAP domain. The ASH domain enables it to interact with Rab5. The C-terminal end has a CAAX region that can be prenylated to facilitate its membrane localization.

5-Phosphatase II is located on the Golgi where it may function in the trafficking of vesicles to both the ER and to the early endosome.

Inositol polyphosphate 5E-phosphatase (INPP5E)

Inositol polyphosphate 5E-phosphatase (INPP5E), which is also known as Type IV 5-phosphatase, is widely expressed where it functions to remove the 5-phosphate from PtdIns4,5P_2, PtdIns3,5P_2 and PtdIns3,4,5P_3.

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN)

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a lipid phosphatase that is mutated in many human cancers. Its gene is a tumour suppressor, which functions in cell migration, proliferation and survival. PTEN inactivates the 3-phosphorylated lipid second messengers that operate within the PtdIns 3-kinase signalling cassette. It functions to remove the phosphate from the 3-position of PtdIns3,4P_2 and PtdIns3,4,5P_3 (Module 2: Figure phosphoinositide metabolism).

PTEN is a redox-sensitive enzyme, which may contribute to a positive-feedback loop that enhances redox signalling (Module 2: Figure plasma membrane ROS formation). It is inhibited by H_2O_2, which induces Cys-124 in the active site to form a disulphide bond with Cys-71. This disulphide bond formation is specifically reversed by thioredoxin. A reversible inactivation of PTEN may thus contribute to the accumulation of PtdIns3,4,5P_3 (Module 2: Figure plasma membrane ROS formation), which may thus help to promote proliferation by switching off the metabolism of the 3-phosphorylated lipid second messengers. This reversible inactivation of PTEN contributes to the accumulation of PtdIns3,4,5P_3 by setting up a positive-feedback loop, since the formation of this lipid messenger is responsible for stimulating the production of H_2O_2 (Module 2: Figure plasma membrane ROS formation).

There are additional enzymes that hydrolyse the 3-phosphorylated position. Transmembrane phosphatase with tensin homology (TPTE) is localized to the plasma membrane, but appears not to have phosphatase activity. TPTE and PTEN homologous inositol lipid phosphatase (TPIP) occurs as α and β isoforms. The TPIPα isoform hydrolyses PtdIns3,4,5P_3, PtdIns3,5P_2, PtdIns3,4P_2 and PtdIns3P. It has N-terminal transmembrane domains that appear to localize the protein to the ER.

PTEN is a potent tumour suppressor that is frequently inactivated in many different cancers, e.g. endometrial, prostate, mammary carcinomas, melanomas and thyroid tumours. When PTEN is inactivated in neurons, there is a progressive increase in cell size and increased phosphorylation of protein kinase B (PKB) that create cerebellar abnormalities resembling those seen in human Lhermitte–Duclos disease (LDD).

Germline mutations in the *PTEN* gene have been implicated in the development of Cowden's disease and Bannayan–Zonana syndrome, where there is an increased risk of breast and thyroid cancers. An increase in the expression of PTEN by the tumour suppressor p53 may contribute to p53-induced apoptosis.

Myotubularins

There is a large family of myotubularins that function as lipid phosphatases that dephosphorylate PtdIns3P and also PtdIns3,5P_2 (Module 2: Figure phosphoinositide metabolism). They may also act on PtdIns3,5P_2. Mammalian cells express 15 myotubularins. The founding member is MTM1 and the remainder are MTMR2–MTMR14. These enzymes have a phosphatase domain, and a GRAM domain, which associates with membranes and a C-terminal coiled-coil domain that links these enzymes to other proteins. One of the isoforms, MTMR6, appears to have a specific role in the PtdIns3P signalling cassette. Hydrolysis of PtdIns3P may control the activity of the Ca^{2+}-activated intermediate-conductance (IK) channel ($K_{Ca}3.1$), and this may be of particular significance for the relationship between K^+ channels and cell proliferation.

These enzymes are of interest because mutations in MTM1 are responsible for X-linked recessive myotubular myopathy and MTMR2 is mutated in Charcot-Marie-Tooth disease 4B. Missense mutations in MTMR14 have also been described in centronuclear myopathy (CNM).

Oculocerebrorenal syndrome of Lowe (OCRL)

Oculocerebrorenal syndrome of Lowe (OCRL) is a Rho-GAP-domain-containing enzyme that can hydrolyse both inositol phosphates (Ins1,4,5P_3 and Ins1,3,4,5P_4) and the phosphoinositides PtdIns4,5P_2 and PtdIns3,4,5P_3. OCRL also contains an ASH [ASPM (abnormal spindle-like microcephaly-associated protein/SPD2 (spindle pole body2)/hydin] domain, which is thought to provide a binding site for microtubules. One of the functions of OCRL is to control membrane and protein trafficking during endocytosis where it operates primarily at the interface between the *trans*-Golgi network (TGN) and the endosomes.

A deficiency of this enzyme is responsible for Lowe's oculocerebrorenal (OCRL) syndrome. Some patients with Dent's disease also carry mutations in OCRL.

Synaptojanin

There are two synaptojanins: synaptojanin 1 (SJ1) and synaptojanin 2 (SJ2).

Synaptojanin 1 (SJ1)

Synaptojanin 1 (SJ1) is a multi-functional inositol polyphosphate 5-phosphatase that has an important role in the process of endocytosis (Module 4: Figure endocytosis). There are two lipid phosphatase domains arranged in tandem: an N-terminal Sac1 domain, which dephosphorylates PtdIns3P and PtdIns4P, and an adjacent 5-phosphatase that acts on PtdIns4,5P_2 and PtdIns3,4,5P_3. The C-terminal region has a proline-rich domain (PRD) that targets synaptojanins to the SH3 domains of dynamin, endophilin and amphiphysin on clathrin-coated pits (Module 4: Figure scission of endocytic vesicles). Alternative splicing of this region gives rise to the SJ1-145 and SJ1-170 isoforms. The former is strongly expressed in presynaptic terminals. SJ1 has a primary role in coat removal in the final stages of endocytosis and is particularly important for synaptic vesicle recycling. Since SJ1 is distributed throughout the neuron, it also plays a role in postsynaptic endocytosis.

The activity of SJ1 is regulated by a phosphorylation/dephosphorylation cycle operated by the dual-specificity tyrosine-phosphorylation regulated kinase 1A (DYRK1A) and calcineurin (CaN) respectively.

Synaptojanin 2 (SJ2)

Synaptojanin 2 (SJ2) has a catalytic domain that closely resembles that of synaptojanin 1 (SJ1). Despite this close similarity, SJ2 does seem to have some distinct functions. Like SJ1, SJ2 has also been implicated in clathrin-mediated endocytosis.

Proline-rich inositol polyphosphate 5-phosphatase (PIPP)

The proline-rich inositol polyphosphate 5-phosphatase (PIPP) has N- and C-terminal proline-rich domains. There also is a SKICH domain responsible for attaching PIPP to the plasma membrane where it acts to hydrolyse PtdIns4,5P_2 and PtdIns3,4,5P_3. It can also hydrolyse Ins1,4,5P_3 and Ins1,3,4,5P_4. It is highly expressed in the brain where it appears to function in neurite extension.

Skeletal muscle and kidney enriched inositol phosphatase (SKIP)

As its name implies, skeletal muscle- and kidney-enriched inositol phosphatase (SKIP) is strongly expressed in skeletal muscle and kidney, but is also found in heart and brain. It's C-terminal SKICH domain attaches it to the plasma membrane and to membranes in the perinuclear region. It acts to hydrolyse both PtdIns4,5P_2 and PtdIns3,4,5P_3 and has been implicated in insulin signalling.

Sac3/Fig4

The suppressor of actin 3 (Sac3), which is the mammalian orthologue of Fig4 in yeast, is a SAC domain lipid phosphatase that removes the 5-phosphate from PtdIns3,5P_2 to form PtdIns3P (see step 13 in Module 2: Figure phosphoinositide metabolism). Sac3/Fig4 is part of the PAS complex and has an important structural role in that it also helps to facilitate the kinase activity of PIKfyve that operates in the PtdIns3,5P_2 signalling cassette (Module 2: Figure PIKfyve activation).

Mutations in Sac3/Fig3 have been linked to Charcot-Marie-Tooth disease 4J.

Inositol phosphate metabolism

The Ca^{2+}-mobilizing messenger function of inositol 1,4,5-trisphosphate ($InsP_3$) is terminated through its metabolism by a complex inositol phosphate metabolic pathway (Module 2: Figure inositol phosphate metabolism) that has two main functions. Firstly, it produces free inositol that is resynthesized to PtdIns, which can be reused for further signalling. Secondly, it generates an assortment of inositol phosphates, some of which contribute to the multipurpose inositol polyphosphate signalling pathway.

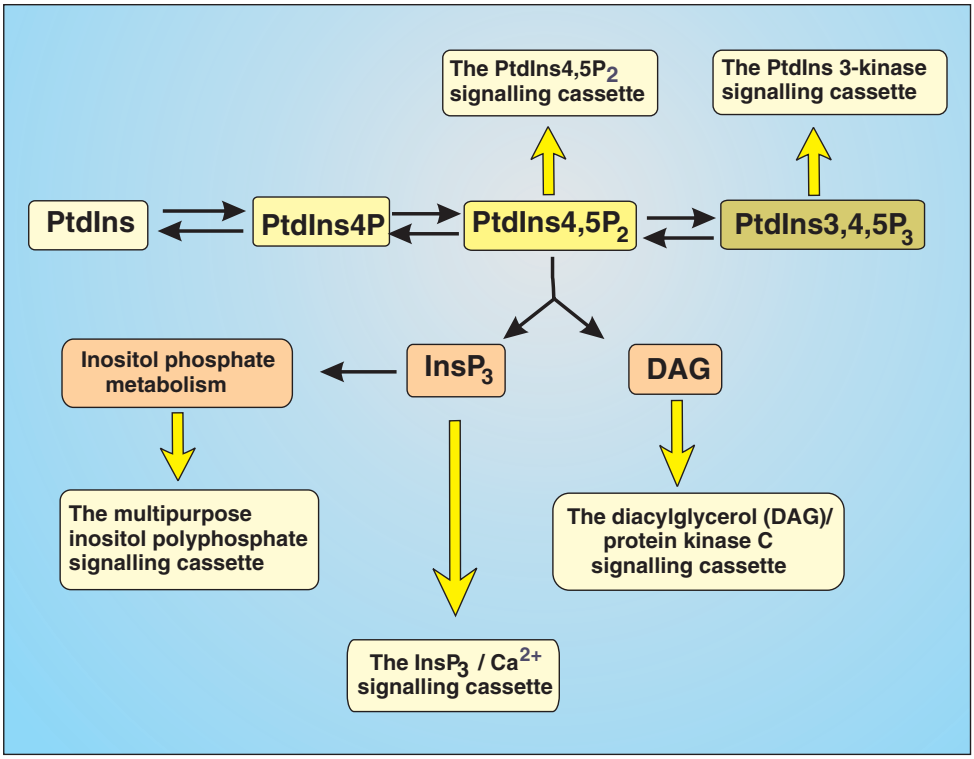
The Ins1,4,5P_3 that enters the metabolic pathway is metabolized via two pathways. It is dephosphorylated by Type I inositol polyphosphate 5-phosphatase (step 1) to form Ins1,4P_2 or it can be phosphorylated by $InsP_3$ 3-kinase to form Ins1,3,4,5P_4 (step 4). Both Ins1,4P_2 and Ins1,3,4,5P_4 are putative messengers in the multipurpose inositol polyphosphate signalling pathway. The Ins1,4P_2 is sequentially dephosphorylated to free inositol. The Ins1,3,4,5P_4 is dephosphorylated to Ins1,3,4P_3, which occupies an important position in the metabolic pathway in that either it is dephosphorylated down to inositol, as part of an inositol recycling pathway, or it can be phosphorylated further to form additional inositol polyphosphates. The inositol phosphates in the pink boxes in Module 2: Figure inositol phosphate metabolism have been implicated as intracellular messengers. $InsP_3$ mobilizes internal Ca^{2+} and the putative functions of the others are described in the multipurpose inositol polyphosphate signalling pathway.

Metabolism of the inositol phosphates is carried out by a large number of inositol phosphate kinases and phosphatases (Module 2: Figure inositol phosphate metabolism):

Inositol polyphosphate 5-phosphatase (INPPs)

This large family of enzymes, which are products of multiple genes and splice variants, removes the 5-phosphate

Module 2: | Figure phosphoinositide signalling systems



PtdIns metabolism spawns a variety of signalling cassettes.
The metabolism of PtdIns creates a number of lipid and inositol phosphate derivatives that operate a variety of signalling cassettes. PtdIns4,5P_2 is a nodal point for a number of signalling cassettes. It is the precursor that is hydrolysed to generate the second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). It is phosphorylated further to form the lipid second messenger PtdIns3,4,5P_3. The localized turnover of PtdIns4,5P_2 also has a signalling role within the PtdIns4,5P_2 signalling cassette that regulates a variety of processes such as the cytoskeleton, control of the processes of membrane trafficking and exocytosis and the regulation of ion channels and exchangers. Finally, the $InsP_3$ enters a complex pathway of inositol phosphate metabolism that generates components of the multipurpose inositol polyphosphate signalling cassette.

group from inositol lipids and, in some cases, can also act on inositol phosphates. All members of the family share a central conserved catalytic domain:

- 5-Phosphatase I
- 5-Phosphatase II
- Oculocerebrorenal syndrome of Lowe (OCRL)
- Src homology 2 (SH2) domain-containing inositol phosphatase (SHIP)
- Proline-rich inositol polyphosphate 5-phosphatase (PIPP)
- Skeletal muscle and kidney enriched inositol phosphatase (SKIP)
- Synaptojanins

5-Phosphatase I

The 5-phosphatase I functions to hydrolyse the inositol phosphates Ins1,4,5P_3 and Ins1,3,4,5P_4 (Steps 1 in Figure 2 inositol phosphate metabolism). If this enzyme is deleted, there is an increase in the level of $InsP_3$, and this was associated with a transformed phenotype. This enzyme is inhibited following phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Type I, which hydrolyses the inositol phosphates, is directed to the membrane by isoprenylation of the C-terminal region. If these membrane attachment sites are removed, allowing the enzyme to disperse into the cytosol, $InsP_3$ metabolism is severely curtailed. It seems that much of the $InsP_3$ is metabolized close to its site of action at the plasma membrane.

Src homology 2 (SH2) domain-containing inositol phosphatases (SHIP)

The Src homology 2 (SH2) domain-containing inositol phosphatases (SHIP1 and SHIP2) hydrolyse Ins1,3,4,5P_4 and PtdIns3,4,5P_3. The SHIPs, which come in different forms, are found in haematopoietic cells, where they play a role in inhibiting signalling. In B cells, for example, the FcγRIIB receptor has an immunoreceptor tyrosine-based inhibitory motif (ITIM) to which SHIPs attach causing a reduction in Ca^{2+} signalling by hydrolysing PtdIns3,4,5P_3 and thereby reducing the stimulatory effect of the Tec tyrosine kinase family on phospholipase Cγ (Module 2: Figure ROS effects on Ca^{2+} signalling). SHIPs perform a similar function in mast cells (Module 11: Figure mast cell inhibitory signalling). SHIP1 functions to localize PIP_3 at the front of the cell during neutrophil chemotaxis (Module 11: Figure neutrophil chemotaxis).

Inositol polyphosphate 1-phosphatase

Steps 2 in Module 2: Figure inositol phosphate metabolism. This enzyme dephosphorylates both Ins1,3,4P_3 and Ins1,4P_2. This enzyme has been implicated in the

signalling pathways of compensatory hypertrophy and is up-regulated in human colorectal cancer.

Inositol monophosphatase
Steps 3 in Module 2: Figure inositol phosphate metabolism. The free inositol that is formed by this enzyme is then used to resynthesize PtdIns, which is returned to the plasma membrane, where it can be reused to function as the precursor for the phosphoinositide signalling lipids (Module 2: Figure phosphoinositide metabolism). This inositol monophosphatase is inhibited by lithium (Li^+), which thus acts to reduce the supply of inositol (Module 2: Figure $InsP_3$/DAG recycling). This ability of Li^+ to lower the level of inositol is the basis of an inositol depletion hypothesis to account for its action in controlling manic-depressive illness.

Ins1,4,5P_3 3-kinase
Step 4 in Module 2: Figure inositol phosphate metabolism. This kinase is one of the major pathways for metabolizing the second messenger Ins1,4,5P_3. There are three isoforms of this enzyme ($InsP_3$ 3-kinase A, B and C). Both type A and type B are activated by Ca^{2+}/calmodulin, with the type B being the most sensitive. This enzyme is also sensitive to other signalling pathways in that both forms are inhibited by PKC. On the other hand, protein kinase A (PKA) activates type A but inhibits type B.

Inositol polyphosphate 4-phosphatase
Steps 5 in Module 2: Figure inositol phosphate metabolism. A multifunctional phosphatase capable of dephosphorylating both Ins3,4P_2 and Ins1,3,4P_3, as well as the inositol lipid PtdIns3,4P_2. This enzyme might be a regulator of cell proliferation, since it is absent in hyperproliferative megakaryocytes that lack the transcription factor GATA-1.

Inositol polyphosphate 3-phosphatase
Steps 6 in Module 2: Figure inositol phosphate metabolism. A multifunctional phosphatase capable of dephosphorylating both Ins1,3P_2 and PtdIns3P.

Ins1,3,4P_3 6-kinase
Step 7 in Module 2: Figure inositol phosphate metabolism.

Ins1,3,4,6P_4 5-kinase
Step 8 in Module 2: Figure inositol phosphate metabolism.

Ins3,4,5,6P_4 1-kinase
Step 9 in Module 2: Figure inositol phosphate metabolism. This enzyme is responsible for inactivating the Ins3,4,5,6P_4 that regulates chloride channels. It is an unusual enzyme in that it has alternative positional specificity (5/6-kinase activity against Ins1,3,4P_3). The enzyme is the same as the Ins1,3,4,5,6P_5 1-phosphatase.

Ins1,3,4,5,6P_5 1-phosphatase
Step 10 in Module 2: Figure inositol phosphate metabolism. The enzyme is equivalent to the Ins 3,4,5,6P_4 1-kinase.

Ins1,4,5,6P_4 3-kinase
Step 11 in Module 2: Figure inositol phosphate metabolism.

Ins1,3,4,5,6P_5/InsP_6 3-phosphatase
Steps 12 in Module 2: Figure inositol phosphate metabolism. This enzyme is also known as the multiple inositol phosphate phosphatase (MIPP). Its function is somewhat unclear because it is located within the lumen of the ER, apparently insulated from its two substrates.

Ins1,3,4,5,6P_5 2-kinase
Step 13 in Module 2: Figure inositol phosphate metabolism. The production of InsP_6 occurs primarily through the phosphorylation of Ins1,3,4,5,6P_5 by a 2-kinase.

InsP_6 kinase
Steps 14 in Module 2: Figure inositol phosphate metabolism. This kinase adds an additional phosphate to that already present on the 5-position to produce InsP_7 (PP-InsP_5). This kinase can run in reverse and is thus potentially capable of reforming ATP. The same enzyme may act on Ins1,3,4,5,6P_5 to produce PPInsP_4. There are three isoforms (InsP_6K1–InsP_6K3). InsP_6K2 has been implicated in the action of interferons on cell growth and apoptosis.

Diphosphoinositol phosphate phosphohydrolase (DIPP)
Steps 15 in Module 2: Figure inositol phosphate metabolism. This enzyme dephosphorylates both PP-InsP_5 (InsP_7) and $[PP]_2$-InsP_4 (InsP_8), during which it removes the β-phosphate from the diphosphate groups.

PP-InsP_5 kinase
Step 16 in Module 2: Figure inositol phosphate metabolism. This kinase adds a further phosphate to PP-InsP_5 to form $[PP]_2$-InsP_4, but the positional specificity of the latter remains to be established. Like the InsP_6 kinase, this enzyme is capable of forming ATP by running in a reverse mode.

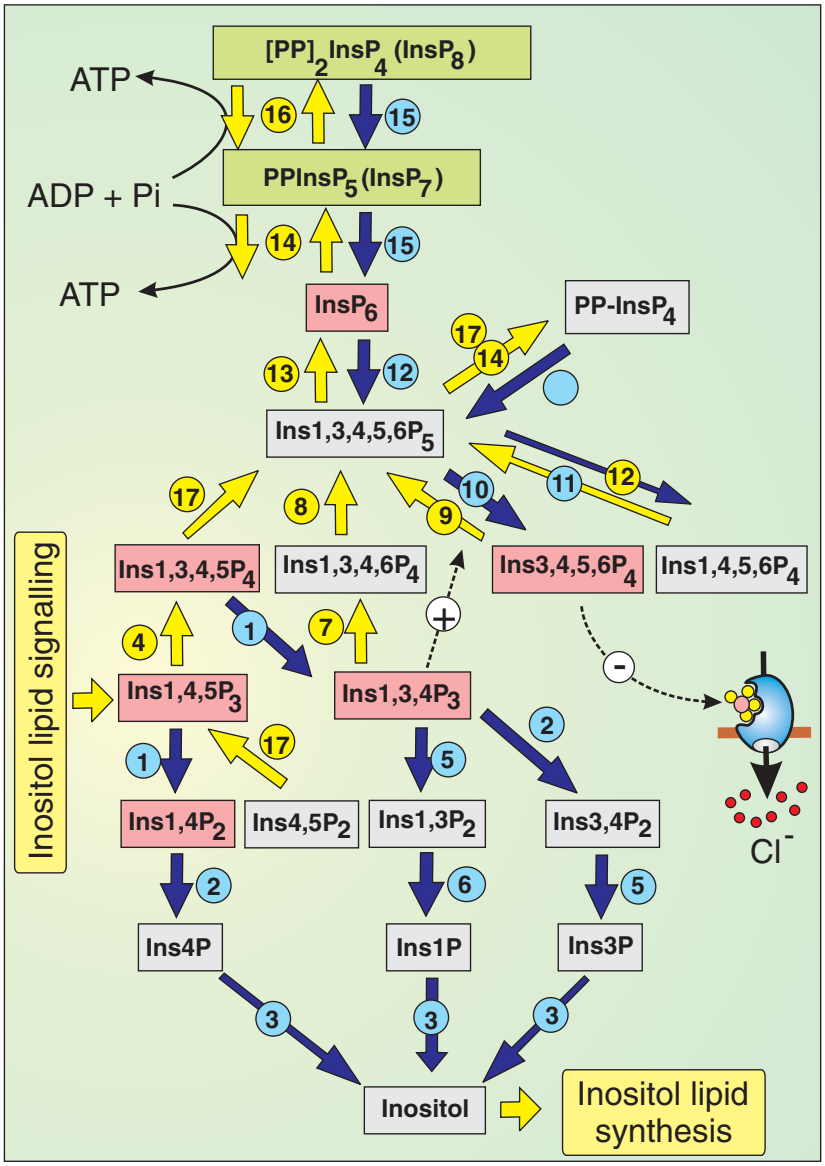
Mammalian inositol phosphate multikinase (mIPMK)
Steps 17 in Module 2: Figure inositol phosphate metabolism. This mammalian inositol phosphate multikinase (mIPMK) is a multifunctional kinase capable of the following phosphorylations:

- Ins4,5P_2 → Ins1,4,5P_3
- Ins1,4,5P_3 → Ins1,3,4,5P_4
- Ins1,3,4,5P_4 → InsP_5
- InsP_5 → PP-InsP_4

Inositol 1,4,5-trisphosphate (InsP_3)/Ca^{2+} signalling cassette

This signalling cassette uses the second messenger inositol 1,4,5-trisphosphate (InsP_3) to mobilize Ca^{2+} from internal stores. When external stimuli engage receptors on the cell surface, they activate the enzyme phospholipase C (PLC), which hydrolyses PtdIns4,5P_2 to yield InsP_3 and

Module 2: | Figure inositol phosphate metabolism



Inositol phosphate metabolism.
The major input into this metabolic system is the Ins1,4,5P_3 released from the membrane following receptor-activated inositol lipid signalling. One of the functions of this pathway is to form free inositol that is used for inositol lipid synthesis. The pathway also generates additional inositol phosphates (highlighted in pink) that have putative signalling functions. See the text for further details of the enzymes responsible for Steps 1-17.

diacylglycerol (DAG) (Module 2: Figure $InsP_3$ and DAG formation). This is a bifurcating signalling pathway in that $InsP_3$ generates a Ca^{2+} signal, whereas DAG functions to stimulate protein kinase C (PKC). The $InsP_3$ released from the membrane diffuses into the cytosol where it engages the $InsP_3$ receptors ($InsP_3$Rs) to release Ca^{2+} from the endoplasmic reticulum.

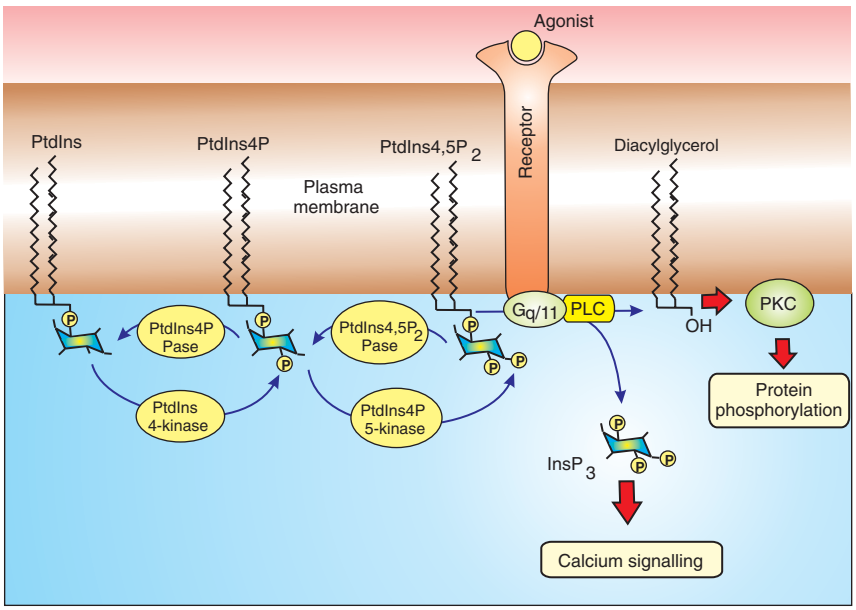
The operation of this $InsP_3/Ca^{2+}$ signalling cassette can be divided into separate components:

- PtdIns conversion into the precursor lipid PtdIns4,5P_2
- Hydrolysis of PtdIns4,5P_2 to generate $InsP_3$ and DAG
- Metabolism of $InsP_3$ and DAG and the resynthesis of PtdIns
- Inositol 1,4,5-trisphosphate ($InsP_3$) and Ca^{2+} release

PtdIns conversion into the precursor lipid PtdIns4,5P_2

The precursor PtdIns4,5P_2, which is hydrolysed to give $InsP_3$, is part of the complex inositol lipid metabolic pathway (Module 2: Figure phosphoinositide metabolism). The synthesis of the lipid precursor PtdIns4,5P_2 from PtdIns is controlled by two substrate cycles involving lipid kinases and phosphatases (Module 2: Figure $InsP_3$ and DAG formation). In the first step, the hydroxy group at the 4-position of the inositol ring is phosphorylated to form PtdIns4P. In the next step, the PtdIns4P is phosphorylated further to yield the precursor PtdIns4,5P_2. The next step is the

Module 2: | Figure $InsP_3$ and DAG formation



Agonist-dependent formation of the second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG).
The inositol lipids that function in signalling are embedded in the inner leaflet of the plasma membrane. The precursor lipid is phosphatidylinositol (PtdIns), which is successively phosphorylated, first on the 4-position to form PtdIns4P and then on the 5-position to form PtdIns4,5P_2. Activated cell-surface receptors are coupled through the G protein $G_{q/11}$ to phospholipase C (PLC) that hydrolyses PtdIns4,5P_2 to generate inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ activates Ca^{2+} signalling, and diacylglycerol (DAG) stimulates protein kinase C (PKC) to initiate protein phosphorylation (Module 2: Figure PKC structure and activation). An animated version of this figure is available.

agonist-dependent hydrolysis of PtdIns4,5P_2 to generate $InsP_3$ and diacylglycerol (DAG).

Hydrolysis of PtdIns4,5P_2 to generate $InsP_3$ and diacylglycerol (DAG)

External stimuli (e.g. hormones, neurotransmitters and growth factors) gain access to this signalling pathway by activating cell-surface receptors. The latter fall into two main classes, the G protein-coupled receptors (GPCRs) and the protein tyrosine kinase-linked receptors (PTKRs). During the transduction process, the precursor lipid PtdIns4,5P_2 is hydrolysed by phospholipase C (PLC) to produce both $InsP_3$ and DAG. The family of PLCs can be distinguished by the way they are coupled to cell-surface receptors. In general, the GPCRs use the PLCβ isoforms, whereas the receptor tyrosine kinases (RTKs) are coupled to the PLCγ isoforms (Module 2: Figure PLC structure and function).

DAG functions to activate the diacylglycerol (DAG)/protein kinase C (PKC) signalling cassette, whereas the $InsP_3$ diffuses into the cytosol to activate the $InsP_3$ receptors to release Ca^{2+} stored in the endoplasmic reticulum. The signalling function of this bifurcating signalling pathway is curtailed by the metabolism of $InsP_3$ and the resynthesis of PtdIns.

Metabolism of $InsP_3$ and DAG and the resynthesis of PtdIns

The metabolism of $InsP_3$ and DAG and the resynthesis of PtdIns are the OFF reactions that terminate the actions of these two messengers. There are two pathways for diacylglycerol (DAG) metabolism. Similarly, there are two separate mechanisms of inositol 1,4,5-trisphosphate ($InsP_3$) metabolism.

Diacylglycerol (DAG) metabolism

The second messenger DAG is metabolized via two separate pathways. It can be phosphorylated by diacylglycerol (DAG) kinase to form phosphatidic acid (PA) or it is hydrolysed by diacylglycerol (DAG) lipase. The PA is transferred to the endoplasmic reticulum, where it interacts with CTP to form the CDP/DAG complex, which is a precursor in the resynthesis of PtdIns (Module 2: Figure $InsP_3$/DAG recycling).

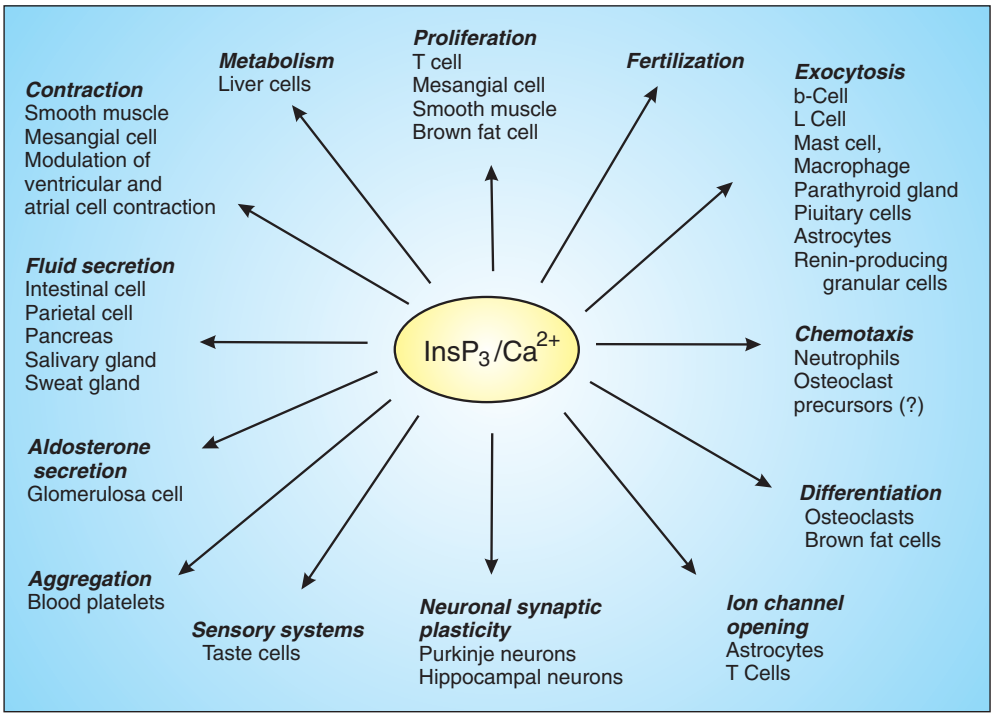
Diacylglycerol (DAG) kinase

Diacylglycerol (DAG) kinase α (DAGKα), which is one of a family of nine mammalian isotypes, has a number of domains, including Ca^{2+}-binding EF-hand motifs and an N-terminal recoverin homology domain that is related to the recoverin family of neuronal Ca^{2+} sensors. These two domains appear to function as a unit during Ca^{2+}-induced activation of DAGKα. In response to an increase in Ca^{2+}, DAGKα translocates to the membrane, where it phosphorylates DAG to phosphatidic acid (PA) (Module 2: Figure $InsP_3$/DAG recycling). DAG kinase functions in the scission of COPI-coated vesicles that bud off from the Golgi (Module 4: Figure COPI-coated vesicles).

Diacylglycerol (DAG) lipase

A diacylglycerol (DAG) lipase is responsible for removing one of the fatty acid tails from the *sn*-position of DAG

Module 2: | Figure $InsP_3/Ca^{2+}$ signalling functions



$InsP_3/Ca^{2+}$ signalling functions.

The mobilization of Ca^{2+} by inositol 1,4,5-trisphosphate ($InsP_3$) functions in the control of many different cellular processes in a wide range of cell types.

to form monoacylglycerol (Module 2: Figure $InsP_3$/DAG recycling). This hydrolysis of DAG, which is a Ca^{2+}-sensitive process, may represent the primary mechanism for removing the DAG that is produced following the hydrolysis of inositol lipids.

The 2-arachidonylglycerol (2-AG) formed by DAG lipase is one of the endocannabinoids that has multiple messenger functions.

Inositol 1,4,5-trisphosphate ($InsP_3$) and Ca^{2+} signalling

The primary function of inositol 1,4,5-trisphosphate ($InsP_3$) is to function as a second messenger to release Ca^{2+} from the internal stores. The 1,4,5-trisphosphate receptors ($InsP_3Rs$) located on the endoplasmic reticulum respond to $InsP_3$ by releasing puffs of Ca^{2+} (Module 3: Figure $InsP_3R$ activation). These $InsP_3Rs$ are sensitive to both $InsP_3$ and Ca^{2+} and can thus function as co-incident detectors (Module 2: Figure Ca^{2+}-induced Ca^{2+} release). This $InsP_3/Ca^{2+}$ signalling system controls many different cellular processes in a large number of different cell types (Module 2: Figure $InsP_3/Ca^{2+}$ signalling functions).

Inositol 1,4,5-trisphosphate ($InsP_3$) metabolism

The inositol 1,4,5-trisphosphate ($InsP_3$) that is formed during signalling enters the pathways of inositol phosphate metabolism from which it emerges as free inositol. It is dephosphorylated by Type I inositol polyphosphate 5-phosphatase (Steps 1 in Module 2: Figure inositol phosphate metabolism) to form $Ins1,4P_2$ or it can be phosphorylated by $InsP_3$ 3-kinase to form $Ins1,3,4,5P_4$ (Step 4). These two products then enter a complex metabolic pathway that plays an important role in recycling the inositol headgroup. Cells have access to three separate sources of inositol: recycling the second messenger $InsP_3$, *de novo* synthesis from glucose 6-phosphate or uptake of dietary inositol circulating in the plasma (Module 2: Figure $InsP_3$/DAG recycling). Drugs such as lithium and valproate may control manic-depressive illness by reducing the supply of inositol by inhibiting the inositol monophosphatase and inositol synthase respectively. The supply of free inositol is one of the essential precursors for the synthesis of PtdIns.

Synthesis of PtdIns

The two second messengers that are formed during phosphoinositide signalling can be recycled back to the precursor lipid $PtdIns4,5P_2$ through a series of steps (Module 2: Figure $InsP_3$/DAG recycling):

1. Conversion of PtdIns into the precursor lipid $PtdIns4,5P_2$.
2. The agonist-dependent activation of phospholipase C (PLC), which hydrolyses $PtdIns4,5P_2$ to generate the second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG).
3. The $InsP_3$ is recycled back to free inositol by a complex metabolic pathway illustrated in Module 2: Figure inositol phosphate metabolism.

4. DAG is phosphorylated by the Ca^{2+}-sensitive enzyme DAG kinase to form phosphatidic acid (PA) or it is hydrolysed by DAG lipase to form monoacylglycerols (MAGs) such as 2-arachidonylglycerol (2-AG), which is one of the endocannabinoids. The 2-AG is hydrolysed by monoacylglycerol lipase (MAGL) to glycerol and arachidonic acid.
5. PA is transferred from the plasma membrane to the ER where it interacts with CTP to form CDP/DAG by a CDP/DAG synthetase. The inositol, which can be produced through three mechanisms (*de novo* synthesis, recycling or uptake of dietary inositol), is attached to CDP/DAG by the PtdIns synthetase [cytidine diphosphate (CDP)/diacylglycerol (DAG):*myo*-inositol 3-phosphatidyltransferase] located on the endoplasmic reticulum (ER) to form PtdIns.
6. The PtdIns is then transported from the ER back to the plasma membrane by a PtdIns transfer protein (PITP). Cells express two PITPs, an α and a β isoform, produced by separate genes. The latter appears to be the housekeeping isoform that is essential for cell survival, whereas the α isoform has a more specialized function. When PtdIns is added back to the plasma membrane, it can once again be converted through the two phosphorylation reactions to maintain the supply of the PtdIns4,5P_2 that is the precursor for the phosphoinositide signalling pathway.
7. Cells can also use plasma inositol, which is taken up by a sodium-dependent *myo*-inositol cotransporter-1 (SMIT1).

Phospholipase C (PLC)

Phospholipase C (PLC) hydrolyses the lipid precursor PtdIns4,5P_2 to produce both InsP_3 and DAG. It is made up of five subclasses, which have variable isoforms PLCβ (β1–β4), PLCδ (δ1–δ4), PLCγ (γ1 and γ2), PLCε and PLCζ (Module 2: Figure PLC structure and function). All forms of the enzyme have an absolute requirement for Ca^{2+} that plays a critical role in the catalytic site. The PtdIns4,5P_2 is cleaved through two sequential reactions: first, the phosphodiester bond is cleaved to DAG and inositol 1,2-cyclic phosphate, the latter is then hydrolysed to give the acyclic InsP_3 that is released into the cytoplasm. The domain structure of the different PLC isoforms reveals a number of common structural features related to the way the enzyme associates with the membrane and functions to hydrolyse PtdIns4,5P_2. The catalytic domain is made up from the X and Y regions. They have at least two potential lipid-binding domains: the pleckstrin homology (PH) domain and the C2 domain (Module 6: Figure modular lipid-binding domains). In the case of PLCδ1, the enzyme may first associate with the membrane through its PH domain, which has a high affinity for PtdIns4,5P_2. Further interactions may then occur through the C2 domain, which has an extensive interface with the catalytic domain and may thus enable the catalytic site to integrate itself into the membrane to hydrolyse the lipid. The main difference between these PLC isoforms concerns the way in which they are activated.

Phospholipase Cβ (PLCβ)

The PLCβ isoforms are mainly activated by G protein-coupled receptors (GPCRs). PLCβ1 and PLCβ3 are fairly ubiquitous, whereas PLCβ2 and PLCβ4 have more limited tissue distributions.

PLCβ isoform distribution and function

PLCβ1 is highly concentrated in brain (pyramidal cells of hippocampus, Purkinje cells and granule cells). The knockout phenotype is characterized by seizures leading to sudden death. These seizures are similar to those seen during epilepsy. Many of the defects are found in the central nervous system (CNS). Activation of PLC by muscarinic receptors is suppressed in the temporal lobe, cerebellum and hippocampus, and this could decrease the inhibitory tone, leading to the seizures.

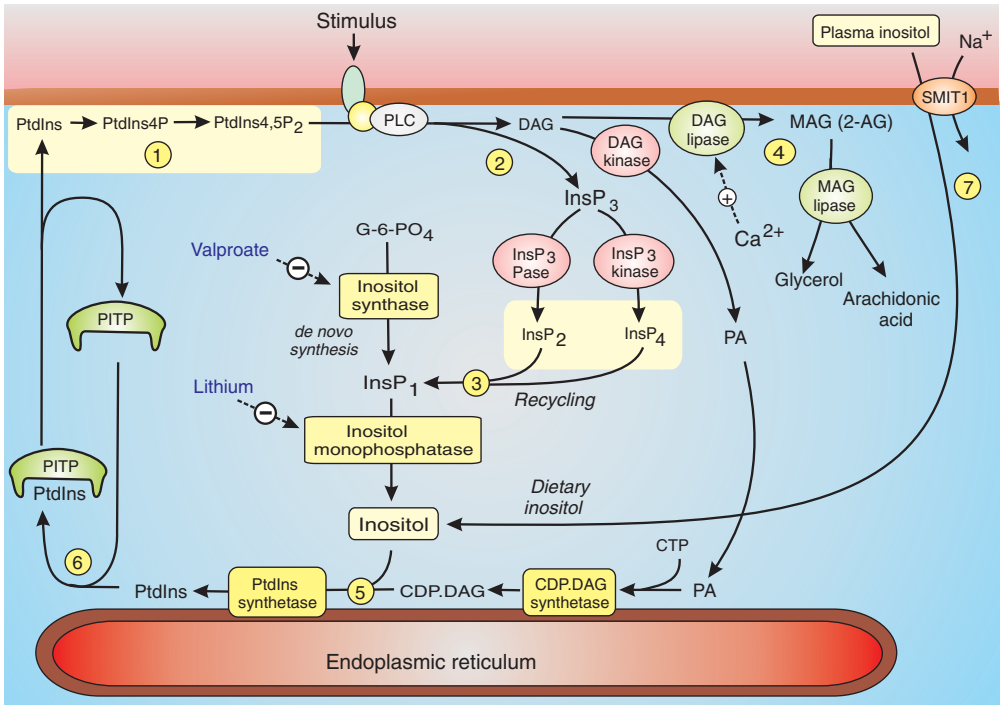
PLCβ2 is mainly expressed in cells of the immune system. Knockout mice show some disruption of chemokine signalling. For example, neutrophils fail to respond to the chemoattractant fMet-Leu-Phe with the usual changes in PLC activation, Ca^{2+} release and superoxide radical ($O_2^{-\bullet}$) production. However, the response to lipopolysaccharide (LPS) is normal. Despite this absence of PLC activation in leucocytes, chemotactic responses are enhanced, suggesting that this signalling pathway may normally antagonize the signalling pathways normally used to control chemotaxis.

PLCβ3 is found mainly in brain, parotid, smooth muscle and liver. Disruption of this gene is lethal, with the mice dying by day 2.5. The embryos are highly disorganized and have low cell numbers, suggesting a role for this isoenzyme in cell division. This isoform has two sites (Ser-26 and Ser-105) that are phosphorylated by cyclic GMP-dependent protein kinase (PKG) resulting in a decrease in enzyme activity (Module 7: Figure smooth muscle cell cyclic GMP signalling).

PLCβ4 is found in cerebellum and granule cells. Knockout mice appear to have defects in the cerebellum, resulting in poor motor co-ordination due to a decrease in PLC stimulation through metabotropic glutamatergic and muscarinic receptors. There also are defects in the processing of visual information.

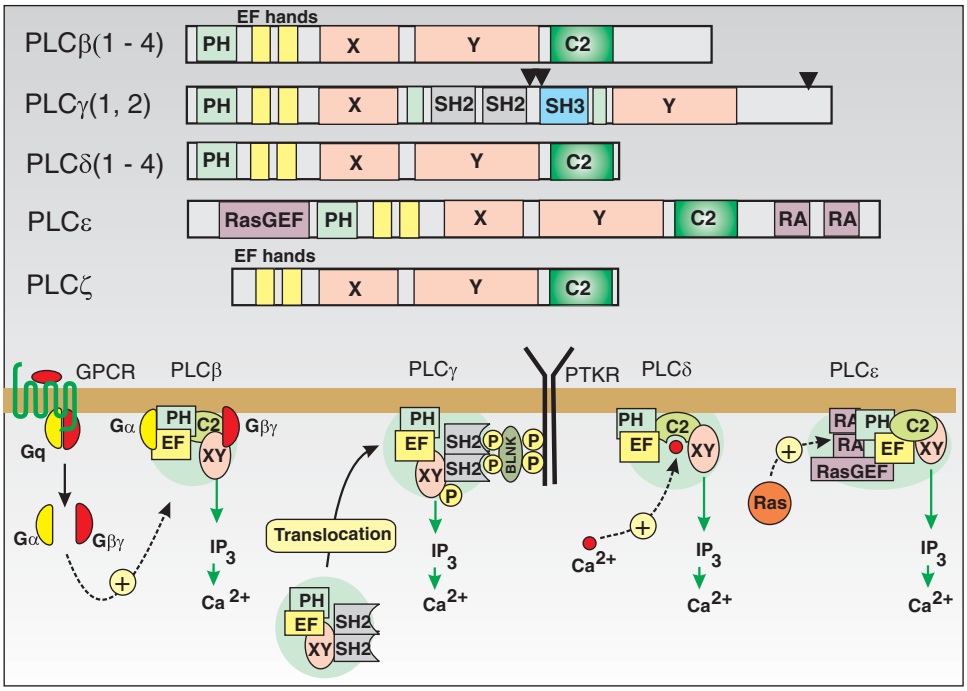
The primary activation mechanism of the PLCβ isoforms is through the G_q family of heterotrimeric G proteins (G_q, G_{11}, G_{14}, G_{15} and G_{16}) (Module 2: Figure PLC structure and function). While G_q and G_{11} are found in most tissues, the other three are restricted to cells of haematopoetic origin. The function of these G proteins is complicated because the PLCβ isoforms are sensitive to both the α and βγ components of the heterotrimeric complex. PLCβ1 and PLCβ4 are most sensitive to stimulation through α subunits, whereas βγ is more effective at activating PLCβ2 and PLCβ3. This sensitivity to βγ subunits may explain the pertussis-toxin-sensitive stimulation of PLC by the G_i family of G proteins. For example, the adenosine A_1, muscarinic M_2, somatostatin and μ-, δ- and κ-opioid receptors can couple to PLC through G_i and G_o. Of the five Gβ and 11 Gγ subunits, there appears to be little specificity with regard to the activation of PLC.

Module 2: | Figure $InsP_3$/DAG recycling



Inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) recycling.
The cell has access to three sources of inositol. It can be synthesized *de novo* from glucose 6-phosphate (G-6-PO_4) by an inositol synthase, it can come into the cell from the plasma using a sodium-dependent *myo*-inositol cotransporter-1 (SMIT1) or it can be obtained by recycling $InsP_3$. Agonist-dependent inositol lipid metabolism occurs through a series of steps as outlined in the text.

Module 2: | Figure PLC structure and function



The domain structure and activation mechanisms of PLC isoforms.
The pleckstrin homology (PH) domain, four EF-hand domains, the catalytic X and Y domains, and the C2 domain are a common feature of most of the PLC isoforms. The two PLCγ isoforms differ from the others by lacking a C2 domain and by having additional domains such as the Src homology 2 (SH2) and Src homology 3 (SH3) domains located between a split PH domain. The black triangles indicate the sites of tyrosine phosphorylation that are important for the activation of PLCγ. PLCε also has additional domains related to its activation by Ras, such as the Ras association motifs (RA) and the Ras guanine nucleotide exchange factor (RasGEF). The bottom panel illustrates how the various PLC isoforms are activated by different mechanisms. Not included in this figure is the action of PLCζ, which is unusual in that it is activated at fertilization following its injection into the oocyte (Module 8: Figure mammalian fertilization).

In general, Gβγ is less efficacious than Gα in stimulating PLC.

The two G protein subunits interact with PLCβ at different sites. The α subunit interacts with a region within the long C-terminal tail, whereas the βγ subunit interacts with a site within the Y region of the catalytic site (Module 2: Figure PLC structure and function).

PLCβ also contains GAP (GTPase-activating protein) activity that stimulates the intrinsic GTPase activity of the α subunit. When an agonist binds to the GPCRs, it induces a conformational change that is transmitted to the underlying G protein, causing it to dissociate. At this time the GDP bound to the α subunit is exchanged for GTP. The active GTP/α subunit complex can then activate PLCβ and this activation process is terminated when the intrinsic GTPase activity of the α subunit hydrolyses GTP back to GDP allowing the GDP/α subunit to once again bind to the βγ subunit to form the inactive complex. The intrinsic GTPase activity of the α subunit is rather low, but is greatly enhanced by two mechanisms. There is a GAP activity associated with the long C-terminal tail of PLCβ. Therefore PLCβ plays a direct role in terminating its own activity. In addition, there are regulators of G protein signalling (RGS) proteins. There are about 20 of these RGS proteins, of which RGS2–RGS4 seem particularly effective in interacting with $G\alpha_q$. The GTPase activity of $G\alpha_q$ is enhanced 25-fold by RGS4. There is considerable discrimination with regard to how individual RGS proteins bind to specific receptor/$G\alpha_q$ complexes enabling them to exert an agonist-specific inhibitory mechanism. This mechanism could explain the different types of Ca^{2+} signals observed in hepatocytes following stimulation with different agonists.

Modulation of PLCβ by other signalling pathways

In general, the phosphorylation of PLCβ, particularly isoforms 2 and 3, by protein kinase A (PKA) results in inhibition of the enzyme.

Phospholipase Cγ (PLCγ)

PLCγ functions predominantly in early development and in the signalling pathways that control cell proliferation. PLCγ1 knockout mice die at embryonic day 9, by which time the embryos appear normal, albeit somewhat smaller.

PLCγ is characterized by having a large insert between the X and Y domains (Module 2: Figure PLC structure and function). This insert contains an additional pleckstrin homology (PH) domain, which is itself split by another insert containing two Src homology 2 (SH2) domains and a single Src homology 3 (SH3) domain. The SH2 domains play a critical role in the activation of PLCγ because they provide a docking module that enables the enzyme to translocate to the membrane to dock to phosphorylated tyrosine residues on activated receptors or associated scaffolding proteins. This tyrosine phosphorylation occurs during activation of protein tyrosine kinase-linked receptors (PTKRs) or the non-receptor protein tyrosine kinases (e.g. Src, Syk, Btk, Lck, Fyn). This sequence of events is well illustrated by the activation of PLCγ1 during activation of the T cell receptor (Module 9: Figure TCR signalling) or PLCγ2 following B-cell antigen receptor (BCR) activation (Module 9: Figure B cell activation). This translocation to the cell surface has two important consequences for the activation of the enzyme. Firstly, it brings the enzyme close to its substrate in the membrane. Secondly, by interacting with tyrosine kinases, PLCγ1 is itself phosphorylated on tyrosine residues located at positions 771, 783 and 1254 (black triangles in Module 2: Figure PLC structure and function). Phosphorylation at Tyr-783 is particularly important for switching on enzyme activity. The PLCγ2 isoform is activated by phosphorylation of tyrosine residues 753 and 759, mainly by the tyrosine kinase Btk (Module 2: Figure ROS effects on Ca^{2+} signalling).

The enzymatic activity of PLCγ is also stimulated by the PtdIns3,4,5P_3 produced by the PtdIns 3-kinase signalling pathway and thus represents a major point of interaction between these two signalling pathways. This interaction is particularly important during lymphocyte activation, where the Tec tyrosine kinase family are activated by PtdIns3,4,5P_3 to phosphorylate and activate both PLCγ1 and PLCγ2. Both isoforms bind strongly to this highly charged lipid through the N-terminal PH domain, and this interaction enhances enzymatic activity.

The Src homology 3 (SH3) domain, which binds to proline-rich sequences, may enable PLCγ to bind to other components, such as the cytoskeleton and the protein dynamin.

Phospholipase Cδ (PLCδ)

Less is known about the activation of PLCδ. Unlike PLCβ and PLCγ, PLCδ appears not to be regulated by receptors directly. Once PLCδ1 has associated with the membrane, it appears to be activated by an elevation in cytosolic Ca^{2+}. This sensitivity to changes in intracellular Ca^{2+} is thought to depend on the C2 domain (Module 2: Figure PLC structure and function).

Crystallographic studies of PLCδ1 have begun to reveal how PLC enzymes function to cleave their lipid substrates that are embedded in the plasma membrane.

Phospholipase Cε (PLCε)

Phospholipase Cε (PLCε) differs from the other isoforms by having two Ras-association domains (RA) at its C-terminus (Module 2: Figure PLC structure and function). It binds specifically to the GTP-bound forms of Ha-Ras and Rap1A. The enzyme is targeted to the membrane through its ability to bind to these G proteins. It also has a Cdc25 homology domain that is a guanine nucleotide exchange factor (GEF) motif for Rap1, which enables it to translocate to the perinuclear region following epidermal growth factor (EGF) stimulation, where it can activate the mitogen-activated protein kinase (MAPK) signalling pathway. There is evidence that cyclic AMP acting through the exchange protein activated by cAMP (EPAC) can stimulate Rap1 to activate PLCε (Module 2: Figure cyclic AMP signalling) and this mechanism has been implicated in the control of autophagy (Module 11: Figure autophagy signalling mechanisms).

Phospholipase Cζ (PLCζ)

Phospholipase Cζ (PLCζ) has a restricted expression in that it is only found in mammalian sperm. PLCζ lacks the N-terminal PH domain (Module 2: Figure PLC structure and function), which means that there is no obvious mechanism for it to bind to phospholipids. PLCζ has a sensitivity to Ca^{2+} that is 100-fold greater than that of PLCδ1. Both the C2 and the tandem EF-hands are necessary for PLCζ to hydrolyse PtdIns4,5P_2 to generate the InsP_3 that plays a central role in sperm-induced oocyte activation during fertilization (Module 8: Figure mammalian fertilization).

Diacylglycerol (DAG)/protein kinase C (PKC) signalling cassette

The diacylglycerol (DAG) that is formed when PtdIns4,5P_2 is hydrolysed by phospholipase C (PLC) remains within the plane of the membrane where it acts as a lipid messenger to stimulate some members of the protein kinase C (PKC) family (Module 2: Figure InsP_3 and DAG formation). This PKC family contains a number of isoforms with diverse structures and activation mechanisms. The PKC signalling function is equally diverse, and it has proved difficult to pin down its precise function in the regulation of specific cellular processes.

Protein kinase C (PKC)

The protein kinase C family has been divided into three subgroups (Module 2: Figure PKC structure and activation):

- Conventional PKCs (cPKCs: α, β1, β2 and γ) contain C1 and C2 domains
- Novel PKCs (nPKCs: δ, ε, η and θ) contain a C1 and a C2-like domain
- Atypical PKCs (aPKCs: ζ , ι and λ) contain a truncated C1 domain

The activation of these different PKC families is complex in that it depends on a number of processes such as priming, translocation and association with scaffolding proteins. The priming process is driven by a sequence of multisite phosphorylation events that have to occur before the enzyme can perform its signalling function. The newly synthesized proteins are inactive and undergo a priming process that begins when phosphoinositide-dependent protein kinase 1 (PDK1), which is part of the PtdIns 3-kinase signalling pathway, phosphorylates a site in the C4 domain (Module 2: Figure PKC structure and activation). This is then followed by intramolecular autophosphorylation reactions of two further sites in the C-terminal tail. The primed enzyme is now ready to perform its signalling function by responding to both diacylglycerol (DAG) and Ca^{2+}. The cPKCs and most of the nPKCs are lipid-sensitive enzymes in that they are activated by DAG that binds to the C1 domain. This C1 domain is also responsible for binding the tumour-promoting phorbol esters. In the case of the cPKCs, Ca^{2+} also plays an important role as a cofactor that binds to the C2 domain to increase its affinity for acidic phospholipids and is thus responsible for inducing the translocation of cPKCs to the plasma membrane.

The activation of the cPKC isoforms follows a set sequence of events that begins with the hydrolysis of PtdIns4,5P_2 to form DAG and inositol 1,4,5-trisphosphate (InsP_3). The InsP_3 releases Ca^{2+}, which initiates the activation process by binding to the C2 domain to induce a conformational change that greatly enhances the membrane affinity of cPKC and thus promotes its translocation to the membrane where it is activated when it makes contact with DAG. The pseudosubstrate (PS) domain swings away from the active site, which is now free to phosphorylate its substrates.

The nPKCs, which are also activated by DAG, have a much slower activation process because the Ca^{2+}-dependent facilitation of membrane translocation is absent.

Protein kinase Cζ (PKCζ)

This is one of the atypical PKC isoforms (aPKC), which fails to respond to diacylglycerol (DAG) or Ca^{2+}, but is sensitive to low levels of ceramide (Module 2: Figure sphingomyelin signalling). PKCζ is also activated following phosphorylation by kinases from other signalling pathways. For example, PKCζ is activated by phosphoinositide-dependent kinase 1/2 (PDK1/2) (Module 2: Figure PtdIns 3-kinase signalling). In some cells, it is responsible for activating nuclear factor κB (NF-κB). PKCζ can also phosphorylate the glucose transporter in skeletal muscle (Module 7: Figure skeletal muscle E-C coupling).

Protein kinase Cθ (PKCθ)

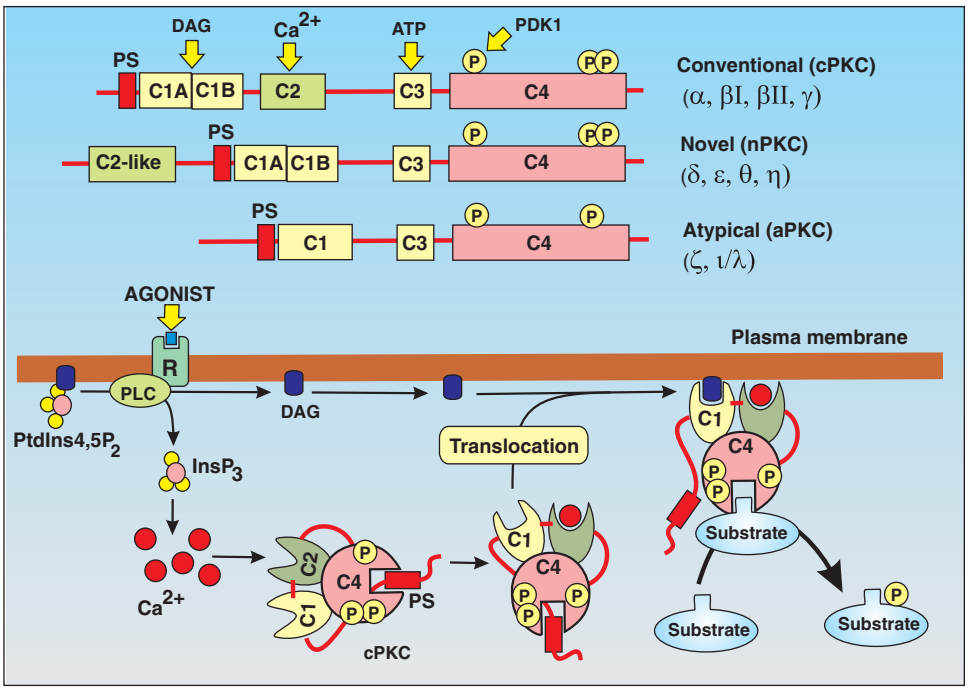
Protein kinase Cθ (PKCθ) is one of the novel PKCs (nPKCs) that plays an important role in lymphocyte activation where it is concentrated in the central region of theimmunological synapse (Module 9: Figure immunological synapse structure). The PKCθ functions by activating the nuclear factor κB (NF-κB) signalling pathway by phosphorylating the scaffolding protein CARMA1 (Module 9: Figure TCR signalling).

PKC signalling functions

The different PKC isoforms function to regulate a wide range of cellular processes:

- PKCε functions to control the N-type Ca^{2+} channel (Module 3: Figure Ca_V2 channel family).
- PKC is one of the kinases responsible for phosphorylating the transcription factor p53 (Module 4: Figure p53 domains).
- PKC functions to regulate the calmodulin (CaM)-binding properties of proteins such as neuromodulin and neurogranin.
- Protein kinase C functions in the modulation of $Ca_V1.2$ L-type channels from heart muscle (Module 3: Figure $Ca_V1.2$ L-type channel).

Module 2: | Figure PKC structure and activation



Structure of PKC isoforms and activation mechanism of the conventional protein kinase C (cPKC) isoforms.
The nine genes that code for protein kinase C (PKC) fall into three families. Conventional PKCs (cPKCs) have a domain structure that has an N-terminal pseudosubstrate (PS) domain followed by regulatory domains (C1 and C2), which are responsible for binding the diacylglycerol (DAG) and Ca^{2+} responsible for membrane targeting, an ATP-binding C3 domain and the C4 catalytic domain. The C1 region is present as a tandem repeat (C1A and C1B). The PS domain has an autoinhibitory function in that it associates with the catalytic site on C4, thus preventing it from phosphorylating its substrates. Novel PKCs (nPKCs) have a similar domain structure, except that they have a C2-like domain that does not bind Ca^{2+}. Atypical (aPKCs) also lack a C2 domain, and the C1 domain is small and fails to bind DAG. The illustration at the bottom indicates how cPKC is activated, as described in the text.

Phosphoinositide lipid signalling molecules

Some of the phosphoinositides derived from the metabolism of PtdIns (Module 2: Figure phosphoinositide metabolism) have important signalling functions that are often located in specific cellular membranes (Module 2: Figure localized inositol lipid signalling). One of the most versatile systems is the PtdIns4,5P_2 signalling cassette. PtdIns4,5P_2 is the precursor used to generate second messengers such as InsP_3, DAG and PtdIns3,4,5P_3. The latter is a particularly important messenger because the formation of PtdIns3,4,5P_3 drives the PtdIns 3-kinase signalling system that controls a great variety of cellular processes (Module 2: Figure PtdIns 3-kinase signalling). In addition, PtdIns4,5P_2 is a messenger in its own right in that localized formation of this lipid can control endocytosis, actin remodelling, ion channels and phagocytosis (Module 2: Figure localized inositol lipid signalling).

There is increasing evidence that some of the other lipids may also play signalling roles as described in the PtdIns3P signalling cassette, the PtdIns4P signalling cassette, the PtdIns5P signalling cassette and the PtdIns3,5P_2 signalling cassette. In the case of the latter, the PtdIns3,5P_2 in the late endosome activates the TRPML1 channels to produce the Ca^{2+} signals necessary to trigger the membrane fusion events that form the lysosomes.

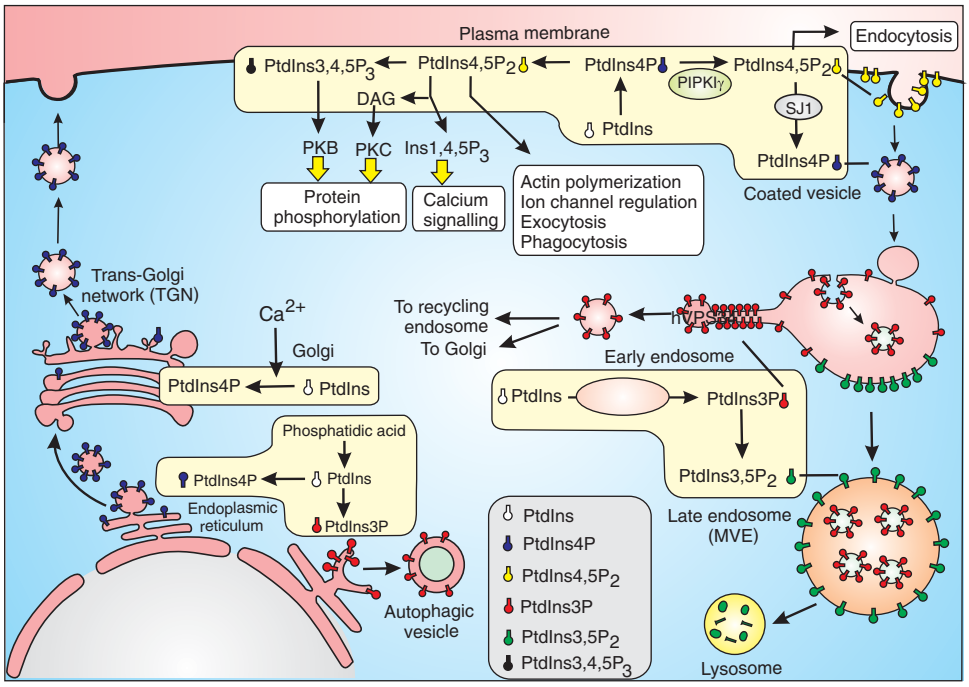
PtdIns4,5P_2 signalling cassette

The phosphoinositide PtdIns4,5P_2 is widely distributed throughout the cell. Not only is it found in the plasma membrane, but also it occurs in the Golgi, endosomes, endoplasmic reticulum (ER) and within dense structures within the nucleus. This lipid has two main roles. Firstly, it is hydrolysed to form InsP_3 and diacylglycerol (DAG) or it can be phosphorylated further to form the lipid messenger PtdIns3,4,5P_3 (Module 2: Figure localized inositol lipid signalling). Secondly, PtdIns4,5P_2 can also function as a lipid messenger (Module 2: Figure PtdIns4,5P_2 signalling). Such a signalling role is supported by the observation that cell stimulation can result in highly localized increases in PtdIns4,5P_2. The localized formation of this lipid messenger can be activated by the monomeric G proteins and in particular the family of Rho proteins. For example, Rac and Rho can function to target PtdIns4P 5-kinase I to the plasma membrane. In addition, Rho-GTP can activate this kinase directly to form PtdIns4,5P_2 (Module 2: Figure Rho signalling).

There is increasing evidence that a variety of cellular processes are activated in response to a change in the level of this lipid:

- PtdIns4,5P_2 regulation of actin remodelling

Module 2: | Figure localized inositol lipid signalling



Multiple roles of inositol lipids in cell regulation

Inositol lipids located in the plasma membrane and in various organelles have multiple roles in cell regulation. At the plasma membrane, they generate various signalling molecules, whereas in cellular organelles they orchestrate many of the events associated with membrane and protein trafficking.

- PtdIns4,5P_2 regulation of membrane trafficking and endocytosis
- PtdIns4,5P_2 regulation of exocytosis
- PtdIns4,5P_2 activation of phospholipase D
- PtdIns4,5P_2 regulation of ion channels and exchangers
- PtdIns4,5P_2 function in focal adhesions
- PtdIns4,5P_2 regulation of phagocytosis

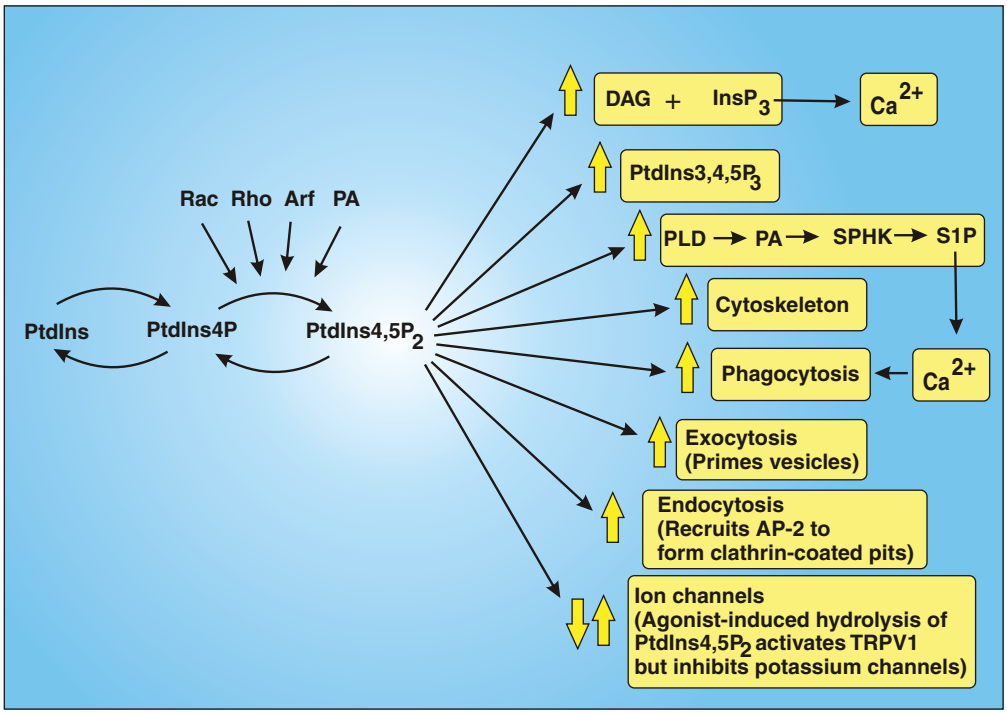
PtdIns4,5P_2 regulation of actin remodelling

There are a number of cellular processes, such as chemotaxis, locomotion, phagocytosis, shape change and cytokinesis, where there are rapid changes in the cytoskeleton in response to external stimuli such as growth factors and cytokines. Inositol lipids play a significant role in transmitting information from the plasma membrane to a variety of cell signalling systems (Module 2: Figure localized inositol lipid signalling). The formation of PtdIns4,5P_2 functions to control actin remodelling (Module 4: Figure actin remodelling). Its main actions are to uncap the barbed end of actin filaments and to facilitate actin nucleation and polymerization by controlling a variety of actin regulatory proteins. A critical component of the signalling network is the Rho family of monomeric G proteins (Arf, Rho, Cdc42 and Rac) that relay information from various signalling pathways such as the PtdIns 3-kinase signalling pathway to the effector molecules that are directly responsible for remodelling the actin network. One of the signalling molecules is PtdIns4,5P_2.

The signalling cascade begins when external signals acting through guanine nucleotide exchange factors (GEFs) stimulate members of the Rho family of G proteins by catalysing the exchange of GDP for GTP. The active G proteins can then have a number of functions. For example, one function of Rac-GTP is to activate actin polymerization (Module 2: Figure Rac signalling). Similarly, Cdc42 also stimulates actin remodelling (Module 2: Figure Cdc42 signalling). The consequence of this remodelling varies depending on which G protein is operating. For example, the Cdc42 and Rac polymerization of actin results in the formation of filopodia and ruffles respectively (Module 4: Figure actin remodelling).

The formation of PtdIns4,5P_2 contributes to actin remodelling by acting on a number of the molecules that control actin polymerization such as the Wiskott-Aldrich syndrome protein (WASP) and the related Wiskott-Aldrich syndrome protein (WASP) verprolin homologous (WAVE) proteins. WASP and WAVE relay information from the upstream signalling elements to the downstream cytoskeletal regulators. Perhaps the most important example of the latter is the actin-related protein 2/3 (Arp2/3) complex (containing seven strongly associated subunits of which two are the actin-related proteins Arp2 and Arp3) that is responsible for catalysing the polymerization of actin. PtdIns4,5P_2 appears to have two actions. It binds to and activates WASP and to Scar and thus contributes to the relay of information to Arp2/3. In addition, it can alter the activity of various proteins that modify the structure of actin. For example, it is an indirect activator of Cdc42 and it may loosen the gelsolin caps on the end of the barbed ends, opening up new sites for actin polymerization. The PtdIns4,5P_2 may also induce a conformational change in vinculin that enables it to bind to talin during

Module 2: | Figure PtdIns4,5P_2 signalling



Multiple signalling functions of PtdIns4,5P_2.
PtdIns4,5P_2 has multiple signalling functions in the plasma membrane. It is the lipid precursor used for the generation of second messengers such as $InsP_3$, diacylglycerol (DAG) and PtdIns3,4,5P_3. It functions in the regulation of the phospholipase D (PLD) signalling pathway. In addition, PtdIns4,5P_2 can function as a messenger to control a whole variety of cellular systems, including the cytoskeleton, phagocytosis, exocytosis, endocytosis and ion channels. All of these processes are sensitive to changes in the membrane level of this lipid.

the formation of the focal adhesion complex (Module 6: Figure vinculin function).

PtdIns4,5P_2 function in focal adhesions

The Iγ isoform of PtdIns4P 5-kinase (PtdIns4P 5-K) is highly localized at the focal adhesion complex, where it is tightly bound to talin to regulate the local formation of PtdIns4,5P_2 (Module 6: Figure integrin signalling).

PtdIns4,5P_2 regulation of exocytosis

The synthesis of PtdIns4,5P_2 has been implicated in the ATP-dependent processes of priming vesicles as part of the exocytotic mechanism. The priming process seems to depend upon a number of steps that begin with the PtdIns transfer protein carrying PtdIns to the vesicle membrane where it is phosphorylated to PtdIns4P by PtdIns 4-kinase (PtdIns 4-K) and then to PtdIns4,5P_2 by the PtdIns4P 5-kinase (PtdIns4P 5-K). It is still not clear exactly how this PtdIns4,5P_2 functions to prime the vesicle for exocytosis.

The ability of the neuronal Ca^{2+} sensor-1 (NCS-1) to facilitate exocytosis may depend on its ability to activate the PtdIns 4-kinase (PtdIns 4-K).

PtdIns4,5P_2 regulation of membrane trafficking and endocytosis

PtdIns4,5P_2 plays a role in endocytosis by targeting adaptor protein 2 (AP2) and various clathrin-associated sortin proteins (CLASPs) (Module 4: Figure cargo sorting proteins) to endocytic vesicles to provide a scaffold to bind clathrin to form clathrin-coated pits (Module 4: Figure endocytosis). The PtdIns4P 5-kinase Iγ is recruited to the plasma membrane, where it phosphorylates PtdIns4P to PtdIns4,5P_2, which recruits the adaptor protein AP-2 to form the clathrin-coated pits.

PtdIns4,5P_2 regulation of phagocytosis

When macrophages engulf foreign particles, PtdIns4P 5-kinase (PtdIns4P 5-K) Iα is recruited to the plasma membrane at the phagosome cup, where it induces a local pulse of PtdIns4,5P_2, which is then rapidly degraded by phospholipase C (PLC) to leave behind diacylglycerol (DAG). This local formation of PtdIns4,5P_2 seems essential for the early process of phagocytosis.

The localized transient increase in PtdIns4,5P_2 may function in the initial recruitment of actin to the phagocyte.

PtdIns4,5P_2 activation of phospholipase D

Phospholipase D (PLD), which catalyses the hydrolysis of phosphatidylcholine (PC) to generate phosphatidic acid (PA), exists as two isoforms, PLD1 and PLD2. The PLD1 is primarily located on vesicles associated with the endosomal/lysosomal pathway, whereas PLD2 is mainly found on the plasma membrane. An interaction between PtdIns4P 5-kinase α (PtdIns4P 5-Kα) and the PLDs ensures that there is a local generation of PtdIns4,5P_2 that

plays an important role in regulating PLD activity (Module 2: Figure PLD signalling).

PtdIns4,5P_2 regulation of ion channels and exchangers

A number of ion channels and exchangers appear to be regulated by PtdIns4,5P_2. In some cases, the hydrolysis of PtdIns4,5P_2 results in channel opening as occurs for the transient receptor potential (TRP) vanilloid 1 (TRPV1) channel, whereas the TRPC6 channel, TRP melastatin 4 (TRPM4), TRP melastatin 7 (TRPM7) and TRP melastatin 8 (TRPM8) channels are closed (Module 2: Figure PtdIns4,5P_2 regulation of TRP channels). In the case of the inward rectifying K^+ channels, lipid hydrolysis results in channel opening (Module 2: Figure PtdIns4,5P_2 regulation of K^+ channels). Modulation of the TRPV1 channel is particularly important in hyperalgesia (Module 10: Figure nociception).

Much of the information on this regulatory function of PtdIns4,5P_2 has emerged from studies on K^+ channels. Stimulation of phospholipase C (PLC) by G protein-coupled receptors (GPCRs) causes a reduction in the level of PtdIns4,5P_2, which results in a reduction in the activity of some of the voltage-dependent K^+ (K_v) channels:

- The human *ether-a-go-go*-related (HERG) K^+ channel (K_v11.1 channel)
- The K_v7.1 channel, which is coded for by the KCNQ1 gene, is regulated by PtdIns4,5P_2. This inositol lipid keeps the channel open, but when PtdIns4,5P_2 is hydrolysed by angiotensin II, the channel closes. Such lipid regulation is seen in adrenal zona glomerulosa cells (Module 7: Figure glomerulosa cell signalling) and also in the heart. Mutation of KCNQ1 causes long QT) syndrome (LQT).
- The heteromultimer KCNQ2/3 is responsible for the M channel in neurons and has been linked to a form of epilepsy known as benign familial neonatal convulsions.
- KCNQ4, which codes for K_v7.4 (Module 3: Table voltage-dependent K^+ channels), is the delayed rectifier that controls K^+ efflux from sensory hair cells of the inner ear. KCNQ4 mutations cause autosomal dominant nonsyndromic deafness type 2 (DFNA2).

PtdIns 3-kinase signalling

The PtdIns 3-kinase signalling cassette, which generates the lipid second messenger phosphatidylinositol 3,4,5-tetrakis phosphate (PtdIns3,4,5P_3), has multiple functions in regulating a wide range of cellular processes such as the control of glycogen metabolism, lipid synthesis, protein synthesis, gene transcription and cell growth, inflammation, cytoskeletal rearrangement and apoptosis (Module 2: Figure PtdIns 3-kinase signalling). With regard to the latter, the PtdIns 3-kinase signalling cassette has a special function in cell survival through its contribution to the hormonal modulation of apoptosis and by enhancing the activity of the target of rapamycin (TOR). PtdIns 3-kinase signalling is one of the major pathways used by the insulin receptor to regulate energy uptake and storage.

This lipid signalling pathway has a special relationship to Ca^{2+} signalling in that it can regulate the way in which external signals can maintain information flowing through the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette.

Operation of the PtdIns 3-kinase signalling cassette can be considered in two parts:

- Generation and metabolism of the 3-phosphorylated lipid messengers
- Mode of action of the 3-phosphorylated lipid messengers

One of the important functions of this pathway is to regulate cell proliferation. Since a number of human cancers are found to have mutations in certain components of this pathway, there is considerable interest in the relationship between PtdIns 3-kinase signalling and cancer. A decline in the activity of this signalling pathway is the cause of insulin resistance that leads to diabetes (Module 12: Figure insulin resistance).

Generation and metabolism of the 3-phosphorylated lipid messengers

Cells contain a number of phosphoinositides carrying a phosphate on the 3-position (Module 2: Figure phosphoinositide metabolism). One of these is PtdIns3,4,5P_3, which is the main second messenger operating in the PtdIns 3-kinase signalling pathway. This highly phosphorylated lipid is generated in cells following activation of either G protein-coupled or tyrosine kinase-coupled receptors (Module 2: Figure PtdIns 3-kinase signalling).

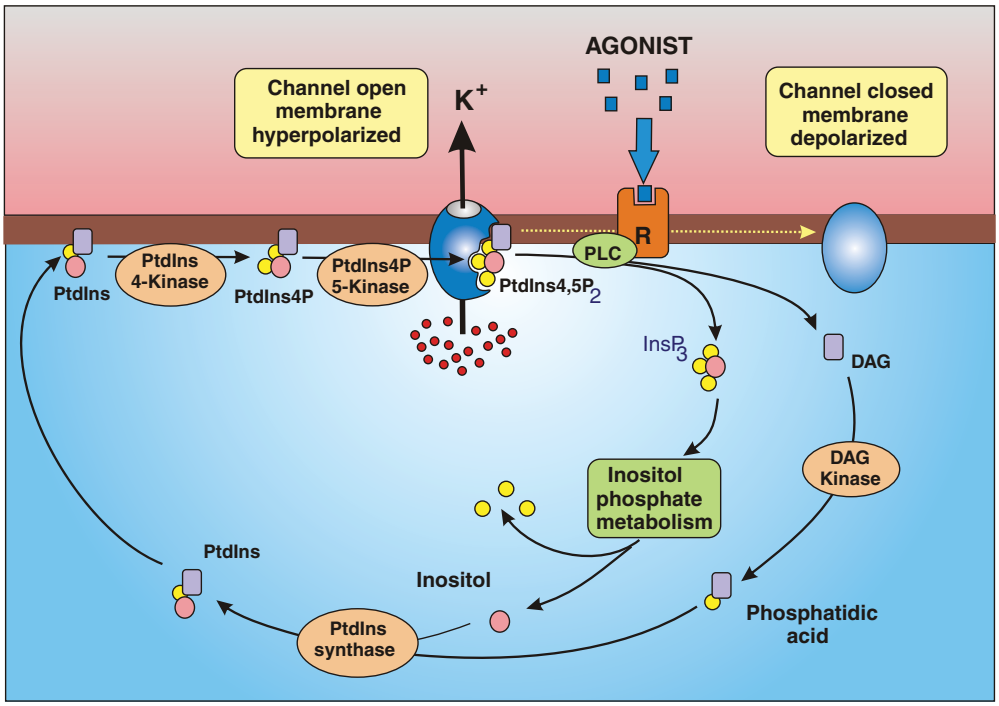
These 3-phosphorylated lipid messengers are metabolized either by the type II inositol polyphosphate 5-phosphatases or by phosphatase and tensin homologue deleted on chromosome 10 (PTEN).

Mode of action of the 3-phosphorylated lipid messengers

The lipid messengers PtdIns3,4P_2 and PtdIns3,4,5P_3 act within the plane of the plasma membrane by binding to a great variety of target proteins (Module 2: Figure PtdIns 3-kinase signalling). Most of these downstream targets are soluble proteins that translocate to the membrane by binding to the lipid messengers through various lipid-binding domains [e.g. pleckstrin homology (PH), Phox homology (PX), C2 domains and basic amino acid regions] (Module 6: Figure modular lipid-binding domains). Once drawn on to the membrane, these target proteins are activated and function as effectors to control a large number of cellular processes:

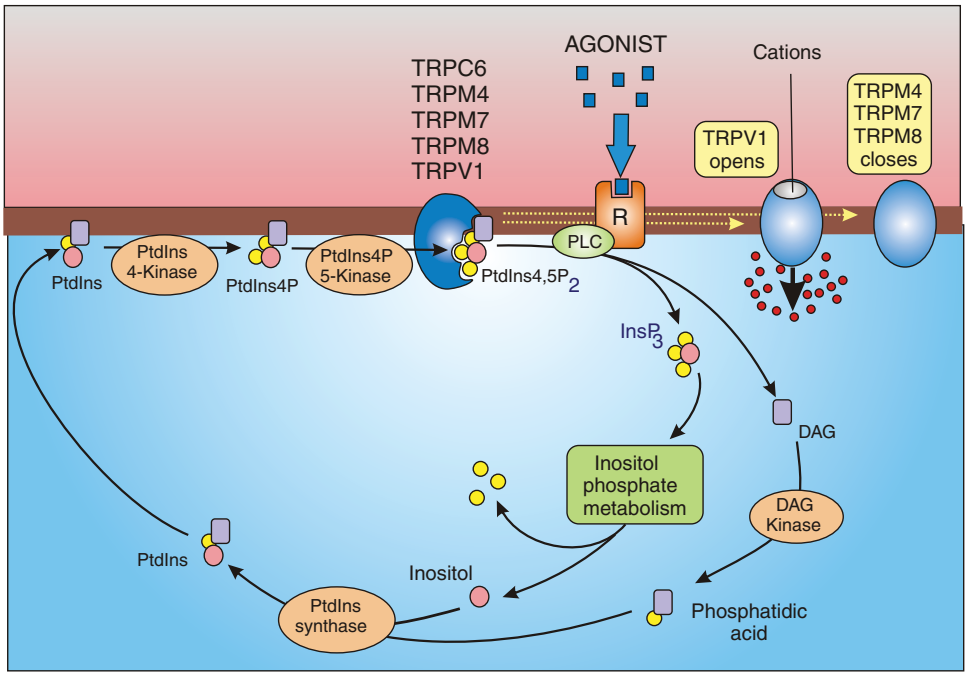
- Contribution to liver cell signalling mechanisms to control glycogen metabolism (Module 7: Figure liver cell signalling).
- Functions in the insulin control of skeletal muscle glycogen synthesis (Module 7: Figure skeletal muscle E-C coupling).
- Functions in white fat cells to control lipid metabolism (Module 7: Figure lipolysis and lipogenesis).
- Functions in the control of cell proliferation (Module 9: Figure growth factor signalling).

Module 2: | Figure PtdIns4,5P_2 regulation of K^+ channels



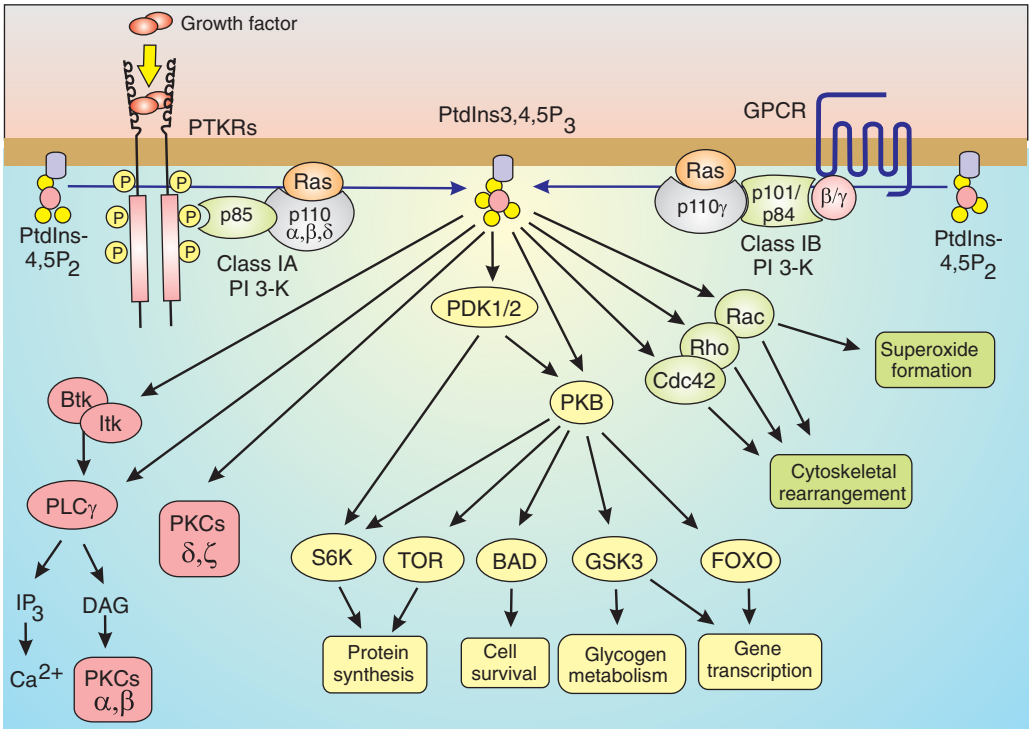
Regulation of K^+ channels by agonist-dependent hydrolysis of PtdIns4,5P_2.
A number of different K^+ channels are activated when they bind to PtdIns4,5P_2. Upon stimulation by agonists that are coupled to phospholipase C (PLC), this lipid is hydrolysed to $InsP_3$ and diacylglycerol (DAG), and this removal causes the channel to shut. The channel will open again when another PtdIns4,5P_2 molecule associates with the lipid-binding site. The supply of PtdIns4,5P_2 is maintained by the resynthesis of PtdIns and its rephosphorylation by PtdIns 4-kinase and then by PtdIns4P 5-kinase (Module 2: Figure $InsP_3$/DAG recycling).

Module 2: | Figure PtdIns4,5P_2 regulation of TRP channels



Regulation of TRP channels by agonist-dependent hydrolysis of PtdIns4,5P_2.
A number of transient receptor potential (TRP) channels are opened (TRPM4, TRPM7 and TRPM8) or closed (TRPV1) when they are bound to PtdIns4,5P_2. Upon stimulation by agonists that are coupled to phospholipase C (PLC), this lipid is hydrolysed, and its removal causes the channel to open (TRPV1) or closed (TRPM4, TRPM7 and TRPM8). The channel returns to the resting state when PtdIns4,5P_2 re-associates with the lipid-binding site. The supply of PtdIns4,5P_2 is maintained by the resynthesis of PtdIns and its sequential phosphorylation by PtdIns 4-kinase and PtdIns4P 5-kinase. The operation of this signalling system is particularly important in nociception (Module 10: Figure nociception).

Module 2: | Figure PtdIns 3-kinase signalling



The PtdIns 3-kinase signalling pathway.

The precursor lipid PtdIns4,5P_2 is phosphorylated on the 3-position by Class I PtdIns 3-kinases (PtdIns 3-Ks) to generate the lipid second messenger PtdIns3,4,5P_3. The Class 1A enzyme has regulatory subunits such as p85 that attaches the catalytic p110 subunits to the phosphorylated tyrosine residues on the cytoplasmic domains of activated growth factor receptors. The Class 1B enzymes (p110γ), which are activated by G protein-coupled receptors (GPCRs), translocate to the membrane by binding to the Gβγ subunit. When brought into the vicinity of the membrane, these PtdIns 3-kinases form the 3-phosphorylated lipid messenger PtdIns3,4,5P_3, to regulate a large number of processes. It binds to other signalling components such as Btk and PLCγ (shown in pink). It stimulates phosphoinositide-dependent kinase 1/2 (PDK1/2) and protein kinase B (PKB), which activate a large number of downstream targets (yellow). It also activates monomeric G proteins (Rac, Rho and Cdc42) to stimulate both cytoskeletal rearrangement and superoxide radical ($O_2^{-\bullet}$) formation (shown in green).

- PtdIns 3-kinase signalling in cardiac hypertrophy is an example where this signalling system carries out multiple roles in the same cell (i.e. inhibits apoptosis, activates protein synthesis and facilitates a programme of foetal gene transcription (Module 12: Figure hypertrophy signalling mechanisms).
- Modulation of InsP_3-induced Ca^{2+} release by phosphorylation of the InsP_3 receptor, and this could provide a possible mechanism for the action of insulin in liver cells (Module 7: Figure liver cell signalling).
- PtdIns3,4,5P_3 activates the monomeric G proteins Rac (Module 2: Figure Rac signalling), Rho (Module 2: Figure Rho signalling) and Cdc42 (Module 2: Figure Cdc42 signalling).
- Formation of osteoclast podosomes (Module 7: Figure osteoclast podosomes).
- Contributes to the amplification of the early polarity signalling during neutrophil chemotaxis (Module 11: Figure neutrophil chemotactic signalling).
- Functions as a regulator of autophagy (Module 11: Figure autophagy).

Phosphoinositide-dependent kinase 1 (PDK1)

One of the main functions of phosphoinositide-dependent kinase 1 (PDK1) is to phosphorylate protein kinase B (PKB), which translocates to the membrane, where it binds to PtdIns3,4,5P_3 (Module 2: Figure PtdIns 3-kinase signalling). This binding alters the conformation of PKB so that critical sites become available to PDK1. Like PKB, PDK1 has pleckstrin homology (PH) domains that also bind to the 3-phosphorylated lipid messengers that serve to activate their kinase activity. PDK1 is also responsible for phosphorylating and activating other signalling molecules such as ribosomal S6 protein kinase 1 (S6K1) and atypical protein kinase Cζ (PKCζ).

Protein kinase B (PKB)

Protein kinase B (PKB), which is also known as Akt, is a serine/threonine protein kinase that functions in the PtdIns 3-kinase signalling pathway (Module 2: Figure PtdIns 3-kinase signalling). PKB has three members (PKBα, PKBβ and PKBγ) that are activated through a two-stage process. Firstly, it translocates to the membrane by binding to either PtdIns3,4P_2 or PtdIns3,4,5P_3 through pleckstrin homology (PH) domains. The latter appears to be particularly important when cells are studied *in vivo*. The next stage depends upon its interaction with phosphoinositide-dependent kinase 1 (PDK1), which then completes the activation process by phosphorylating PKB on Thr-308. In addition, a DNA-dependent protein

kinase (previously called PDK2) phosphorylates Ser-473. The activated PKB then functions to stimulate a variety of molecular targets, including glycogen synthase kinase-3 (GSK-3) (which mediates the effect of insulin on glycogen metabolism) and the pro-apoptotic factor Bad. PKB also plays a role in redox signalling in apoptosis.

PKB is a key player in cell growth control, where it functions by phosphorylating tuberous sclerosis 1 and 2 (TSC1/2), which integrate a number of inputs that control the activity of the target of rapamycin (TOR) (Module 9: Figure target of rapamycin signalling). PKB can also translocate into the nucleus, where it phosphorylates the Forkhead box O (FOXO) transcription factors (Module 4: Figure FOXO control mechanisms) that play an important role in regulating the cell cycle.

A reduction in PKB activity by various mechanisms seems to be responsible for the onset in insulin resistance (Module 12: Figure insulin resistance).

Ribosomal S6 protein kinase 1 (S6K1)

This kinase plays an important role in regulating protein synthesis by phosphorylating the S6 ribosomal protein (a component of the 40S ribosomal subunit), which then enhances the translation of those mRNA transcripts that contain a polypyrimidine tract at the 5' transcriptional start site. The control of S6K1 is complex in that it depends upon a priming step that is followed by series of phosphorylation events at multiple sites that are sensitive to a number of kinases. The priming step depends on Ca^{2+}, which appears to act by opening up the enzyme so that it becomes sensitive to phosphorylation by different kinases. One of these is phosphoinositide-dependent kinase 1 (PDK1), which carries out the wortmannin-sensitive phosphorylation. In addition there are rapamycin-sensitive sites phosphorylated by the target of rapamycin (TOR) (Module 9: Figure target of rapamycin signalling).

Glycogen synthase kinase-3 (GSK-3)

One of the primary functions of insulin is to act through glycogen synthase kinase-3 (GSK-3) to stimulate the conversion of glucose into glycogen by increasing the activity of glycogen synthase (Module 7: Figure skeletal muscle E-C coupling). GSK-3 is a proline-directed protein kinase that usually requires a priming kinase to add a phosphate to its substrate before it can carry out further phosphorylations. Casein kinase I (CKI) often acts as the priming kinase as it does for β-catenin in the Wnt signalling pathway (Module 2: Figure Wnt canonical pathway).

GSK-3 also has a number of other signalling functions:

- It phosphorylates nuclear factor of activated T cells (NFAT) and thus contributes to the NFAT shuttle (Module 4: Figure NFAT activation).
- It contributes to the Wnt signalling pathway (Module 2: Figure Wnt canonical pathway).
- It functions in dorsoventral specification during development (Module 8: Figure dorsoventral specification).
- It is one of the kinases that phosphorylates the transcription factor p53 (Module 4: Figure p53 domains).
- It contributes to Myc degradation.
- It phosphorylates the neuron-specific microtubule-associated protein tau, which forms tangles in neurons during the onset of Alzheimer's disease (Module 12: Figure amyloid plaques and tangles).
- It plays a significant role in cardiac gene transcription, where it has an important role in the NFAT shuttle (Module 12: Figure hypertrophy signalling mechanisms).
- GSK-3 activity can be regulated by disrupted in schizophrenia 1 (DISC1), which is mutated in some patients with schizophrenia. When mutated, DISC1 alters both the structure and function of fast-spiking interneurons (Module 12: Figure schizophrenia).

Tec tyrosine kinase family

All members of this tyrosine kinase family are cytosolic, but they translocate rapidly to the membrane through pleckstrin homology (PH) domains that are particularly sensitive to PtdIns3,4,5P_3. A prominent member of this family is Bruton's tyrosine kinase (Btk). Once bound to the membrane, these enzymes are also phosphorylated by Src family tyrosine kinases such as Lyn and Src. Activation thus requires both translocation to the membrane and phosphorylation on tyrosine residues. One of the functions of these Tec tyrosine kinases is to enhance Ca^{2+} signalling by maintaining the activity of phospholipase Cγ (PLCγ). This enhancement of PLCγ activity by the Tec kinases represents a major point of interaction between the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette and the PtdIns 3-kinase signalling cassette.

Bruton's tyrosine kinase (Btk)

Bruton's tyrosine kinase (Btk) is one of the non-receptor protein tyrosine kinases (Module 1: Figure non-receptor tyrosine kinases) that plays an important role in B cells where it functions to activate PLCγ2 (Module 9: Figure B cell activation). It has a similar mode of action in the mast cell FcεRI signalling pathway (Step 7 in Module 11: Figure FcεRI mast cell signalling).

Inactivation of Btk results in Bruton's type X-linked agammaglobulinaemia.

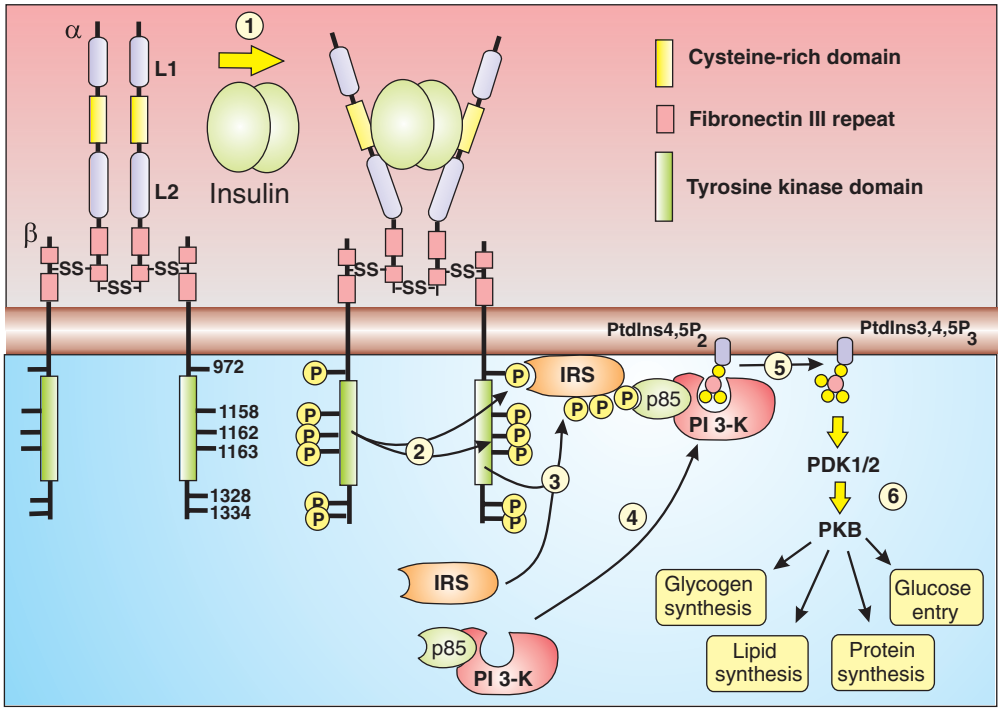
Inducible T cell kinase (Itk)

As for Bruton's tyrosine kinase (Btk) in B cells, inducible T cell kinase (Itk) has a similar function in controlling the maturation of T cells by activating phospholipase Cγ1 (PLCγ1) (Module 9: Figure TCR signalling).

Insulin receptor

Insulin has a major role to play in regulating a variety of cellular processes, with particular emphasis on the regulation of energy uptake and storage. The action of insulin is carried out by the insulin receptor, which is a disulphide-linked homodimer that belongs to the large family of receptors that have tyrosine kinase domains (Module 1: Figure tyrosine kinase-linked receptors). An important component of the signal transduction mechanism used by the insulin receptor is the insulin receptor substrate (IRS),

Module 2: | Figure insulin receptor



Activation of the PtdIns 3-kinase signalling pathway by the insulin receptor.
The insulin receptor is a homodimer that is connected together by disulphide bonds. Each monomer consists of an α-chain, which is extracellular and has the insulin-binding region. The β-chain has a single membrane-spanning region with a large intracellular region that contains the tyrosine kinase domain that is critical for the process of signal transduction that proceeds through a sequence of events as described in the text.

which is a classical scaffolding protein (Module 6: Figure IRS domain structure).

The function of the insulin receptor depends upon the activation of the PtdIns 3-kinase signalling pathway that occurs through the sequence of events shown in Module 2: Figure insulin receptor:

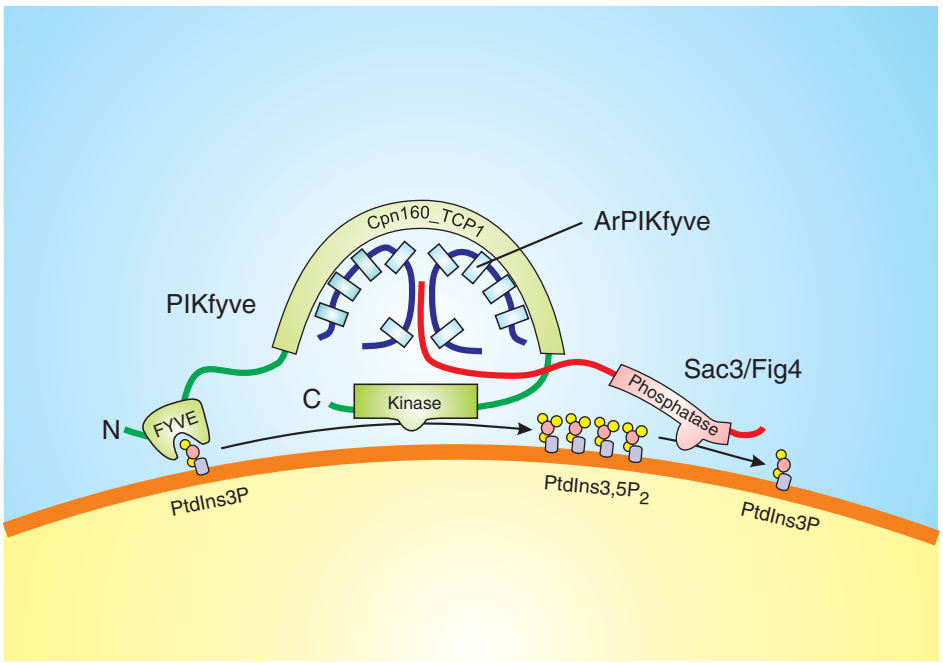
1. Insulin binds to the extracellular domain to induce a conformational change in the receptor resulting in the activation of the intracellular tyrosine kinase domains.
2. Once activated, the tyrosine kinase domains undergo autophosphorylation whereby they phosphorylate up to six tyrosine residues on the opposite β chain.
3. The insulin receptor substrate (IRS), which is a scaffolding domain (Module 6: Figure IRS domain structure), attaches itself to juxtamembrane phosphotyrosine residue 972. Once it is drawn into the vicinity of the receptor, the tyrosine kinase domain also phosphorylates IRS on multiple residues.
4. The phosphotyrosine residues on IRS then function as docking sites for Class IA PtdIns 3-kinase (PI 3-K).
5. The PI 3-K then phosphorylates PtdIns4,5P_2 to form the lipid second messenger PtdIns3,4,5P_3.
6. The PtdIns3,4,5P_3 then activates the PtdIns 3-kinase signalling pathway (Module 2: Figure PtdIns 3-kinase signalling) that controls a number of cellular processes:
   - It stimulates lipogenesis in white fat cells (Module 7: Figure lipolysis and lipogenesis).
   - It stimulates glucose uptake and glycogen synthesis in skeletal muscle cells (Module 7: Figure skeletal muscle E-C coupling).
   - It stimulates glycogen synthesis in liver cells (Module 7: Figure liver cell signalling).

The onset of diabetes (i.e. Type 2 diabetes) begins when cells become resistant to the action of insulin in energy uptake and storage.

PtdIns3,5P_2 signalling cassette

The PtdIns3,5P_2 signalling cassette functions in membrane and protein trafficking and particularly in the events that occur during the early endosome maturation to lysosome (see step 10 in Module 4: Figure membrane and protein trafficking). The early endosome is rich in PtdIns3P that is converted into PtdIns3,5P_2 (Module 2: Figure localized inositol lipid signalling), which then orchestrates the trafficking events responsible for the transformation of the early endosome into the multivesicular endosome (MVE) that end up as lysosomes. The PtdIns3,5P_2 seems to act by stimulating TRPML1 channels to create the local domains of Ca^{2+} necessary to activate the endosome fusion to form the lysosomes. The formation of PtdIns3,5P_2 is carried out by PhosphoInositide Kinase for five position containing a Fyve finger (PIKfyve), which has a number of protein interaction domains that enables it to interact with a number of other proteins to form a functional PIKfyve–ArPIKfyve–Sac3

Module 2: | Figure PIKfyve activation



PIKfyve-ArPIKfyve-Sac3 (PAS) core complex
The PIKfyve inositol lipid kinase, which phosphorylates PtdIns3P to PtdIns3,5P_2, functions within a PAS complex containing the scaffolding protein ArPIKfyve and the Sac3/Fig4 phosphatase. The latter hydrolyses PtdIns3,5P_2 back to PtdIns3P. Information for this drawing was taken from Figure 6 in Ikonomov et al. 2009.

(PAS) complex (Module 2: Figure PIKfyve activation). The N-terminal FYVE domain binds to the substrate PtdIns3P. The Cpn160_TCP1 domain interacts with the associated regulator of PIKfyve (ArPIKfyve), which forms a dimer and functions as a scaffolding protein to assemble the PAS complex. The C-terminal kinase domain is then positioned to catalyse the conversion of PtdIns3P into PtdIns3,5P_2. The suppressor of actin 3 (Sac3), which is the mammalian orthologue of Fig4 in yeast, is a SAC domain lipid phosphatase that removes the 5-phosphate from PtdIns3,5P_2 to form PtdIns3P. Sac3 is part of the PAS complex and has an important structural role in that it also helps to facilitate the kinase activity of PIKfyve.

Mutations in Sac3/Fig3 have been linked to Charcot-Marie-Tooth disease 4J.

PtdIns3P signalling cassette

PtdIns3P, which is formed by the phosphorylation of PtdIns by the Class III PtdIns 3-kinase, has a specific role to play in controlling a number of cellular processes.

One of the main functions of PtdIns3P is to regulate intracellular vesicle trafficking especially at the level of the early endosome (Module 2: Figure localized inositol lipid signalling). Most of the PtdIns3P is found on internal membranes and particularly those that function in vesicle trafficking:

- This function is particularly evident in the endosome vesicle fusion to the early endosome (Module 4: Figure endosome vesicle fusion)
- Early endosome sorting and intraluminal vesicle formation (Module 4: Figure intraluminal endosomal vesicle formation)
- Early endosome to plasma membrane trafficking (Module 4: Figure early endosome budding)
- Early endosome to *trans*-Golgi network (TGN) trafficking (Module 4: Figure endosome budding to TGN)
- Phagosome maturation (Module 4: Figure phagosome maturation).
- Formation of PtdIns3P has a role to play in activating the isolation membrane responsible for initiating the events of autophagy (Module 11: Figure autophagy).
- PtdIns3P plays a role in regulating the activity of the intermediate conductance (IK) channel, particularly as part of the relationship between K^+ channels and cell proliferation.

PtdIns3P levels can be reduced through the action of the myotubularin family of 3-phosphatases (MTM1 and MTMR1–MTMR14). These enzymes are of interest because MTM1 mutations are responsible for X-linked recessive myotubular myopathy, MTMR2 mutations occur in Charcot-Marie-Tooth disease 4B and MTMR14 mutations have been linked to centronuclear myopathy (CNM).

PtdIns4P signalling cassette

For a long time, PtdIns4P was considered to be only a precursor of PtdIns4,5P_2, but now there are indications that it might have a signalling role to regulate membrane and protein trafficking. One role is to control coat removal following the formation of clathrin-coated vesicles

(Module 4: Figure scission of endocytic vesicles). Another role is to control trafficking through the endoplasmic reticulum/Golgi transport system (Module 2: Figure localized inositol lipid signalling), where it is formed by at least two PtdIns 4-kinases (PtdIns 4-Ks). The small GTPase ADP-ribosylation factor Arf-1 recruits the PtdIns 4-KIIIβ to the Golgi. At resting levels of Ca^{2+}, this PtdIns 4-KIIIβ is kept inactive when bound to the Ca^{2+}-sensing proteins calneuron-1 or calneuron-2. In response to a local pulse of Ca^{2+}, the inhibitory calneurons are replaced by neuronal Ca^{2+} sensor 1 (NCS-1) that stimulates PtdIns 4-KIIIα to begin to produce the PtdIns4P necessary for vesicle formation.

In addition, the Golgi also has a PtdIns 4-kinase α that produces the PtdIns4P that associates with activating protein 1 (AP-1) to regulate the trafficking of clathrin-coated vesicles through the *trans*-Golgi network.

PtdIns5P signalling cassette

PtdIns5P has been implicated in several signalling events. It may modulate the PtdIns 3-kinase signalling pathway by interfering with the metabolism of the lipid messenger PtdIns3,4,5P_3. It may also have a signalling role within the nucleus to control the response to DNA damage. PtdIns5P located on chromatin may provide an anchor to bind inhibitor of growth family, member 2 (ING2), which then results in acetylation of the p53 tumour suppressor.

Multipurpose inositol polyphosphate signalling pathway

The process of inositol phosphate metabolism generates a large number of inositol phosphates (Module 2: Figure inositol phosphate metabolism). Many of these are metabolic intermediates, but some have been implicated in a variety of control functions:

Ins1,4P_2

There is some evidence to suggest that Ins1,4P_2 may function within the nucleus to activate DNA polymerase. This inositol phosphate has also been implicated in Ca^{2+} signalling and cardiac hypertrophy.

Ins1,3,4P_3

This inositol phosphate has an important signalling function as a negative regulator of the Ins1,3,4,5,6P_5 1-phosphatase (**Module 2: Figure inositol phosphate metabolism**) that controls the level of Ins3,4,5,6P_4, which is an inhibitor of Ca^{2+}-sensitive Cl^- channels.

Ins1,3,4,5P_4

This inositol phosphate, which is formed by phosphorylating Ins1,4,5P_3, has been implicated in the control of Ca^{2+} entry into cells. However, its mode of action is unknown. Some clues concerning its action may come from the identification of $GAP1^{IP4BP}$, which is one of the GTPase-activating protein (GAP) family (Module 2: Table monomeric G protein toolkit). $GAP1^{IP4BP}$ normally associates with the plasma membrane. When Ins1,3,4,5P_4 binds to the membrane-anchoring domain of $GAP1^{IP4BP}$, it causes this GAP to come off the membrane. A closely related $GAP1^m$ is located on the ER.

Ins3,4,5,6P_4

Ins3,4,5,6P_4 functions as an inhibitor of the Ca^{2+}-sensitive Cl^- channels (CLCAs) in epithelial cells, which regulate salt and fluid secretion, cell volume homoeostasis and electrical excitability in neurons and smooth muscle cells. It appears to act by preventing Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) from activating the channel.

Ins3,4,5,6P_4 is formed by an Ins1,3,4,5,6P_5 1-phosphatase, which is activated by Ins1,3,4P_3 (**Module 2: Figure inositol phosphate metabolism**).

InsP_6

There are a number of suggestions concerning the possible messenger role of InsP_6. Cells contain high levels of InsP_6 (approximately 15–100 μM), and much of this is probably not in solution, but is probably attached to the phospholipids in membranes through electrostatic interactions with bivalent cations. The level of InsP_6 does not change much during acute stimulation, but its level can vary over the long term, as occurs during the cell cycle and during cellular differentiation. The various proposed messenger functions outlined below may thus depend upon highly localized fluctuations in specific cellular compartments.

Trafficking of vesicles

InsP_6 may inhibit clathrin cage assembly by binding to adaptor protein (AP)-2 and -3. Such a role in membrane trafficking is also consistent with the observation that InsP_6 binds to synaptotagmin by competing with the inositol lipid-binding site. It therefore seems that InsP_6 may function as a negative regulator of endocytic vesicle traffic.

Such a possibility may explain the observation that GRAB, which is a guanine nucleotide exchange factor that acts on Rab3A, interacts with InsP_6 kinase which converts InsP_6 into InsP_7.

Endocytosis

In insulin-secreting β-cells, InsP_6 appears to promote dynamin I-mediated endocytosis through a mechanism that depends upon protein kinase C (PKC), which may act to inhibit the phosphoinositide phosphatase synaptojanin, thereby raising the level of PtdIns4,5P_2 that has been implicated in vesicle dynamics.

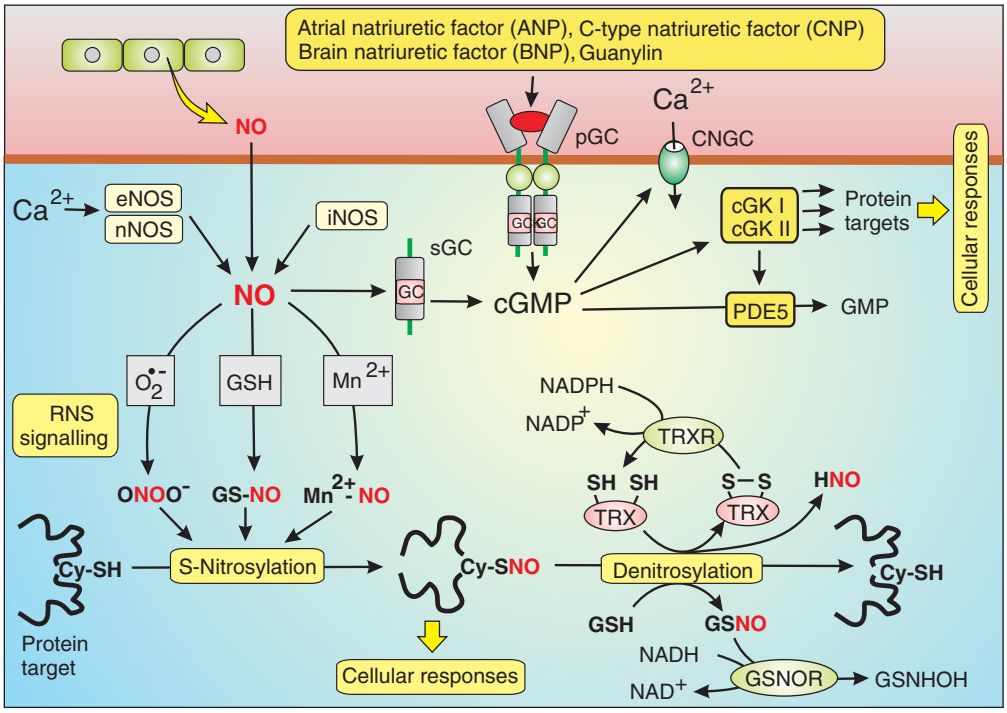
Regulation of Ca^{2+} channels

InsP_6 is present in many brain regions and appears to be elevated following neural activity. It may act by stimulating adenylyl cyclase to produce cyclic AMP, which then increases the activity of L-type Ca^{2+} channels. Alternatively, it may activate L-type Ca^{2+} channels through an inhibition of protein phosphatases. In vascular smooth muscle cells, InsP_6 appears to act through a protein kinase C (PKC)-dependent pathway.

Regulation of gene transcription

Studies on yeast have revealed that ARG82, which is an Ins1,4,5P_3 6-kinase that phosphorylates Ins1,4,5P_3 within

Module 2: | Figure NO and cyclic GMP signalling



Nitric oxide (NO) and cyclic GMP (cGMP) signalling pathways.
The nitric oxide (NO) signal can either diffuse in from other cells as a paracrine signal or it can be generated within the cell by different NO synthases (NOSs). The NO has two main actions. It can stimulate soluble guanylyl cyclase (sGC) to form the messenger cyclic GMP (cGMP), which can act through cyclic nucleotide-gated channels (CNGCs) to promote Ca^{2+} entry or it can activate cyclic GMP-dependent protein kinase (cGK). Cyclic GMP is also formed by a plasma membrane guanylyl cyclase (pGC), which is part of the single membrane-spanning receptor activated by a variety of peptides such as atrial natriuretic peptide (ANP), brain type natriuretic factor (BNP), C-type natriuretic factor (CNP) and guanylin. The other main action is through reactive nitrogen species (RNS) signalling mechanisms that depend upon an S-nitrosylation reaction, which is reversed by denitrosylation reactions.

the nucleus to form Ins1,4,5,6P_4, functions as a transcriptional regulator.

Regulation of mRNA export from the nucleus
Studies on yeast indicate that InsP_6 formed by an inositol polyphosphate kinase located on the nuclear pores may facilitate the export of mRNA from the nucleus.

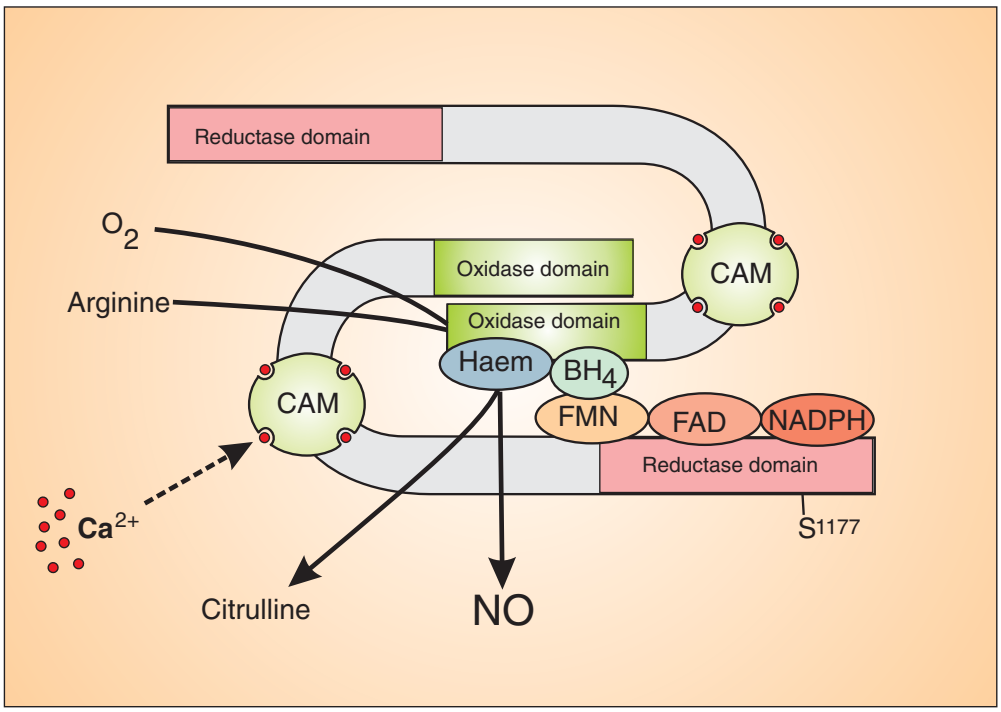
PP-InsP_4
This diphosphorylated inositol phosphate has been considered as an orphan signal, as its function is unknown. Its level declines in response to either cyclic AMP or cyclic GMP.

InsP_7 (PP-InsP_5)
This diphosphoinositide, which is formed by the phosphorylation of InsP_6 by an InsP_6 kinase (see Step 14 in Module 2: Figure inositol phosphate metabolism), has been implicated in a number of functions. It may serve as an energy store as has been suggested for InsP_8 ([PP]$_2$-InsP_4). InsP_7 has been shown to regulate insulin signalling by inhibiting the activity of protein kinase B (PKB), which has a central role in the insulin receptor pathway responsible for regulating a large number of insulin-sensitive processes (Module 2: Figure insulin receptor). Through this inhibitory effect on insulin signalling, InsP_7 may contribute to the onset of insulin resistance (Module 12: Figure insulin resistance).

InsP_8 ([PP]$_2$-InsP_4)
InsP_8 is a diphosphoinositide that may function as a high-energy store in that it can donate a high-energy phosphate to ADP to form ATP.

Nitric oxide (NO)/cyclic GMP signalling pathway
Nitric oxide (NO) is a highly diffusible messenger, which passes rapidly through cell membranes. It can act as a second messenger within its cell of origin or it can diffuse across membranes to act on neighbouring cells as a paracrine signalling agent. NO synthesis is carried out by nitric oxide synthase (NOS), which comes in three different forms: neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS). These different isoforms share a similar NO synthetic reaction mechanism, which uses L-arginine as a substrate and O_2 and NADPH as co-substrates to form NO. NOS regulation is very different for the three isoforms and is partly dependent on the way they are located in different parts of the cell. The action of NO is complex in that it can transmit information in markedly different ways. One of its actions is mediated through the cyclic GMP signalling pathway, where it stimulates soluble guanylyl cyclase to produce the cyclic GMP that can modify the properties of ion channels, protein phosphatases or cyclic nucleotide phosphodiesterase. NO can also act through the

Module 2: | Figure NO synthase mechanism



Nitric oxide synthase (NOS) reaction mechanism.
The two nitric oxide synthase (NOS) monomers are lined up alongside each other so that the reductase domain of one functions together with the oxidase domain of its neighbour. The enzyme dimer also functions as a scaffold to organize the other components of the reaction mechanism such as the bound cofactors [flavin-adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), haem and tetrahydrobiopterin (BH_4)] and the tightly bound prosthetic group calmodulin (CaM). The formation of NO is driven by an NADPH-dependent electron flux that passes from the reductase towards the oxidase domain. The attached haem is the terminal electron acceptor, which binds the oxygen that is inserted into arginine to form the hydroxyarginine that decays to release NO. One of the important regulators of NOS is calmodulin, which is constitutively active in inducible NOS (iNOS), but requires an elevation of Ca^{2+} for both neuronal NOS (nNOS) and endothelial NOS (eNOS). One consequence of increasing the concentration of Ca^{2+} in cells is therefore to increase the formation of NO.

reactive nitrogen species (RNS) signalling pathways, whereby the NO alters the activity of a variety of protein targets through a nitrosylation reaction. This diverse NO/cyclic GMP signalling pathway operates to control the following cellular processes:

- NO/cyclic GMP and smooth muscle relaxation
- NO/cyclic GMP and synaptic plasticity
- NO/cyclic GMP and cardiac hypertrophy

NO synthesis

Nitric oxide (NO) synthesis is carried out by NO synthase (NOS), of which there are three isoforms named either after the tissues where they were first discovered, i.e. neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) or by the way in which they are controlled, i.e. inducible nitric oxide synthase (iNOS) (Module 2: Figure NO and cyclic GMP signalling). The expression of these enzymes is not as restricted as their names imply, but are widely expressed and can coexist in many cell types. Even though these isoforms are regulated differently and have different cellular locations, they all seem to use the same NO synthetic reaction mechanism.

The excessive production of NO can have pathological consequences and has been linked to various disease states such as Huntington's disease, Alzheimer's disease and hypertension.

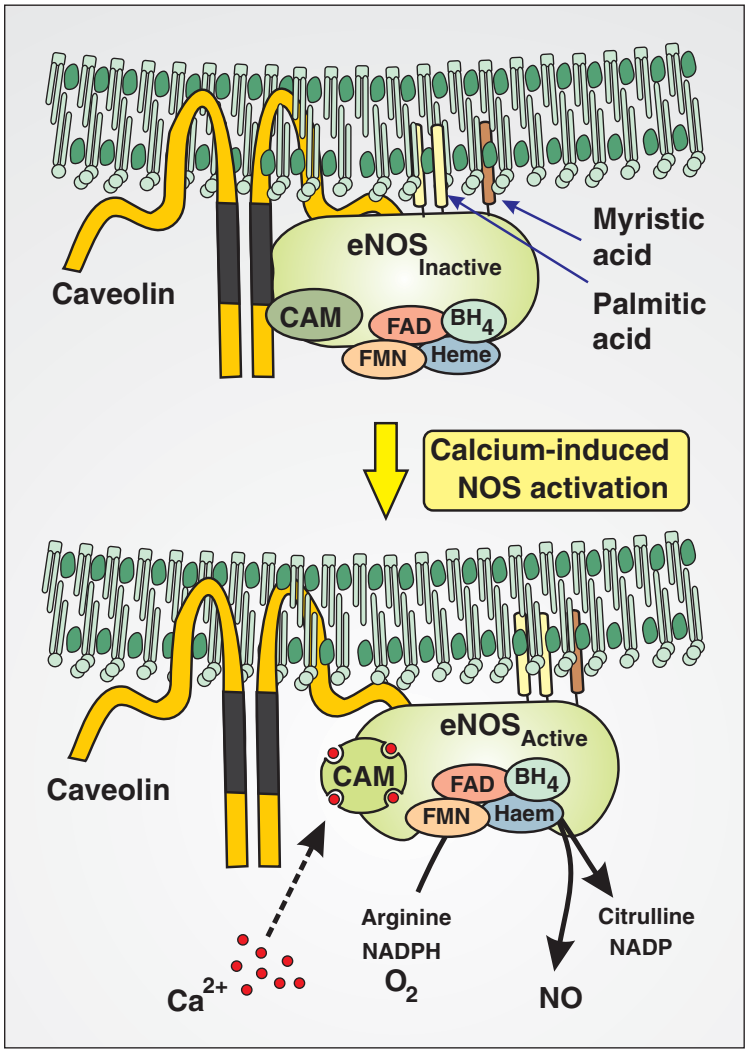
NO synthetic reaction mechanism

The different nitric oxide synthase (NOS) enzymes function as homodimers, which are arranged in a head-to-head orientation with the N-terminal oxidase domain of one monomer lined up alongside the C-terminal reductase domain of its neighbour. The substrates for the enzymatic reaction mechanism are L-arginine, oxygen and NADPH, which combine to form citrulline with the liberation of NO (Module 2: Figure NO synthase mechanism). NOS regulation is complicated because each isoform appears to be regulated by different mechanisms.

Endothelial nitric oxide synthase (eNOS)

As its name implies, endothelial nitric oxide synthase (eNOS) was first described in endothelial cells, where it generates NO in response either to agonists such as acetylcholine and bradykinin that elevate Ca^{2+} or to blood flow-induced shear stress. It is now evident that eNOS is expressed in many different cell types (lung epithelial cells, blood platelets, cardiac myocytes and hippocampal neurons). It has a complex regulation, which is very dependent on its attachment to caveolin, one of the proteins in caveolae (Module 6: Figure caveolae molecular organization), where it contributes to their signalling function. One of the key regulators of eNOS is Ca^{2+}, which acts through

Module 2: | Figure eNOS activation



Ca^{2+}-dependent activation of endothelial nitric oxide synthase (eNOS).
An important feature of eNOS is its location on the membrane of caveolae (Module 6: Figure caveolae molecular organization). Its membrane localization is facilitated by an N-terminal myristic acid and by two palmitic acid residues attached to two cysteine residues (Cys-15 and Cys-26), whereas its association with the caveolae depends upon its attachment to caveolin, which is responsible for keeping the enzyme inactive under resting conditions. There appears to be a competition between caveolin and calmodulin (CaM) for the caveolin-binding site (amino acids 350-358) on eNOS. In the absence of Ca^{2+}, caveolin dominates, but when Ca^{2+} increases and binds to CaM, the latter relieves the inhibitory effect of caveolin, and the enzyme is activated to generate NO from arginine using oxygen and NADPH as co-substrates.

calmodulin (CaM) to stimulate the enzyme to release NO (Module 2: Figure eNOS activation).

The NO released from endothelial cells diffuses out to regulate smooth muscle cell contraction and hence controls blood pressure, smooth muscle cell proliferation, aggregation of blood platelets and leucocyte adhesion. Studies on NO/cyclic GMP and cardiac hypertrophy have revealed that expression of eNOS in endothelial cells can inhibit hypertrophy in neighbouring cardiac cells. Given its central role in regulating so many cellular processes, alterations in the endothelial production of NO have been implicated in many disease states, such as hypertension, diabetes and hypercholesterolaemia.

Inducible nitric oxide synthase (iNOS)

Inducible nitric oxide synthase (iNOS), which is also known as immunocyte NOS, was first described in macrophages, where its expression is up-regulated by inflammatory mediators. Although macrophages are the main cells that express iNOS, it is also found in other cell types (cardiac cells, vascular smooth muscle cells and glial cells). Unlike the other isoforms, the activation of iNOS does not require an elevation of Ca^{2+}. However, iNOS does bind calmodulin (CaM), which is essential for its activation. Since the enzyme is constitutively active, its primary regulation depends upon its induction by inflammatory receptors such as those that respond to interferon-γ (IFN-γ) or lipopolysaccharide (LPS). As large amounts of enzyme are produced, this is a high-output pathway capable of delivering NO for prolonged periods as part of the cells defence against invading micro-organisms. As such, it does not strictly function as a messenger. However, the large production of NO at the sites of inflammation will spill over to affect neighbouring cells. The large up-regulation

of iNOS may account for the fall in blood pressure during endotoxic shock.

Neuronal nitric oxide synthase (nNOS)

Neuronal nitric oxide synthase (nNOS) was first described in neurons, but has since been found in other cell types such as skeletal muscle, which has an alternatively spliced variant that has a 34-amino-acid insert between exons 16 and 17. In both neurons and muscle, nNOS is closely associated with the plasma membrane where it binds to various proteins through its PDZ domains. In neurons, nNOS binds to the postsynaptic density (PSD) proteins such as PSD-93 and PSD-95 (Module 10: Figure postsynaptic density), whereas in skeletal muscle it interacts with α1-syntrophin (a binding partner of dystrophin).

Cyclic GMP signalling pathway

The cyclic GMP signalling pathway (Module 2: Figure NO and cGMP signalling) is governed by the second messenger cyclic GMP, which is synthesized by guanylyl cyclase (GC). The latter comes in two different forms: there are the soluble GC (sGC) and membrane-bound particulate guanylyl cyclases (pGCs). Many of the signalling functions of cyclic GMP are carried out by cyclic GMP-dependent protein kinase (cGK). In addition, cyclic GMP can act directly to open cyclic nucleotide-gated channels. Cyclic GMP hydrolysis is carried out by a cyclic GMP-specific phosphodiesterase (PDE5). Through these different signalling pathways, cyclic GMP functions to regulate a diverse collection of cellular processes. There are those where cyclic GMP mediates the action of NO:

- NO/cyclic GMP and smooth muscle relaxation
- NO/cyclic GMP and synaptic plasticity
- NO/cyclic GMP and cardiac hypertrophy

In those cases where cyclic GMP is formed by the pGC, cyclic GMP acts independently of NO to regulate cellular processes such as phototransduction (Module 10: Figure phototransduction overview). Certain strains of *Escherichia coli,* which secrete the STa toxin, increase intestinal secretion and cause diarrhoea by activating the cyclic GMP signalling pathway by stimulating the particulate guanylyl cyclase C (pGC-C) receptor that is normally activated by guanylin (Module 7: Figure intestinal secretion).

NO/cyclic GMP and synaptic plasticity

The enzymes responsible for NO formation and its action are richly expressed in the nervous system. Although the precise function of NO is still debated, there are indications that it might function in synaptic plasticity by contributing to cerebellar cell long-term potentiation (LTP) at the parallel fibre/Purkinje cell synapse.

Guanylyl cyclase (GC)

Guanylyl cyclase (GC) is the enzyme that synthesizes cyclic GMP from ATP (Module 2: Figure NO and cyclic GMP signalling). It comes in two main forms, soluble GC (sGC), which is activated by NO, and membrane-bound particulate guanylyl cyclases (pGCs). The latter belongs to a group of single membrane-spanning receptors that use an enzyme to transduce information. In this case, it is the guanylyl cyclase region of the cytoplasmic domain that functions both as a transducer and an amplifier to generate the second messenger cyclic GMP.

Cyclic GMP hydrolysis

The enzyme that reverses the second messenger action of cyclic GMP by hydrolysing it to GMP is the cyclic GMP-specific phosphodiesterase (PDE5), which can be activated by cGKI, thus setting up a negative-feedback loop. Excessive signalling through the cyclic GMP signalling pathway will thus be curtailed through this ability of cyclic GMP to enhance its own hydrolysis. PDE5 is of considerable interest as it is the target of Viagra.

Cyclic GMP-dependent protein kinase (cGK)

There are two cyclic GMP-dependent protein kinases (cGKs): cGKI and cGKII. The cGKI comes in two alternatively spliced forms, cGKIα and cGKIβ. These cGKs are serine/threonine protein kinases that exist as homodimers that are held together by leucine zippers in their N-terminal domains, which fold over to inhibit the catalytic domain. The binding of cyclic GMP to a regulatory site induces a conformational change that relieves this inhibition, thus enabling the catalytic domain to phosphorylate its substrates.

cGK targets

These cGKs are targeted to specific sites in the cell. The N-terminal domain is responsible for targeting cGKIα and cGKIβ to specific cellular regions as is particularly important for smooth muscle relaxation mediated by nitric oxide (NO) and cyclic GMP (Module 7: Figure smooth muscle cell cyclic GMP signalling).

The cGKII has an N-terminal Gly-2 myristic acid residue that serves to target it to the plasma membrane.

cGK protein substrates

The cGMP-sensitive target proteins regulated by cGKs include inositol 1,4,5-trisphosphate receptor-associated cGKI substrate (IRAG), large conductance (BK) channels, cGMP-specific phosphodiesterase (PDE5), cerebellar G substrate, vesicle-associated membrane protein (VASP) and telokin.

Inositol 1,4,5-trisphosphate receptor-associated cGKI substrate (IRAG)

Inositol 1,4,5-trisphosphate receptor ($InsP_3R$)-associated cGKI substrate (IRAG) is located on the endoplasmic reticulum (ER), where it appears to associate with the $InsP_3R$. When it is phosphorylated by cGKIβ, IRAG acts to inhibit channel opening (Module 7: Figure smooth muscle cell cyclic GMP signalling).

Reactive nitrogen species (RNS) signalling

One of the ways by which NO functions in cells is through an S-nitrosylation reaction (Module 2: Figure NO and cGMP signalling). This covalent modification results from the addition of NO to reactive cysteine residues on specific target proteins. NO does not react directly with these

proteins, but is first of all converted into reactive nitrogen species (RNS) that are then responsible for carrying out the nitrosylation reaction. These nitrosylation reactions are reversed by denitrosylation reactions. Many of the functions of NO are carried out by this nitrosylation-sensitive signalling pathway and there is growing evidence for nitrosylation dysfunction in disease.

Reactive nitrogen species (RNS)

The reactive nitrogen species (RNS) that carry out the nitrosylation reaction are formed when NO interacts with various acceptors such as superoxide radical ($O_2^{-\bullet}$), cysteine (Cys), glutathione (GSH) or transition metal ions (M^{n+}, e.g. Fe^{3+} or Cu^{2+}) (Module 2: Figure NO and cGMP signalling):

1. NO + O_2^- → $ONOO^-$ (peroxynitrite)
2. NO + M^{n+} → Mn^{n+}-NO
3. NO + GSH → GS-NO
4. NO + Cys → Cys-NO

The interaction between NO and $O_2^{-\bullet}$ forms the strong oxidant peroxynitrite ($ONOO^-$), which is very much more reactive than the two parent molecules. It can react with electron-rich groups such as protein sulfhydryls as part of the nitrosylation reaction to form the S-nitrosothiols (SNOs). Although peroxynitrite has a brief half-life (approx. 15 ms), it exists for long enough to diffuse through a cell and perhaps also to neighbouring cells enabling it to function as an intra- and inter-cellular messenger.

Low molecular mass thiols, such as glutathione (GSH), can also be nitrosylated by NO to form GS-NO.

Nitrosylation reaction

The S-nitrosylation reaction depends upon the transfer of NO from one of the reactive nitrogen species (RNS) to a peptidyl cysteine thiol group of target proteins (R-SH) (Module 2: Figure NO and cGMP signalling):

1. R-SH + $ONOO^-$ → R-SNO
2. R-SH + M^{n+}-NO → R-SNO
3. R-SH + GS-NO → R-SNO
4. R-SH + Cys-NO → R-SNO

Specificity is determined by the fact that the target proteins (R-SH) have hyperreactive cysteine groups where the thiol moiety exists as a thiolate anion due to the presence of positively charged amino acids in the immediate vicinity of the protein chain.

Denitrosylation reactions

Denitrosylation is carried out by two main denitrosylases (Module 2: Figure NO and cGMP signalling). One mechanism depends on the oxidoreductase thioredoxin (Trx) operating in concert with Trx reductase (TrxR). In its reduced state, the two cysteine residues in the highly conserved -Cys-Gly-Pro-Cys- active site are reduced and the hydrogens are used to release the NO as HNO and to convert the SNO group on the target protein into an SH group. During this denitrosylation reaction, a disulphide bond is formed between the vicinal cysteine residues at the active site. This oxidized Trx is then reduced by NADPH through the activity of the seleno-flavoprotein TrxR.

The other mechanism depends on *S*-nitrosoglutathione reductase (GSNOR), which is also known as GSH-dependent formaldehyde dehydrogenase and Class III alcohol dehydrogenase (ADH3). The GSNOR metabolizes the GSNO, which is one of the reactive nitrogen species (RNS), which is produced through two mechanisms. It is formed when NO interacts with GSH and is also released following the denitrosylation of target proteins (Module 2: Figure NO and cGMP signalling). The GSNOR uses NADH as an electron donor to irreversibly convert GSNO into GSNHOH. In the absence of GSNOR, the level of GSNO increases as does the amount of S-nitrosylated proteins, which suggest that these SNO proteins might be in equilibrium with GSNO and this might account for the fact that certain cellular proteins seem to by constitutively nitrosylated.

Redox signalling

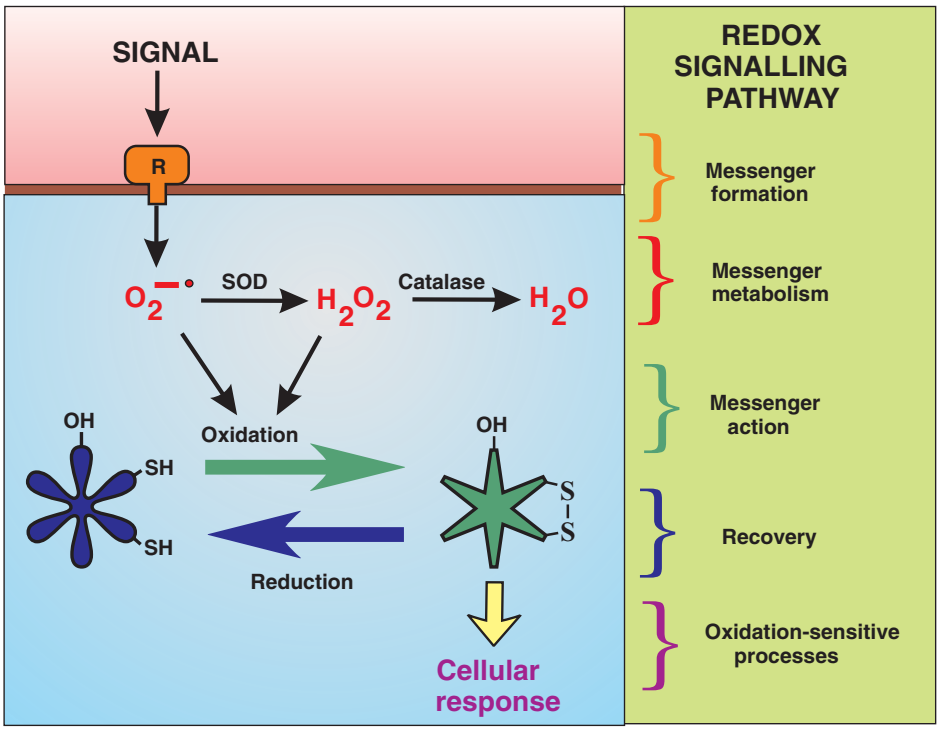
Cells have evolved a sophisticated mechanism of intracellular signalling based on localized changes in the oxidation state of specific proteins. The internal environment of cells is normally highly reduced. Certain forms of stress are associated with an increase in the oxidative state, and this can induce apoptosis. It is also well known that certain phagocytic cells, such as neutrophils, can rapidly generate superoxide radical ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) that are used to kill other cells during inflammatory responses. In addition to these pathological effects, there is increasing evidence that the redox system has been adapted to perform a variety of signalling functions and can modulate the activity of other signalling pathways. As such, they can control many cellular processes, including cell proliferation, apoptosis and cellular senescence. Some of these effects are exerted through a two-way interaction with Ca^{2+} signalling. For example, redox signalling can help to promote the tyrosine phosphorylation events that generate many signalling cascades and it can modulate the activity of the ryanodine receptors (RYRs) and inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) that release Ca^{2+}. Conversely, Ca^{2+} can stimulate redox signalling, particularly within the mitochondrion, indicating that there are dynamic interactions operating between these signalling pathways.

There are two main types of redox signalling. The first type is reactive oxygen species (ROS) signalling, which depends on the formation of ROS. The second type is reactive nitrogen species (RNS) signalling, which is carried out by RNS and is linked to the nitric oxide (NO)/cyclic GMP signalling pathway.

Reactive oxygen species (ROS) signalling

ROS signalling has all the hallmarks of a classical signalling mechanism. The second messengers are the reactive oxygen species (ROS) formed in response to many agonists. Reactive oxygen species (ROS) formation depends on the stimulation of a NADPH oxidase that removes an electron from NADPH and adds it to oxygen to create superoxide radical ($O_2^{-\bullet}$), which is one of the ROS found in cells.

Module 2: | Figure summary of redox signalling



Summary of the main features of redox signalling.
The sequential processes that constitute redox signalling begin with an external signal activating a receptor (R) that then generates reactive oxygen species, such as superoxide radical ($O_2^{-\bullet}$), which is then converted into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). $O_2^{-\bullet}$ and H_2O_2 diffuse into the cell where they exert their messenger action by stimulating the oxidation of specific proteins, sometimes forming an internal disulphide bond. Recovery is carried out by various enzyme systems that return the target protein to its reduced state. The oxidized protein acts to stimulate a variety of cellular processes.

Superoxide dismutase (SOD) rapidly converts the $O_2^{-\bullet}$ into hydrogen peroxide (H_2O_2), which is one of the main messenger molecules used by the redox signalling pathway (Module 2: Figure summary of redox signalling).

Rapid reactive oxygen species (ROS) metabolism ensures that H_2O_2 , like other intracellular messengers, has a short half-life. This metabolism of H_2O_2 is carried out by a range of enzymes, including catalase, glutathione peroxidase (GPx) and peroxiredoxin (Prx). Since the inside of the cell is a highly reducing environment, the last two enzymes can draw upon a large reservoir of reducing equivalents in order to metabolize the ROS. The cell has a large redox buffer capacity in the form of glutathione, which functions to maintain the redox balance in the cell. The fact that H_2O_2 is metabolized so rapidly means that its site of action is highly localized close to its site of production.

The reactive oxygen species (ROS) messenger action of H_2O_2 depends upon its ability to react with the cysteine residues of a certain group of target proteins. The latter are marked out by virtue of having hyperreactive thiol groups that are rapidly oxidized to form a disulphide bond. The recovery from this oxidized state back to a fully reduced thiol group is carried out by the glutaredoxin and/or the thioredoxin systems.

Finally, the oxidized target proteins activate a number of oxidation-sensitive processes that bring about a number of cellular responses such as gene activation, modulation of ion channels and the activity of other signalling pathways [mitogen-activated protein kinase (MAPK) cascade and Ca^{2+} signalling]. It is therefore not surprising to find a role for redox signalling in proliferation and cancer. There is also a strong relationship between redox signalling and schizophrenia (Module 12: Figure schizophrenia).

Reactive oxygen species (ROS)

Reactive oxygen species (ROS) is a collective term that refers to those oxygen species [superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$)] that are more reactive than ground-state oxygen [Module 2: Figure reactive oxygen species (ROS)]. These are sometimes considered synonymous with free radicals, but not all ROS are free radicals. The latter are defined as atoms or molecules that contain one or more unpaired electrons. With regard to signalling, it is the $O_2^{-\bullet}$ and H_2O_2 that appear to be the most important messengers. Also, there are indications that these two messengers may perform different functions.

There are two important sources of reactive oxygen species (ROS): one is at the plasma membrane and the other is within the mitochondria (Module 2: Figure sites of ROS formation).

Superoxide ($O_2^{-\bullet}$)

The superoxide radical ($O_2^{-\bullet}$) that is formed by the one-electron reduction of O_2 is short-lived (half-life of 10^{-6} s) in that it rapidly dismutates into hydrogen peroxide

Module 2: | Table redox signalling components
The major components of the redox signalling pathway

Component	Comment
NADPH oxidases (NOXs)	
NOX1	Inducible enzyme found in colon and smooth muscle
NOX2 ($gp91^{phox}$)	Major NOX in phagocytes
NOX3	Foetal kidney
NOX4	Widespread
NOX5	Brain, spleen and sperm
DUOX1 (dual oxidase 1)	A Ca^{2+}-sensitive isoform
DUOX2 (dual oxidase 2)	A Ca^{2+}-sensitive isoform
NOX/DUOX regulatory factors	
$p47^{phox}$	
$p67^{phox}$	
$p40^{phox}$	
$p22^{phox}$	
NOXO 1	
NOXA 1	
Rac1/Rac2	
ROS metabolism	
Superoxide dismutase (SOD)	
Catalase	Localized in peroxisomes
Glutathione peroxidase (GPx)	Localized in cytosol and mitochondria
Peroxiredoxin (Prx)	
Prx I-IV (2-Cys)	I and II in cytosol; III in mitochondria; IV in endoplasmic reticulum
Prx V (atypical 2-Cys)	
Prx VI (1 Cys)	
Thiol-containing proteins/peptides	
Glutathione (GSH)	
Glutaredoxin (Grx)	
Thioredoxin (Trx)	
Trx-1	
Trx-2	Mitochondria-specific
Reductases	
Glutathione reductase	
Glutaredoxin reductase	
Thioredoxin reductase (TrxR)	
Sulphiredoxin (Srx)	Catalyses reduction of hyperperoxidized proteins

(H_2O_2). This conversion can occur spontaneously, but the reaction is greatly accelerated by the enzyme superoxide dismutase (SOD).

Hydrogen peroxide (H_2O_2)

Much attention is focused on hydrogen peroxide (H_2O_2) because it appears to be the primary messenger molecule functioning in the redox signalling pathway. Since it has no unpaired electrons, H_2O_2 is not a free radical and thus is not a particularly powerful oxidizing agent. This means that it can function as a messenger by diffusing away from its site of action to interact with more distant targets. However, its sphere of influence is restricted by its short half-life, which is determined by the rapid reactive oxygen species (ROS) metabolism of H_2O_2. The H_2O_2 may also act as a paracrine signal that diffuses away from stimulated cells to alter the activity of neighbouring cells. Release of H_2O_2 during wound healing recruits leucocytes as part of the inflammatory response.

Hydroxyl radical ($OH^{\bullet}$)

While H_2O_2 is relatively benign, it can be converted into the highly toxic hydroxyl radical ($OH^{\bullet}$) through a reduction process catalysed by transition metals (Fe^{3+} or Cu^{2+}). $OH^{\bullet}$ has a half life of 10^{-9} s indicative of its very high reactivity in that it reacts immediately and indiscriminately with the first molecule it finds. Much of the oxidative damage cause by ROS is mediated by $OH^{\bullet}$.

Superoxide dismutase (SOD)

A family of metalloproteinases that converts superoxide radical ($O_2^{-\bullet}$) into hydrogen peroxide (H_2O_2) (Module 2: Figure plasma membrane ROS formation):

$$2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

There are four families of SOD enzymes:

- Copper/zinc-containing superoxide dismutases (CuZnSODs)
- Manganese-containing superoxide dismutases (MnSODs)
- Copper-containing superoxide dismutases (CuSODs)
- Iron-containing superoxide dismutases (FeSODs)

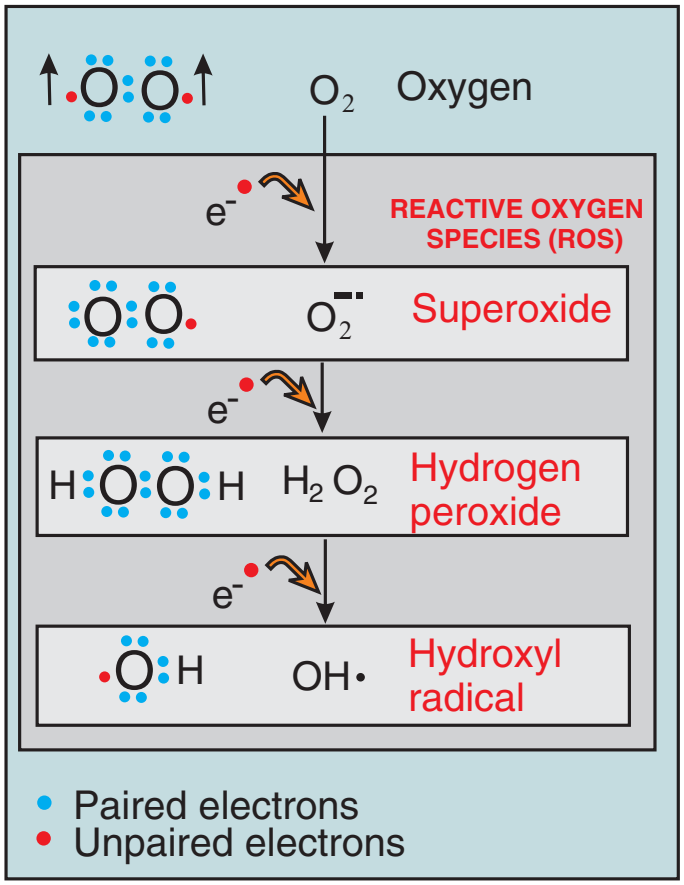
Expression of MnSOD is regulated by the FOXO3a transcription factor (Module 4: Figure FOXO control mechanisms).

Mutation of SOD is the cause of amyotrophic lateral sclerosis (ALS), which is a debilitating and progressive neurological disease.

Reactive oxygen species (ROS) formation

Reactive oxygen species (ROS) are formed at two main sites: there is plasma membrane reactive oxygen species (ROS) formation and mitochondrial reactive oxygen species (ROS) formation (Module 2: Figure sites of ROS formation). This production of ROS appears to be highly localized suggesting the existence of reactive oxygen species (ROS) microdomains.

Module 2: | Figure reactive oxygen species (ROS)



Formation and metabolism of the reactive oxygen species (ROS).
To understand the properties of reactive oxygen species (ROS) and how they are formed, it is best to begin with oxygen. Oxygen is a strong oxidizing agent in that it has two unpaired electrons (e^-; red dots), which have parallel spins (i.e. they spin in the same direction as indicated by the two black arrows) and occupy separate π-antibonding orbitals. Given that they have these unpaired electrons, oxygen qualifies as a free radical. However, oxygen is relatively inert because, in order to react with another molecule, it has to accept a pair of electrons with antiparallel spins to fit into the empty spaces in the π orbitals. Because of this restriction, oxygen accepts electrons (e^-) one at a time and this leads to the formation of the different ROS.

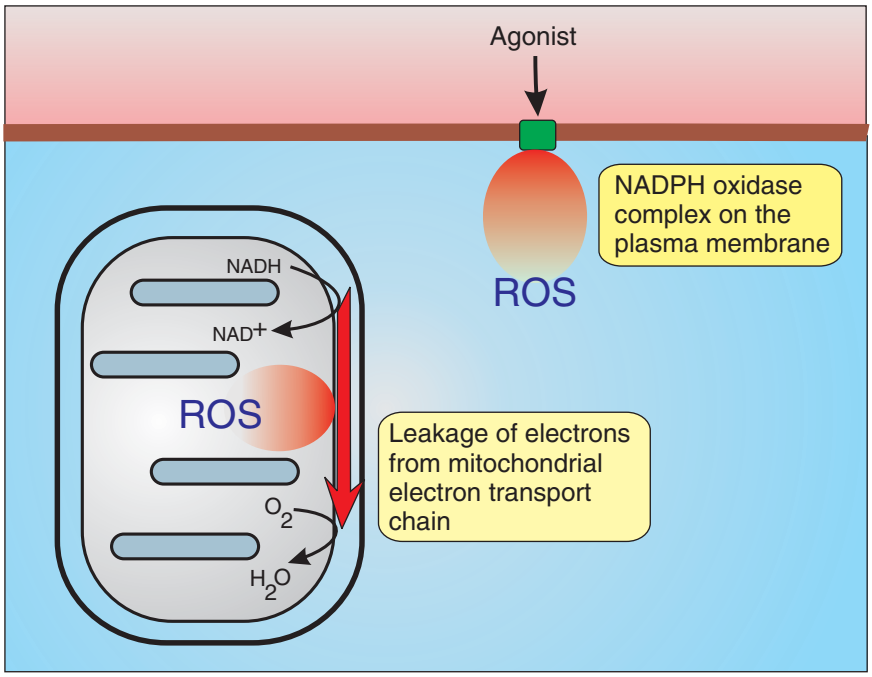
Plasma membrane reactive oxygen species (ROS) formation

A large number of receptors responding to external signals stimulate the formation of reactive oxygen species (ROS), including cytokine receptors (tumour necrosis factor α, interleukin-1 and interferon-γ), growth factor receptors [platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) receptors] and G protein-coupled receptors (GPCRs) (5-hydroxytryptamine, bradykinin, angiotensin II, thrombin and endothelin receptors). Just how all these receptors are coupled to the formation of ROS is still somewhat uncertain, but there is increasing evidence that the mechanism might resemble that found in phagocytic cells.

In phagocytes, external signals acting through cell-surface receptors stimulate the PtdIns 3-kinase signalling pathway to form the lipid messenger PtdIns3,4,5P_3 (Module 2: Figure PtdIns 3-kinase signalling), which then acts through Rac to stimulate NADPH oxidase. This multicomponent enzyme uses NADPH as an electron donor to carry out the first step of ROS formation i.e. the reduction of oxygen to superoxide radical ($O_2^{-\bullet}$) (Module 2: Figure plasma membrane ROS formation). In addition to this activation through Rac, it seems that the enzyme can also be regulated through diacylglycerol (DAG) and Ca^{2+} acting through protein kinase C (PKC), which phosphorylates $p47^{phox}$, one of the cytoplasmic components of NADPH oxidase, to bring about the assembly of the multicomponent complex. Non-phagocytic cells that generate ROS for signalling use a similar, but genetically distinct, NADPH oxidase called Nox1, which plays a significant role in redox signalling in proliferation and cancer.

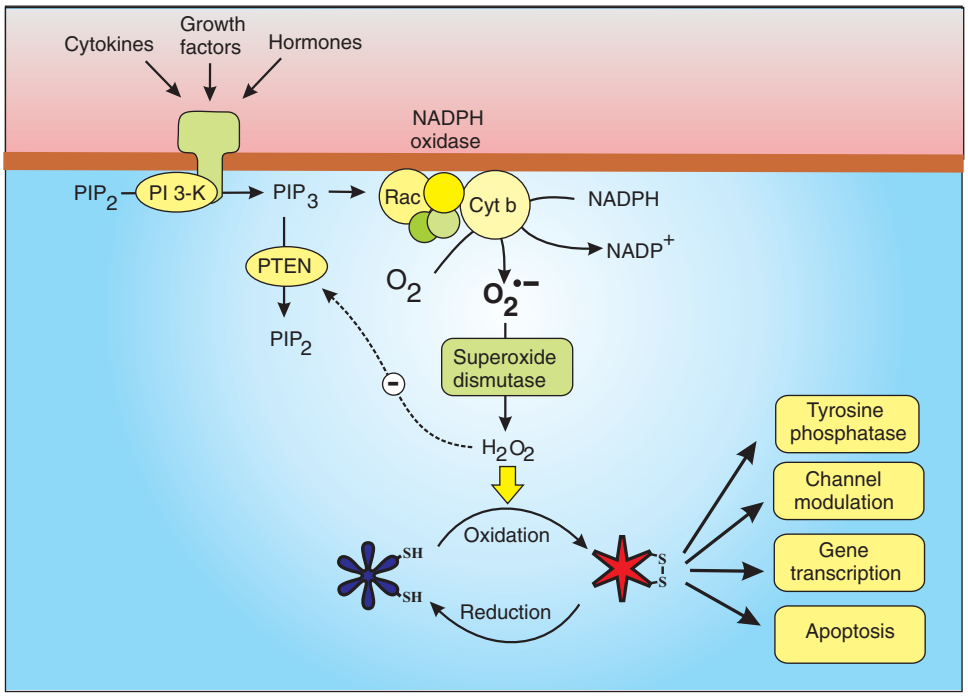
In phagocytes, the $O_2^{-\bullet}$ is released to the outside of the cell, where it is able to attack invading micro-organisms, whereas, in non-phagocytic cells, it functions as a messenger to activate a signalling cascade on the inside of the cell (Module 2: Figure plasma membrane ROS formation). Superoxide dismutase (SOD) rapidly converts the $O_2^{-\bullet}$ into hydrogen peroxide (H_2O_2), which appears to be the primary messenger molecule of this redox signalling pathway.

Module 2: | Figure sites of ROS formation



The two main sites of reactive oxygen species (ROS) formation.
There are two main sites of ROS formation in cells. One is at the level of the plasma membrane, where an NADPH oxidase complex is activated by cell signalling pathways. The other is at the mitochondria, where ROS are produced as a result of electron leakage from the electron transport chain. As indicated, this production of ROS appears as microdomains indicating that ROS signalling might be highly localized in cells.

Module 2: | Figure plasma membrane ROS formation



Receptor-dependent reactive oxygen species (ROS) formation at the plasma membrane.
The formation of reactive oxygen species (ROS) occurs in response to many external signals such as cytokines, growth factors and hormones. In many cases, these signals activate receptors coupled to PtdIns 3-kinase (PI 3-K) which produces PtdIns3,4,5P_3 (PIP_3) that then acts through Rac to stimulate NADPH oxidase at the plasma membrane. An electron is removed from NADPH and transferred to oxygen to form superoxide radical ($O_2^{-\bullet}$). This $O_2^{-\bullet}$ is then transformed by the addition of further electrons by superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2). There is a positive-feedback loop that will amplify the redox signalling pathway because one of the actions of H_2O_2 is to inhibit the enzyme phosphatase and tensin homologue deleted on chromosome 10 (PTEN) that hydrolyses PIP_3.

NADPH oxidase

The NADPH oxidase (NOX/DUOX) family (Module 2: Table redox signalling components) consists of a number of enzymes with different cellular locations.

NOX2, also known as $gp91^{phox}$, has been described best for phagocytes, where it is made up of a number of subunits. The catalytic component is cytochrome b_{558}, which is a heterodimer formed from $gp91^{phox}$ and $p22^{phox}$. In addition to this heterodimer, which is located in the membrane, there are cytoplasmic components (e.g. $p47^{phox}$, $p67^{phox}$, Rap1A and Rac) that play a role in regulating enzyme activity. The DUOX (dual oxidase) enzymes are sensitive to Ca^{2+} and play an important role in interacting with the Ca^{2+} signalling pathway (Module 2: Figure ROS effects on Ca^{2+} signalling).

There is a strong relationship between redox signalling and schizophrenia during which the induction of NOX2 may play an important role in triggering the formation of the peroxynitrite that acts to inhibit NMDA receptor function (Module 12: Figure schizophrenia).

Mitochondrial reactive oxygen species (ROS) formation

Most of the electrons that enter the electron transport chain are transferred to oxygen in an orderly manner, but there is always a 1–2% leakage during which an electron is transferred directly to oxygen to form superoxide ($O_2^{-\bullet}$) and this is the source of mitochondrial reactive oxygen species (ROS) (Module 5: Figure mitochondrial Ca^{2+} signalling). This orderly electron transfer to oxygen occurs during energy metabolism, where oxygen is reduced to water by accepting four electrons (e^-) from cytochrome *c* oxidase:

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

Mitochondrial energy metabolism is inherently dangerous, because the electron transport chain is somewhat leaky in that some of the molecular oxygen is diverted into the formation of the superoxide radical ($O_2^{-\bullet}$), which is then converted into hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\bullet$). These mitochondrial ROS may play an important role in apoptosis by acting synergistically with Ca^{2+} to stimulate the formation of the mitochondrial permeability transition pore (MTP) (Module 5: Figure mitochondrial Ca^{2+} signalling). The formation of mitochondrial ROS appears to be highly localized in that small superoxide flashes have been recorded in single mitochondria. This is another example of reactive oxygen species (ROS) microdomains.

The generation of ROS by mitochondria might be a regenerative process in that a local release of ROS in cardiac myocytes causes a rapid mitochondrial depolarization due to formation of a MTP and a concomitant increase in intrinsic ROS production, i.e. a process of ROS-induced ROS release (RIRR). This RIRR often occurs synchronously and reversibly among long chains of adjacent mitochondria, suggesting a co-operative mechanism.

Redox balance

The redox balance of the cell is maintained by energy metabolism, primarily the pentose phosphate shunt that feeds reducing equivalents into the cell in the form of NADPH. The latter is then used to maintain redox buffers such as glutathione (GSH) (Module 2: Figure GSH/GSSG couple) and thioredoxin (Trx) in their reduced forms. The concentration of GSH in the cytoplasm lies within the 1–10 mM range, with over 99% existing as the reduced GSH form. The 2GSH/GSSG redox couple is thus a measure of the redox balance in the cell. A similar balance exists for the oxidized and reduced forms of TRX. The reduced GSH and Trx are used for a number of reductive processes such as the metabolism of hydrogen peroxide by glutathione peroxidase (GPx) (Module 2: Figure H_2O_2 metabolism) or as a source of reducing equivalents for the glutaredoxin system (Module 2: Figure recovery of protein oxidation). The GSSG (the oxidized form of glutathione) is converted back into GSH by glutathione reductase.

Similar redox control enzymes and buffers are located in both the mitochondria and within the lumen of the endoplasmic reticulum (ER). Like the cytoplasm, the mitochondrial matrix maintains a reducing environment and uses similar enzymatic mechanisms to control the ROS emanating from the electron transport chain. The ER, however, is somewhat different in that the GSH/GSSG ratio is close to 1 and this more oxidizing environment is necessary for the formation of the disulphide bonds that are an integral component of the extracellular proteins that are processed and packaged within the ER.

Since the cytoplasm and the ER lumen have different redox potentials, there is a redox potential gradient across the ER membrane and this might be used to modulate Ca^{2+} signalling by altering the activity of the ion channels that release Ca^{2+}.

Reactive oxygen species (ROS) metabolism

Like all other intracellular signalling molecules, ROS are metabolized rapidly. Superoxide dismutase (SOD) rapidly converts superoxide radical ($O_2^{-\bullet}$) into hydrogen peroxide (H_2O_2), which is then metabolized by a number of enzyme systems including catalase, glutathione peroxidase (GPx) and peroxiredoxin (Prx) (Module 2: Figure H_2O_2 metabolism).

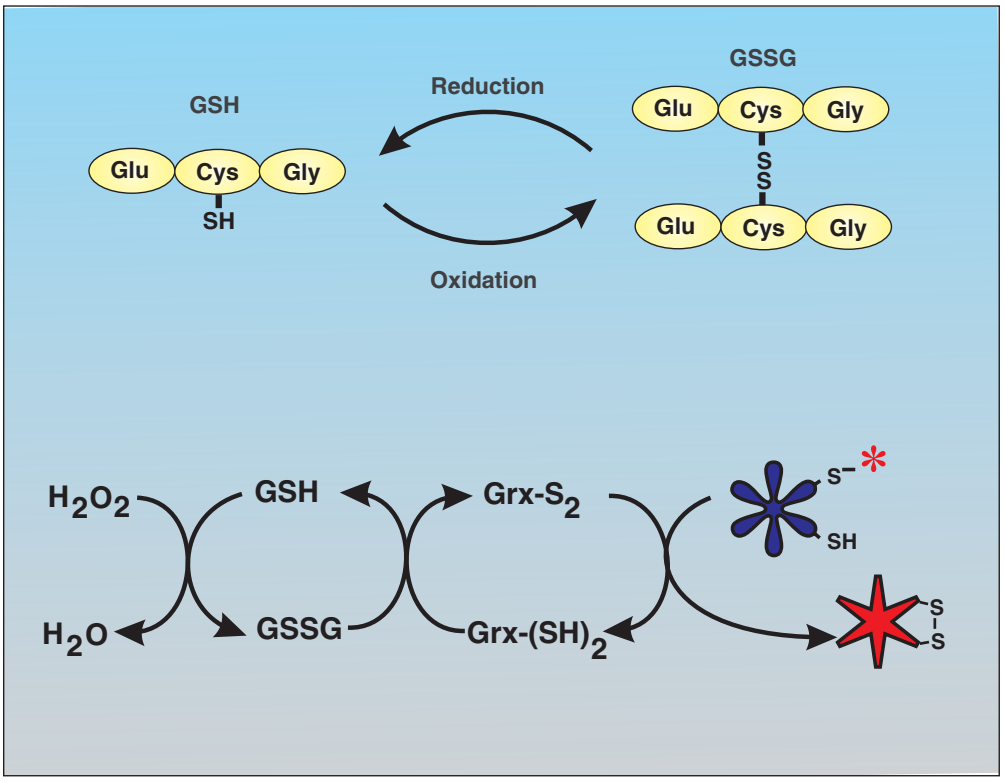
With so many enzyme systems co-operating to metabolize H_2O_2, it is likely that this messenger will have a highly restricted sphere of influence localized to its site of production either at the plasma membrane or within the mitochondrion.

Catalase

Catalase is a haem-containing protein that decomposes hydrogen peroxide (H_2O_2) to water and oxygen (Module 2: Figure H_2O_2 metabolism).

Most of the catalase in cells is found in peroxisomes, thus restricting the role of the enzyme in dealing with the H_2O_2 generated during the redox signalling mechanism at the plasma membrane.

Module 2: | Figure GSH/GSSG couple



The GSH/GSSG redox couple.

GSH is a tripeptide consisting of glutamic acid, cysteine and glycine. In its oxidized state, two molecules of GSH are joined together through a disulphide bond to form GSSG. This is the most abundant redox couple in the cell. The state of this couple can be determined by measuring the half-cell reduction potential (E_{hc}). Under normal reducing conditions, this potential is high, i.e. −240 mV, and this seems to be associated with cell proliferation. Differentiation seems to occur at lower potentials (−200 mV), whereas still lower potentials of −170 mV favour apoptosis. At these lower potentials, where there is an alteration in the redox balance, the build-up of GSSG within the cell can reverse the operation of the glutaredoxin system that functions normally in the recovery of oxidation-sensitive processes. GSSG interacts with reduced glutaredoxin [Grx-$(SH)_2$] to form oxidized Grx-S_2, and this disulphide bond can be transferred to oxidize target proteins.

Glutathione (GSH)

Glutathione (GSH) is a redox buffer that regulates the cellular redox balance (Module 2: Figure GSH/GSSG couple). GSH is a tripeptide consisting of glutamic acid, cysteine and glycine. It is synthesized by two enzymes. First, there is glutamate cysteine ligase (GCL), which is made up of two subunits: a GCL catalytic subunit (GCLC) and a GCL modifier subunit (GCLM). The GCLC carries out an ATP-dependent condensation reaction between cysteine and glutamate to form gamma-glutamylcysteine. Secondly, a GSH synthetase (GSS), which is also known as GSH S-transferase (GST), adds a glycine to the dipeptide gamma-glutamylcysteine to form GSH.

A dysregulation of GSH metabolism has been implicated in schizophrenia (Module 12: Figure schizophrenia).

Glutathione peroxidase (GPx)

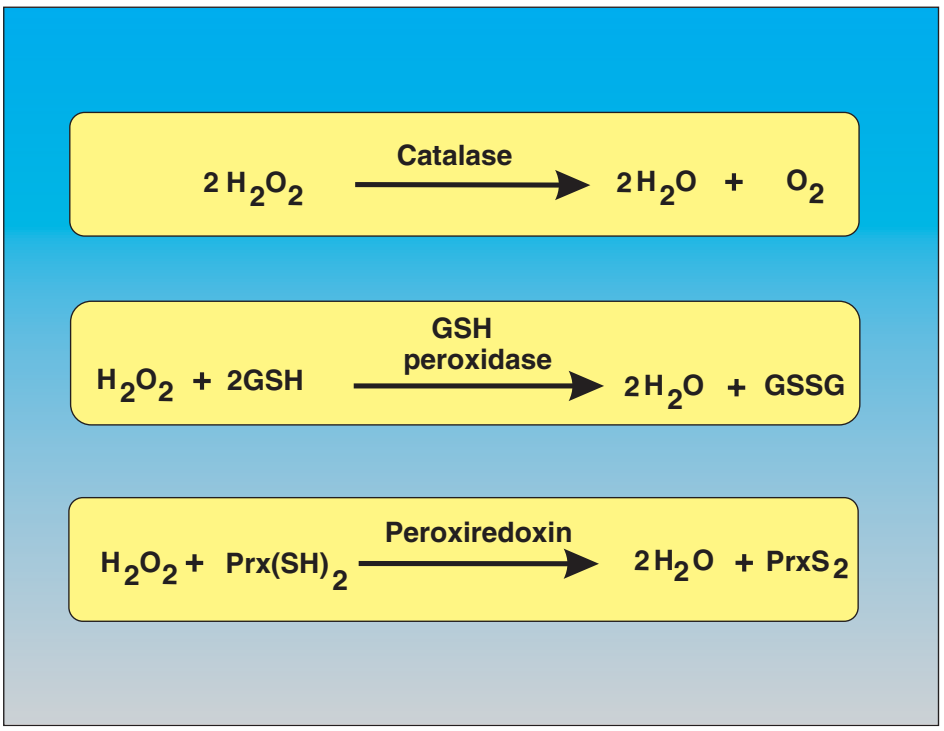
The glutathione peroxidase (GPx) family uses the reducing power of glutathione to convert H_2O_2 into water (Module 2: Figure H_2O_2 metabolism).

Peroxiredoxin (Prx)

The peroxiredoxins (PrxI to PrxIV) are a family of small antioxidant proteins that function to metabolize hydrogen peroxide (H_2O_2) to water, thus curtailing its messenger action. PrxI and PrxII are cytosolic, whereas Prx III is found on mitochondria and PrxIV is on the endoplasmic reticulum. The operation of the catalytic cycle goes through the following steps (Module 2: Figure peroxiredoxin catalytic cycles):

1. H_2O_2 is generated near the plasma membrane when the PtdIns3,4,5P_3 (PIP_3) formed by receptor activation stimulates NADPH oxidase.
2. H_2O_2 interacts with the reduced cysteine residues (Cys-SH) in the N-terminal regions of the thioredoxin (Trx) dimers to form two oxidized sulphenic residues (Cys-SOH).
3. The Cys-SOH can then interact with the conserved Cys-SH on the C-terminal regions of the neighbouring dimer to form two intermolecular disulphides.
4. The Prx disulphide is converted back into the reduced form by Trx, which is regenerated by thioredoxin reductase (Module 2: Figure recovery of protein oxidation).
5. The sulphenic residues formed by Reaction 2 can undergo hyperperoxidation by interacting with further molecules of H_2O_2 to form the sulphinic acid residues.

Module 2: | Figure H_2O_2 metabolism



Hydrogen peroxide (H_2O_2) metabolism by different enzyme systems.
Hydrogen peroxide (H_2O_2) can be metabolized by three main mechanisms. The enzyme catalase, which is restricted to peroxisomes, converts H_2O_2 into water and oxygen. GSH peroxidase uses the reducing power of glutathione (GSH) to convert H_2O_2 into water with the formation of GSSG. The peroxiredoxin (Prx) family is a major player in the metabolism of H_2O_2 through a series of catalytic cycles (Module 2: Figure peroxiredoxin catalytic cycles).

6. This hyperperoxidation reaction can be reversed by a reaction that requires ATP catalysed by the enzyme sulphiredoxin (Srx).
7. The phosphorylated intermediate is reduced back to the reduced form of Prx by the Trx system.
8. The Prx system is very efficient at limiting the size of reactive oxygen species (ROS) microdomains. However, cell-surface receptors are capable of enlarging this microdomain by inducing a local inactivation of Prx. The activated receptor, perhaps acting through the non-receptor protein kinase Src, brings about an inactivation of the Prx molecules in the local vicinity by phosphorylating Prx on Tyr-194 thus enabling the plume of H_2O_2 to spread away from the receptor.

The reducing equivalents derived from thioredoxin (Trx) are used to regenerate Prx-$(SH)_2$:

$$\text{Prx-S}_2 + \text{Trx-(SH)}_2 \rightarrow \text{Prx-(SH)}_2 + \text{Trx-S}_2$$

When Prx1 in mice is knocked out, animals develop haemolytic anaemia and malignant cancers.

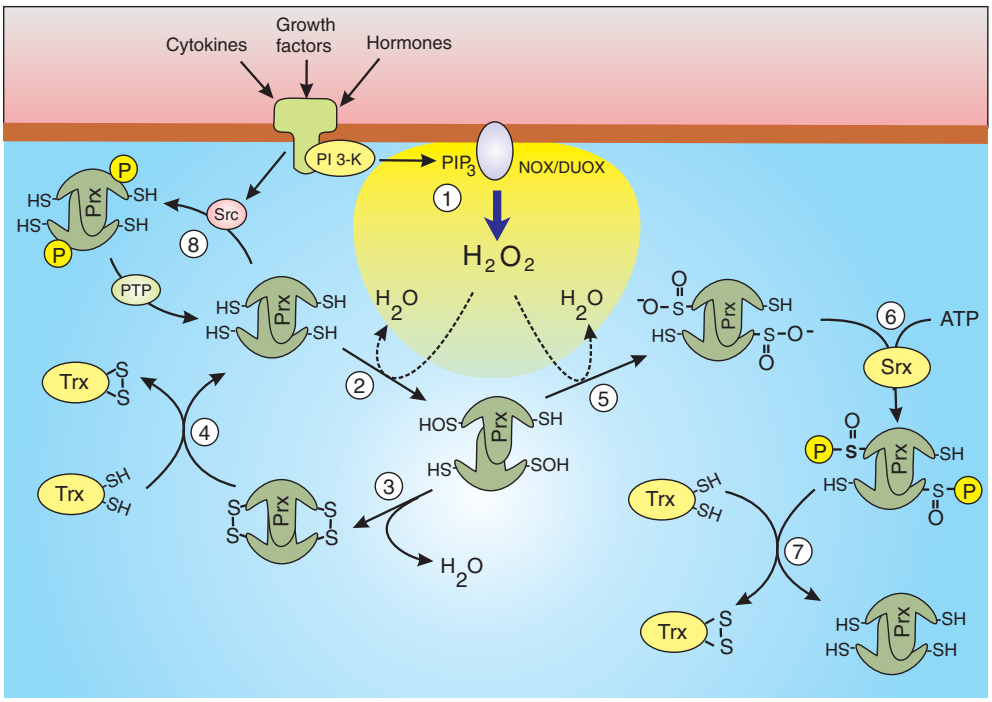
Reactive oxygen species (ROS) messenger action

The primary action of hydrogen peroxide (H_2O_2) is to reversibly oxidize a variety of target proteins with a high degree of specificity (Steps 1–7 in Module 2: Figure reversible and irreversible ROS oxidations):

1. The primary action of H_2O_2 is to oxidize the hyperreactive cysteine to form a sulphenic acid group (-SOH), which can be metabolized further along a number of pathways.
2. The sulphenic acid residue can interact with nitrogen on a neighbouring serine residue to form an intramolecular cyclic sulphenyl amide as occurs during the oxidation of protein tyrosine phosphatases (Module 2: Figure ROS formation and action).
3. The sulphenic acid residue can be converted into an intramolecular disulphide bond with the elimination of water.
4. The sulphenic acid residue can interact with glutathione (GSH) to form an intermolecular disulphide bond.
5. The sulphenic acid residue can undergo hyperperoxidation by interacting with another molecule of H_2O_2 to form a sulphinic acid intermediate (Cys-SO_2H).
6. The sulphinic acid intermediate undergoes further hyperperoxidation to form the sulphonic acid intermediate (Cys-SO_3H).
7. The sulphinic acid group (Cys-SO_2H) can be reduced by a reaction that requires ATP and is catalysed by the enzyme sulphiredoxin (Srx).

The various oxidated intermediates can be converted back into the initial reduced state by either the thioredoxin (Trx) or the glutaredoxin (Grx) system (Module 2: Figure recovery of protein oxidation).

Module 2: | Figure peroxiredoxin catalytic cycles



Metabolism of H_2O_2 by peroxiredoxin.
Peroxiredoxin (Prx) plays a major role in restricting the microdomain of hydrogen peroxide (H_2O_2) that forms beneath the plasma membrane by rapidly removing this messenger through a series of catalytic reactions as described in the text. Information adapted from *Curr. Opin. Cell Biol.*, Vol. 17, Rhee, S.G., Kang, S.W., Jeong, W., Chang, T.-S., Yang, K.-S. and Woo, H.A., Intracellular messenger function of hydrogen peroxide and its regulation by peroxyredoxins, pp. 183-189. Copyright (2005), with permission from Elsevier; see Rhee et al. 2005.

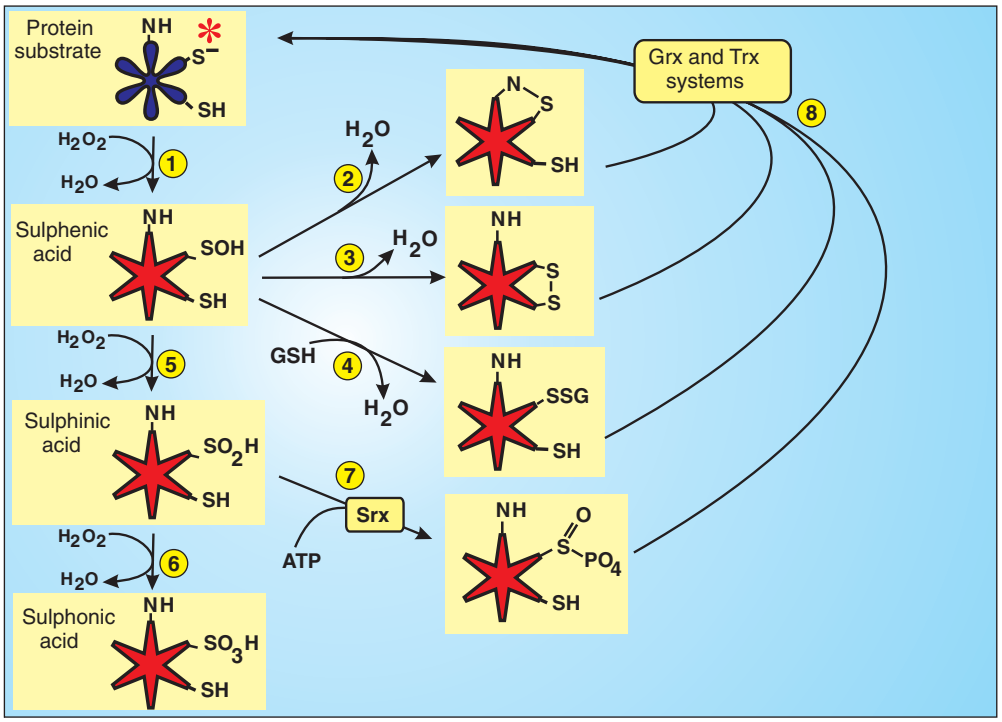
These oxidation processes can occur through a number of mechanisms. What is remarkable about this process is its specificity. Only a subset of proteins are modified, and within these there is a high degree of specificity in that only certain thiols are modified. How is it that an oxidizing agent such as H_2O_2 is able to seek out and selectively modify specific target proteins?The answer lies in the fact that proteins vary considerably in their sensitivity to mild oxidizing agents, such as H_2O_2. Most of the cysteine residues in proteins have a high acidic constant (i.e. pK_a values of approximately 8.5), which means that they are resistant to attack by H_2O_2. However, some of the cysteine residues, particularly those located next to positively charged amino acids, have pK_a values between 4 and 5 and thus exist as a thiolate anion (Cys-S^-), which is very vulnerable to oxidation, and these have been referred to as hyperreactive cysteine residues (the S^- group marked with an asterisk in Module 2: Figure reversible and irreversible ROS oxidations).

The sulphenic acid intermediate is somewhat unstable and can be converted into a number of intermediates by either eliminating water or causing it to interact with GSH (Reactions 2, 3 and 4 in Module 2: Figure reversible and irreversible ROS oxidations). In addition, sulphenic acid can be oxidized further by H_2O_2 to sulphinic acid and sulphonic acid. The formation of sulphinic acid is reversible through a reaction that requires ATP and sulphiredoxin (Srx). However, the final step to form sulphonic acid is irreversible and can result in serious damage and has been implicated in the ageing process. One idea is that this irreversible change may accumulate with time and the progressive damage may result in ageing.

There is a suggestion that the hyperperoxidation reactions that lead to the irreversible oxidation may be avoided by an internal reaction whereby the sulphenic acid is rapidly converted into a sulphenylamide species by interacting with the main-chain nitrogen atom of an adjacent serine residue (Step 2 in Module 2: Figure reversible and irreversible ROS oxidations). The formation of this sulphenylamide intermediate protects against further oxidation, and the enzyme can be reactivated by converting the intermediate back into a thiol group via a mixed disulphide reaction involving GSH.

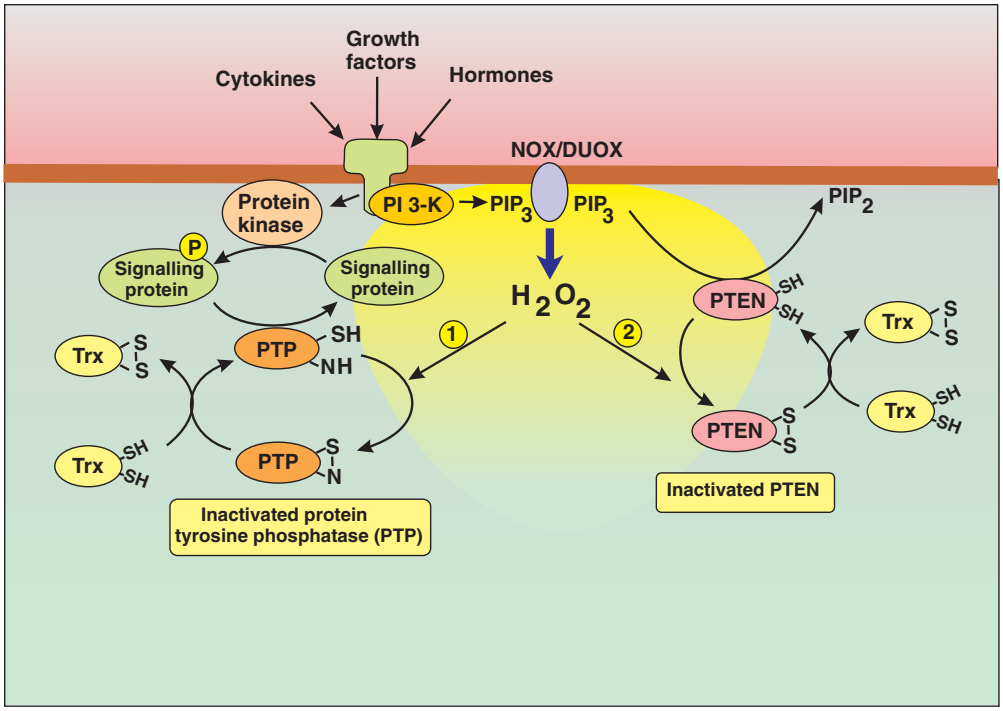
Two of the main signalling molecules whose activities are reduced by oxidation are the protein tyrosine phosphatases and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (Module 2: Figure ROS formation and action). An analysis of tyrosine phosphatase structure and function reveals the presence of a hyperreactive cysteine residue in the catalytic domain that is sensitive to oxidants resulting in inactivation of the enzyme. Other examples of proteins that have such hypersensitive residues include the cell cycle regulatory enzyme Cdc25C, the Ca^{2+}-release channels ryanodine receptors (RYRs) and inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$). The specificity with which this redox signalling system exerts its effects therefore depends on the fact that H_2O_2 will only modify hyperreactive cysteine residues that exist in these target proteins of the different oxidation-sensitive processes.

Module 2: | Figure reversible and irreversible ROS oxidations



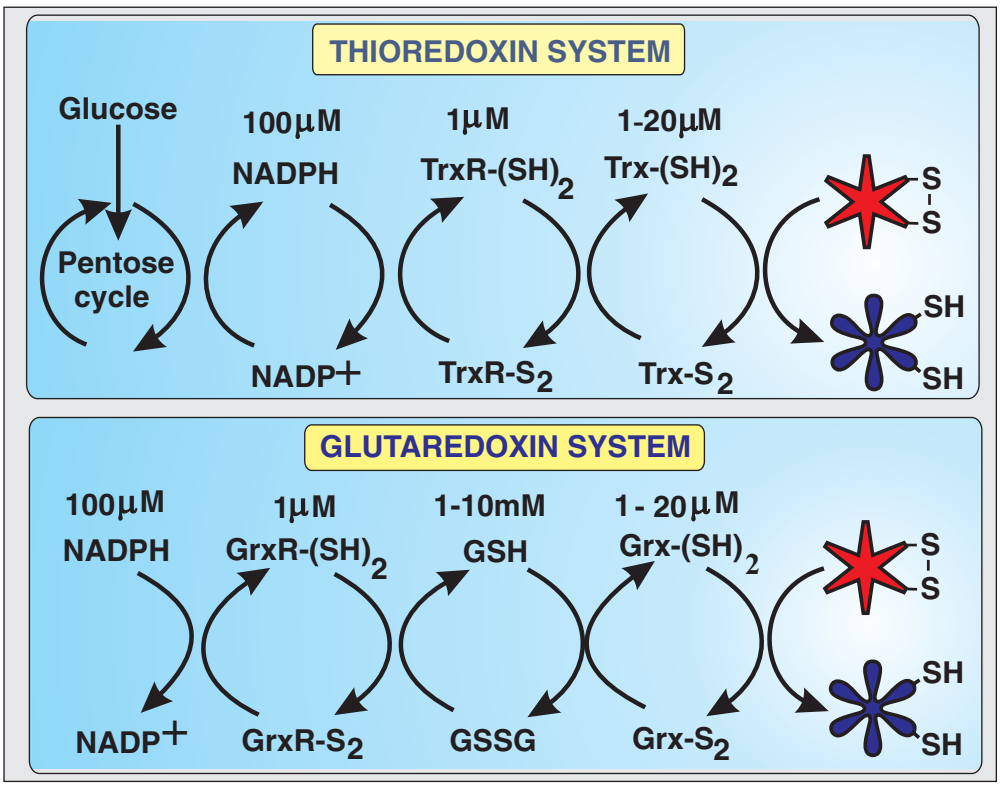
Reactive oxygen species (ROS) messenger action through the reversible oxidation of target proteins.
The target protein (blue) undergoes a variety of oxidative covalent modifications, some of which induce the conformational changes responsible for mediating a variety of oxidation-sensitive processes. The target proteins that are modified by reactive oxygen species (ROS) have a hyperreactive cysteine thiolate anion (asterisk) that is highly sensitive to oxidation by H_2O_2 to initiate a cascade of reactions as described in the text.

Module 2: | Figure ROS formation and action



Formation of H_2O_2 and its action to regulate phosphotyrosine phosphatase (PTP) and phosphatase and tensin homologue deleted on chromosome 10 (PTEN).
The hydrogen peroxide (H_2O_2) formed by the NADPH oxidase (NOX)/DUOX (dual oxidase) complex creates a microdomain in which it can inactivate enzymes involved in various signalling pathways: 1. The phosphotyrosine phosphatase (PTP) that dephosphorylates various signalling proteins is inactivated following its oxidation by H_2O_2; 2. The inositol lipid phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which dephosphorylates the lipid second messenger PtdIns3,4,5P_3, is inactivated following its oxidation by H_2O_2. These two enzymes can be reactivated following their reduction by the thioredoxin (Trx) system (Module 2: Figure recovery of protein oxidation).

Module 2: | Figure recovery of protein oxidation



Recovery of protein oxidation by the thioredoxin and glutaredoxin systems.
These two systems operate through protein disulphide oxidoreductases, which function to reduce the disulphide bond on the oxidized protein (red) back to the reduced thiol groups (blue). Thioredoxin (Trx) system: the active site on Trx is a -Cys-32-Gly-Pro-Cys-35- motif and it is these two cysteine residues in the reduced Trx-$(SH)_2$ form that are responsible for reducing disulphide bonds. Upon transferring the two protons to the substrate protein, the Trx becomes oxidized to Trx-S_2. Before it can operate again, the Trx-S_2 must be converted back into Trx-$(SH)_2$ by thioredoxin reductase (TrxR), which extracts reducing equivalents from the NADPH formed from the pentose cycle. Glutaredoxin (Grx) system: Grx has a -Cys-Pro-Tyr-Cys- motif, which is the active site for the oxidoreduction reaction. Glutaredoxin can act both as a dithiol-disulphide oxidoreductase and as a GSH-disulphide oxidoreductase. The latter action enables Grx to reverse mixed protein disulphides (protein-SSG not shown on the figure) formed when proteins interact with GSH. In order to continue its reducing function, the Grx-S_2 or Grx-SSG must be reduced back to Grx-$(SH)_2$ by its interaction with GSH, which is maintained in a reduced form by glutathione reductase.

Recovery of oxidation-sensitive processes

Like other signalling pathways, there are mechanisms in place for the recovery of oxidation-sensitive processes based on the thioredoxin (Trx) and glutaredoxin (Grx) systems (Module 2: Figure recovery of protein oxidation). Although the two systems have much in common, there are some differences, not least of which are their substrate specificity and the kinds of disulphide bonds that they can reduce. For example, Trx is more effective at reducing protein tyrosine phosphatase 1B than is Grx.

Trx and Grx exist in a reduced or oxidized state, and it is the former that enables them to reduce their substrates. In doing so, they become oxidized and have to be converted back into a reduced state by the Trx and Grx systems respectively. In the case of Trx, this is carried out by thioredoxin reductase. The Grx system is somewhat more complicated in that it depends upon glutathione that is regenerated by a glutathione reductase.

The level of Trx is markedly elevated during rheumatoid arthritis and this may influence the rate of secretion of matrix metalloproteinases (MMPs).

Thioredoxin (Trx)

Thioredoxin (Trx) is a redox buffer that operates together with thioredoxin reductase (TrxR) and NADPH to regulate the redox state of many different proteins (Module 2: Figure recovery of protein oxidation). It is a major dithiol reductase that functions to re-activate signalling proteins such as tyrosine phosphatases and PTEN (Module 2: Figure ROS formation and action). One of its other functions is to re-juvenate the peroxiredoxins (Prxs), which are a family of small antioxidant proteins that function to metabolize hydrogen peroxide (H_2O_2) to water, thus curtailing its messenger action (Module 2: Figure peroxiredoxin catalytic cycles). Trx also plays a role in the denitrosylation reaction (Module 2: Figure NO and cGMP signalling).

One of the functions of Trx is to regulate apoptosis signal-regulating kinase 1 (ASK1). Reduced Trx-$(SH)_2$ is known to bind to ASK1, but when it is oxidized to Trx-S_2, the ASK1 is released, and proceeds to induce apoptosis.

Thioredoxin-2 (Trx-2) is a mitochondrial-specific member of the Trx family. It functions together with mitochondrial thioredoxin reductase 2 (TrxR2) to regulate the

mitochondrial membrane potential and contributes to the inhibition of apoptosis by regulating the nitrosylation levels of the mitochondrial caspases. Activation of the FAS signalling system can increase the activity of Trx2, which switches the balance towards the more active de-nitrosylated form of the caspases.

Expression of the thioredoxin-interacting protein (Txnip), which binds to Trx and inhibits its participation in the denitrosylation reaction, is reduced in schizophrenia and thus contributes to the increase in redox signalling in schizophrenia (Module 12: Figure schizophrenia).

Thioredoxin reductase (TrxR)

Thioredoxin reductase (TrxR) together with thioredoxin (Trx) is an important oxidoreductase system that has a significant role in regulating the redox state (Module 2: Figure recovery of protein oxidation).

Trx reductase is unusual in that it contains selenocysteine (SeCys) located in the C-terminal active site, which has a highly conserved -Gly-Cys-SeCys-Gly- sequence. The N-terminal region contains flavin–adenine dinucleotide (FAD). The enzyme operates by transferring electrons from NADPH to FAD and then on to the active site in the C-terminus.

The level of Trx reductase is greatly increased in various tumour cells, where it may play an important role in inhibiting apoptosis by regulating the activity of Trx that inhibits apoptosis signal-regulating kinase 1 (ASK1). An enhanced level of TrxR may prevent apoptosis by ensuring that the Trx remains reduced to ensure that ASK1 remains inactive.

The ability of this thioredoxin system to reverse redox signalling is inhibited by Ca^{2+}, which acts to convert a large proportion of the reduced Trx-$(SH)_2$ into its oxidized form. Such an action would enhance the growth-promoting activity of the redox signalling system.

Thioredoxin-2 (Trx-2) is a mitochondria-specific member of the Trx family. It functions to regulate the mitochondrial membrane potential and contributes to the inhibition of apoptosis.

Glutathione reductase

Glutathione reductase is responsible for converting oxidized GSSG back into the reduced GSH (Module 2: Figure recovery of protein oxidation):

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

Oxidation-sensitive processes

The redox signalling pathway acts to regulate a variety of oxidation-sensitive processes (Module 2: Figure plasma membrane ROS formation). The cellular proteins that are sensitive to oxidation are those that contain hyperreactive cysteine residues. One of the main functions of redox signalling is to modulate the activity of other signalling systems that contain protein components with such hyperreactive cysteine residues, e.g. Ca^{2+}-releasing channels [inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) and ryanodine receptors (RYRs)], transcription factors, protein tyrosine phosphatases and by activating the mitochondrial permeability transition pore (MTP) to induce apoptosis. As so many key signalling functions are being influenced, it is not surprising to find that there is a role for redox signalling in many cellular processes:

- redox signalling in proliferation and cancer
- redox signalling in apoptosis
- redox signalling and DNA damage
- redox signalling in vascular homoeostasis
- redox signalling and gene transcription
- redox signalling and modulation of Ca^{2+} signalling
- redox signalling in schizophrenia

Redox signalling in proliferation and cancer

One of the main actions of redox signalling is to control growth. During the action of many growth factors there is an increase in the production of hydrogen peroxide (H_2O_2) (Module 2: Figure plasma membrane ROS formation), which facilitates growth factor signalling by inhibiting tyrosine phosphatases and the tumour suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN). The latter inhibits the hydrolysis of PtdIns3,4,5P_3 (PIP_3), which functions in cell migration, proliferation and survival. H_2O_2 inactivates PTEN by inducing a disulphide bond to form between Cys-124 in the active site and Cys-71. This disulphide is specifically reversed by thioredoxin. A reversible inactivation of PTEN may thus contribute to the accumulation of PIP_3, which will thus set up a positive-feedback loop, since the formation of PIP_3 is responsible for stimulating the production of H_2O_2.

Many cancer cells are known to use ROS to control their proliferation. There are five human homologues of Nox1. When Nox1 is overexpressed in fibroblasts, there is an increase in proliferation and tumour formation. The addition of antioxidants can reduce the growth of cancer cells.

Many cancer cells, like a number of stem cells, have enhanced ROS defences in the form of elevated levels of GSH and thioredoxin, which make them particularly resistant to apoptosis. Thioredoxin may also play a role in increasing the expression of the hypoxia-inducible factor 1α (HIF-1α), resulting in an increase in vascular endothelial growth factor (VEGF) and tumour angiogenesis.

Redox signalling in apoptosis

In addition to playing a role in cell proliferation, there is a darker side to reactive oxygen species (ROS) in that they can also activate apoptosis. One of the actions of redox signalling is that it contributes to Ca^{2+}-induced apoptosis at the level of the mitochondria. The uptake of Ca^{2+} and the resulting increase in ROS formation act synergistically to open the mitochondrial permeability transition pore (MTP) (Module 5: Figure mitochondrial Ca^{2+} signalling). ROS can also increase apoptosis by stimulating the acidic sphingomyelinases (SMases) that produce ceramide (Module 2: Figure sphingomyelin signalling).

Cells have different ways of suppressing this ROS-induced apoptosis. One mechanism is carried out by PtdIns 3-kinase signalling during which protein kinase

B (PKB) plays a prominent role through the Forkhead box O 3a (FOXO3a) transcription factor, which acts by increasing the amount of manganese superoxide dismutase (MnSOD) to provide greater protection against ROS (Module 4: Figure FOXO control mechanisms).

An overactive redox signalling system may contribute to the increase in neuronal cell death that characterizes Alzheimer's disease (Module 12: Figure astrocyte-induced neuronal death) and Down's syndrome.

Redox signalling and DNA damage

One of the major pathological consequences of excess ROS formation is DNA damage. When this damage occurs during the G_1 phase of the cell cycle, specific repair mechanisms function to repair the damage and they also induce the process of G_1 checkpoint signalling to DNA double-strand breaks (DSBs) (Module 9: Figure G_1 checkpoint signalling).

Redox signalling in vascular homoeostasis

Hydrogen peroxide (H_2O_2) may function as an endothelium-derived hyperpolarizing factor (EDHF) that diffuses across to relax neighbouring smooth muscle cells. This action may be particularly important in regulating the tone of cerebral arteries.

Redox signalling and gene transcription

The redox signalling pathway has been implicated in the control of gene transcription, particularly with regard to the activation of nuclear factor κB (NF-κB). Whether this activation is due to a direct modulation of the transcription factor by messengers such as H_2O_2 or indirectly through activation of other signalling pathways remains to be determined.

A large number of other transcription factors [activating protein 1 (AP-1), specificity protein 1 (SP1), c-Myb, p53 and Egr-1] are redox-sensitive. Many of these have a highly conserved cysteine residue located within their DNA-binding domains that has to be reduced in order for the factor to bind DNA (Module 4: Figure SRF and AP-1). In theory, therefore, such factors would be inhibited by oxidation. There is a nuclear redox factor 1 (Ref-1) that functions to control transcription by reducing this cysteine.

HDAC oxidation is an important mechanism used to control chromatin remodelling and gene transcription.

Redox factor 1 (Ref-1)

Ref-1 plays a role in the nucleus to promote gene transcription (Module 4: Figure SRF and AP-1) and to protect cells against oxidative stress. There is a possibility that it might also have a function within the cytoplasm to reduce the Rac-1-regulated production of reactive oxygen species (ROS).

Redox signalling and modulation of Ca^{2+} signalling

There are reciprocal interactions operating between the redox and Ca^{2+} signalling pathways. There are Ca^{2+} signalling effects on redox signalling and there are redox signalling effects on Ca^{2+} signalling.

Ca^{2+} signalling effects on redox signalling

One of the actions of Ca^{2+} is to enhance redox signalling by interfering with the recovery of the oxidation-sensitive processes. Ca^{2+} acts by turning down the thioredoxin system by inhibiting the thioredoxin reductase that normally switches off redox signalling.

Redox signalling effects on Ca^{2+} signalling

There are numerous examples of redox signalling acting to enhance Ca^{2+} signalling. For example, the two Ca^{2+} release channels ryanodine receptors (RYRs) and the $InsP_3$ receptors ($InsP_3Rs$) can be activated by oxidation of key cysteine residues. In the case of the latter, the oxidizing agent thimerosal faithfully reproduces the Ca^{2+} transients normally induced by sperm fusion during fertilization (Module 2: Figure thimerosal-induced Ca^{2+} signalling).

Another example is that hydrogen peroxide (H_2O_2) can markedly enhance Ca^{2+} signalling by inhibiting the Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1), which normally acts to keep in check the protein tyrosine phosphorylation cascade that occurs during B cell receptor (BCR) activation (Module 2: Figure ROS effects on Ca^{2+} signalling). This is a reciprocal interaction because it is the increase in Ca^{2+} that activates the formation of H_2O_2 through a Ca^{2+}-dependent activation of dual oxidase (DUOX). This is a good example of the cross-talk that can exist between signalling pathways.

Mitogen-activated protein kinase (MAPK) signalling

Overview

The multifunctional mitogen-activated protein kinase (MAPK) signalling system consists of separate pathways that function to control a number of different cellular processes such as gene transcription, metabolism, motility, cell proliferation, apoptosis, synaptic plasticity and long-term memory. These different downstream effectors are activated by the final MAPK components associated with the three main signalling pathways:

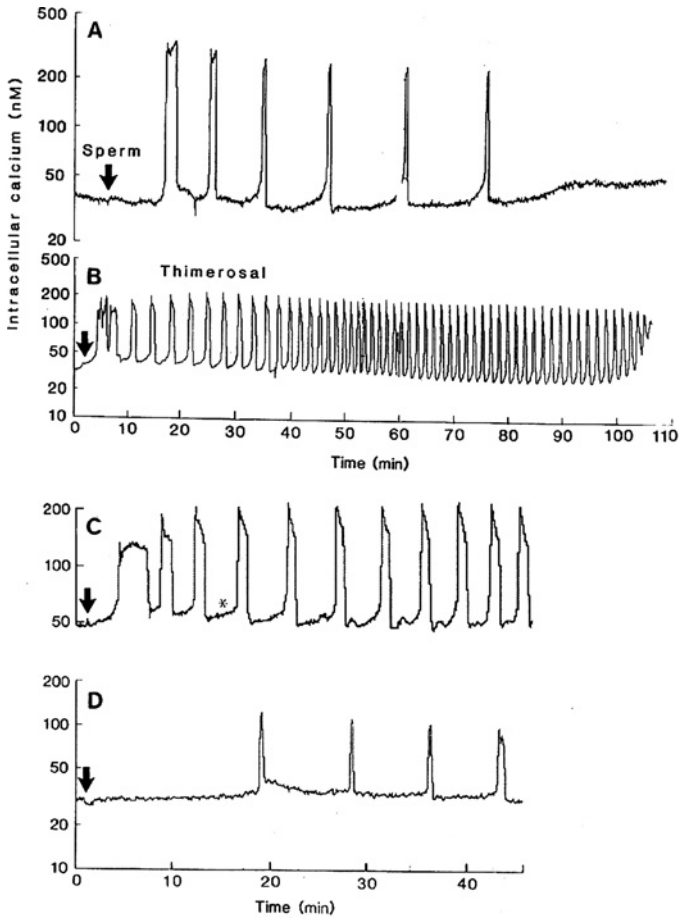
- Extracellular-signal-regulated kinase (ERK) pathway
- c-Jun N-terminal kinase (JNK) pathway
- p38 pathway

These different pathways are assembled by combining components from an extensive mitogen-activated protein kinase (MAPK) signalling toolkit.

The mitogen-activated protein kinase (MAPK) signalling properties such as their spatio-temporal control mechanisms help to explain how they operate to regulate so many cellular processes.

The activity of the MAPK signalling pathway is reversed by the mitogen-activated protein kinase (MAPK) phosphatases.

Module 2: | Figure thimerosal-induced Ca^{2+} signalling



Thimerosal-induced Ca^{2+} oscillations in oocytes.

A. The normal Ca^{2+} oscillation induced by sperm fusion in a mouse oocyte. **B**-**D**. Examples of Ca^{2+} oscillations induced by addition of the oxidizing agent thimerosal at three different concentrations: **B**, 100 μM; **C**, 10 μM; **D**, 1 μM. Reproduced from Cheek, T.R., McGuinness, O.M., Vincent, C., Moreton, R.B., Berridge, M.J. and Johnson, M.H. (1993) Fertilisation and thimerosal stimulate similar calcium spiking patterns in mouse oocytes but by separate mechanisms. Development **119**, 179-189, with permission from The Company of Biologists; see Cheek et al. 1993.

Mitogen-activated protein kinase (MAPK) signalling toolkit

There is an extensive mitogen-activated protein kinase (MAPK) signalling toolkit, which can be divided into different functional components such as the transducers, the MAPK kinase kinases (MAPKKKs), MAPK kinases (MAPKKs), MAPKs, MAPK scaffolding proteins and MAPK target proteins (Module 2: Table MAPK signalling toolkit). Specific components from this toolkit are then assembled into the different signalling pathways (Module 2: Figure MAPK signalling).

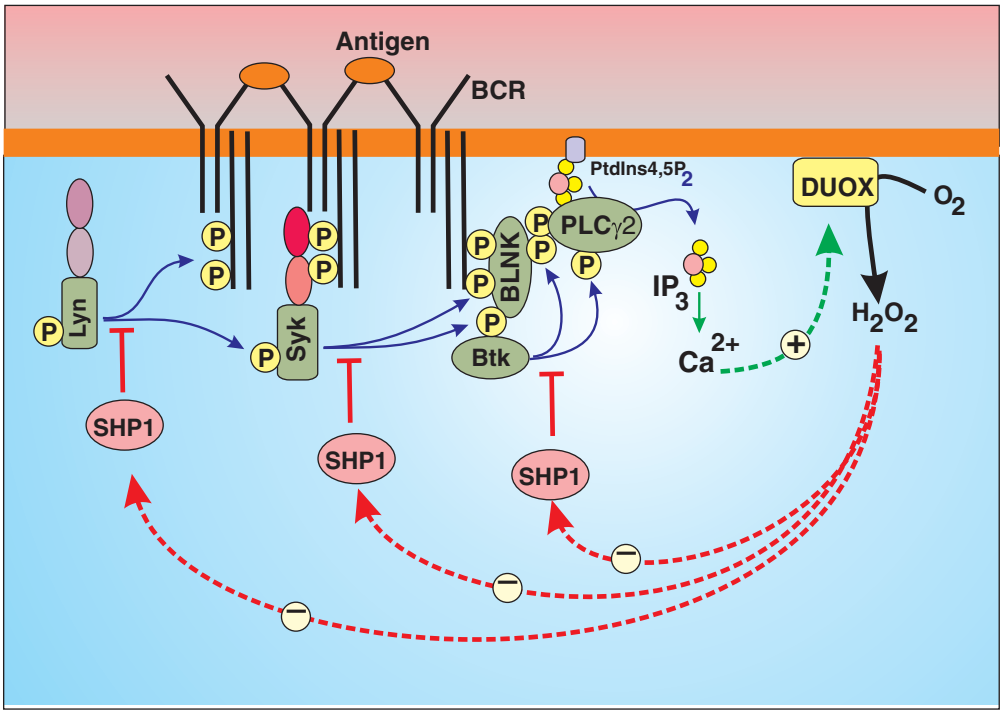
Extracellular-signal-regulated kinase (ERK) pathway

The extracellular-signal-regulated kinase (ERK) pathway is one of the major signalling cassettes of the mitogen-activated protein kinase (MAPK) signalling pathway (Module 2: Figure MAPK signalling). It performs a number of important signalling functions, including the control of cell proliferation and the synaptic plasticity responsible for learning and memory. The main MAPK/ERK kinase kinase (MEKK) components are the Raf family members Raf-1, A-Raf and B-Raf that phosphorylate two serine residues on the MAPK/ERK kinase (MEK) components MEK1/2. The latter are dual-specificity protein kinases that phosphorylate the tyrosine and threonine residues of the characteristic MAPK components ERK1/2 that are responsible for stimulating the downstream effectors, many of which are transcription factors (Module 2: Figure ERK signalling). There is thus a linear transfer of information through a phospho-relay system based on a sequential series of phosphorylation events.

An important feature of this ERK pathway, which can be activated by both protein tyrosine kinase-linked receptors (PTKRs) and by G protein-coupled receptors (GPCRs), is its spatial organization (Module 2: Figure ERK signalling).

In the case of the PTKRs, growth factors such as platelet-derived growth factor (PDGF) usually cause receptor dimerization, which allows the cytosolic tyrosine kinase domains to come together and to phosphorylate each other (Module 1: Figure PDGFR activation). These phosphorylated residues then function as docking motifs to pull in signalling components such as Shc, growth factor receptor-bound protein 2 (Grb2) and

Module 2: | Figure ROS effects on Ca^{2+} signalling



Reciprocal interaction between the ROS and Ca^{2+} signalling pathways during B cell receptor (BCR) activation.
Cross-linking the B cell receptor (BCR) with antigen sets up a protein phosphorylation cascade that begins with Lyn phosphorylating both the receptor and Syk. The latter then binds to the phosphorylated receptor, where it phosphorylates the adaptor protein B cell linker (BLNK) and the tyrosine kinase Bruton's tyrosine kinase (Btk). The latter phosphorylates BLNK and phospholipase Cγ2 (PLCγ2), which associates with BLNK and begins to hydrolyse PtdIns4,5P_2 to form inositol 1,4,5-trisphosphate (IP_3). This phosphorylation cascade is kept in check by the tyrosine phosphatase Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1). One of the functions of the Ca^{2+} released by IP_3 is to set up a positive-feedback loop based on the activation of the Ca^{2+}-sensitive enzyme dual oxidase (DUOX) that generates hydrogen peroxide (H_2O_2). The latter feeds back to inhibit SHP-1, which enables the signalling cascade to work more effectively in generating Ca^{2+} signals. This formation of H_2O_2 is highly localized as a microdomain in the immediate vicinity of the BCR (Module 2: Figure ROS microdomains). This figure is based on information taken from Singh et al. 2005.

Son-of-sevenless (SoS) that then activate the small GTP-binding protein Ras. Activated Ras then interacts with the protein kinase Raf, which initiates the phosphorylation cascade of the ERK pathway. The three components of this pathway (Raf-1, MEK1/2 and ERK1/2) are held in place at the cell surface by the scaffolding protein kinase suppressor of Ras 1 (KSR1). Up to this point, all the signal transduction processes have occurred at the cell surface, and the next information transfer step depends upon the diffusion of the activated enzyme from the cell surface to the nucleus. Once it is phosphorylated, the activated phospho-ERK1/2 leaves the plasma membrane to diffuse into the cytoplasm and then into the nucleus, where it phosphorylates and activates a number of transcription factors (Module 2: Figure ERK signalling). Some of these genes code for MAPK signalling components, such as MAPK phosphatase 1 (MKP-1), which sets up a negative-feedback loop. In addition, phospho-ERK1/2 can also act together with Ca^{2+} to stimulate cytoplasmic phospholipase A_2 ($cPLA_2$). The Ca^{2+} induces the $cPLA_2$ to associate with cytosolic membranes, whereas the ERK1/2 phosphorylates Ser-505 and Ser-727 to stimulate the enzymatic release of arachidonic acid from phospholipid precursors.

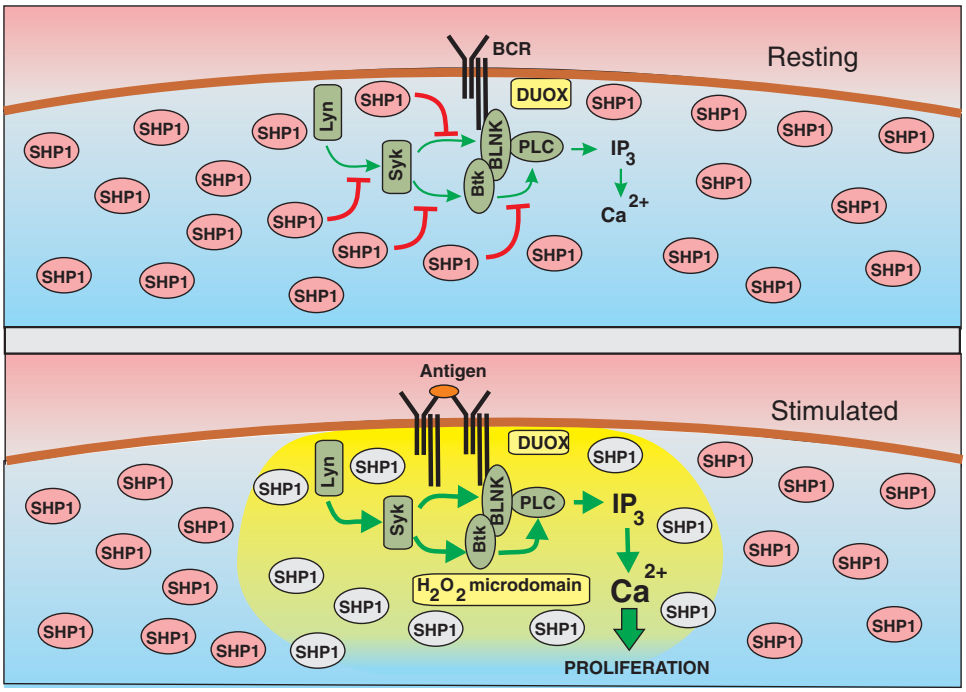
There are a number of putative mechanisms for linking the activation of G protein-coupled receptors (GPCRs) to the ERK pathway. The release of βγ subunits may activate Src, which can then feed into the processes that activate Ras. The arrestins can also function as scaffolds to assemble components of the ERK pathway such as Raf1 and ERK1/2. Alternatively, activation of phosphoinositide hydrolysis by G_q to produce inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) can access the ERK pathway via two mechanisms. The $InsP_3/Ca^{2+}$ can act through proline-rich tyrosine kinase 2 (Pyk2), whereas DAG and Ca^{2+} act through protein kinase C (PKC).

One of the major functions of the ERK pathway is to activate a range of different transcription factors such as cyclic AMP response element-binding protein (CREB) (Module 4: Figure CREB activation) and Elk-1 (Module 4: Figure ETS activation).

This ERK pathway contributes to the control of a large number of cellular processes:

- Regulation of cell proliferation such as T cell activation (Module 9: Figure TCR signalling)
- Cardiac hypertrophy (Module 12: Figure hypertrophy signalling mechanisms)

Module 2: | Figure ROS microdomains



Localized ROS signalling in a microdomain surrounding the B cell receptor.
Under resting conditions, the protein phosphorylation cascade that operates between Lyn, Syk, Bruton's tyrosine kinase (Btk) and phospholipase C (PLC) (Module 2: Figure ROS effects on Ca^{2+} signalling) is inhibited by the tyrosine phosphatase Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1), which is present at very high levels. When the receptor is stimulated by antigen, there is a Ca^{2+}-dependent activation of dual oxidase (DUOX) that creates a microdomain of H_2O_2 (yellow oblong) to inhibit the SHP-1 enzymes in the immediate vicinity of the B cell receptor (BCR). This figure is based on information taken from Singh et al. 2005.

- Synaptic plasticity such as long-term potentiation (LTP) in hippocampal neurons
- Proliferation of endothelial cells during angiogenesis (Module 9: Figure VEGF-induced proliferation)
- Phosphorylation of the transcription factor p53 (Module 4: Figure p53 domains)
- Remodelling the ERK signalling pathway may contribute to the development of polycystic kidney disease (Module 12: Figure polycystins and polycystic kidney disease)
- Activation of phospholipase A_2 (PLA_2) in mast cells (Module 11: Figure mast cell signalling)

c-Jun N-terminal kinase (JNK) pathway

The c-Jun N-terminal kinase (JNK) pathway is one of the major signalling cassettes of the mitogen-activated protein kinase (MAPK) signalling pathway. It functions in the control of a number of cellular processes, including proliferation, embryonic development and apoptosis. The pathway takes its name from the c-Jun N-terminal kinases 1–3 (JNK1–JNK3), which are the MAPKs that interact with the final effectors (Module 2: Figure MAPK signalling). They contain the dual phosphorylation motif Thr-Pro-Tyr, which is phosphorylated following activation of the upstream phosphorylation cascade.

The JNK pathway is activated by a bewildering number of mechanisms. This complexity is evident by the fact that there are 13 MAPK kinase kinases (MAPKKKs) responsible for feeding information into the JNK pathway. The apoptosis signal-regulating kinase 1 (ASK1) is an example of such a kinase that initiates the signalling cascade that leads to JNK activation. One way of trying to cope with this complexity is to examine specific examples such as the activation of JNK by the interleukin-1 receptor (Module 2: Figure JNK signalling).

The JNK pathway can also be activated through G protein-coupled receptors (GPCRs) using G proteins such as $G_{12/13}$. Just how G proteins feed into the cascade is unclear, but it seems that they activate the GTP-binding proteins such as Rac and Cdc42. Alternatively, the arrestins that associate with GPCRs during the process of receptor desensitization may function as a scaffold to bring together components of the JNK pathway such as MKP7 and JNK3.

The MAPK signalling system operates a negative-feedback loop in that some of the genes activated by the JNK pathway code for signalling components such as the scaffold protein JNK-interacting protein 1 (JIP1), which will bind JNK and thus limits its action.

This JNK pathway contributes to the control of a large number of cellular processes:

- Phosphorylation of the transcription factor p53 (Module 4: Figure p53 domains).
- The JNK pathway has been implicated in the mitogen-activated protein kinase (MAPK) signalling in cardiac hypertrophy (Module 12: Figure hypertrophy signalling mechanisms).

Module 2: | Table MAPK signalling toolkit
Mitogen-activated protein kinase (MAPK) signalling toolkit

Component	Comment
Transducers	
GCK	Germinal centre kinase
GLK	GCK-like kinase
HPK1	Haematopoietic progenitor kinase 1
MST1	Mammalian Ste20-like protein kinase
MAPKKKs	
ASK1	Apoptosis signal-regulating kinase 1
ASK2	Apoptosis signal-regulating kinase 2
DLK	Dual leucine-zipper-bearing kinase
MEKK1	MAPK/ERK kinase kinase 1
MEKK2	MAPK/ERK kinase kinase 2
MEKK3	MAPK/ERK kinase kinase 3
MEKK4	MAPK/ERK kinase kinase 4
MLK1	Mixed lineage kinase 1
MLK2	Mixed lineage kinase 2
MLK3	Mixed lineage kinase 3
Mos	
Raf-1	
B-Raf	
PAK	p21-activated kinase
TAK1	TGFβ-activated protein kinase 1
Tpl2	Tumour progression locus 2
MAPKKs	
MEK1	MAPK/ERK kinase 1
MEK2	MAPK/ERK kinase 2
MKK3	MAPK kinase 3
MKK4	MAPK kinase 4
MEK5	MAPK/ERK kinase 5
MKK6	MAPK kinase 6
MKK7	MAPK kinase 7
MAPKs	
ERK1	Extracellular-signal-regulated kinase 1
ERK2	Extracellular-signal-regulated kinase 2
ERK3-related	
ERK3	Extracellular-signal-regulated kinase 3
ERK5	Extracellular-signal-regulated kinase 5
ERK7	Extracellular-signal-regulated kinase 7
ERK8	Extracellular-signal-regulated kinase 8
JNK1	c-Jun N-terminal kinase 1
JNK2	c-Jun N-terminal kinase 2
JNK3	c-Jun N-terminal kinase 3
p38α	
p38β	
p38γ	
p38δ	
MAPK scaffolding proteins	
β-Arrestin-2	
JIP1	JNK-interacting protein 1
JIP2	JNK-interacting protein 2
JIP3	JNK-interacting protein 3
KSR1	Kinase suppressor of Ras 1
KSR2	Kinase suppressor of Ras 2
MP1	MEK partner 1
MKPX	Phosphatase that may also function as a scaffold
SKRP1	Stress-activated protein kinase (SAPK) pathway-regulating phosphatase 1
MAPK target proteins	
ATF2	Activating transcription factor 2
Cytoplasmic PLA_2	Cytoplasmic phospholipase A_2
ETS	
Elk-1	
c-Jun	
Jun-B	
Jun-D	
MAPKAP-kinase	MAPK-activated protein kinase
MEF2	Myocyte enhancer factor 2
MSK1	Mitogen- and stress-activated protein kinase 1
MSK2	Mitogen- and stress-activated protein kinase 2
SAP-1	Stomach cancer-associated protein tyrosine phosphatase-1

Module 2 | Table continued

Component	Comment
MAPK phosphatases	
MKP-1	MAPK phosphatase-1 (Module 2: Figure ERK signalling)
MKP-2	MAPK phosphatase-2
MKP-3	MAPK phosphatase-3, acts specifically to dephosphorylate ERK2
MKP-4	MAPK phosphatase-4
MKP-5	MAPK phosphatase-5
VHR	VH1-related
hVH3/B23	
hVH5/M3/6	
PAC1	
Pyst2	
STEP	Striatal-enriched protein tyrosine phosphatase; dephosphorylates ERK in neurons (Module 10: Figure medium spiny neuron signalling).

Apoptosis signal-regulating kinase 1 (ASK1)

Apoptosis signal-regulating kinase 1 (ASK1) is part of the mitogen-activated protein kinase (MAPK) signalling toolkit (Module 2: Table MAPK signalling toolkit) where it functions as one of the MAPK kinase kinases (MAPKKKs) that operates within the c-Jun N-terminal kinase (JNK) pathway (Module 2: Figure MAPK signalling). One of the stimuli capable of activating ASK1 is tumour necrosis factor α (TNFα) (see step 2 in Module 2: Figure NF-κB activation).

The activity of ASK1 can also be modulated by at least two other signalling mechanisms. First, the reduced form of the redox buffer thioredoxin (Trx) binds to ASK1 and inhibits its activity. Following ROS formation, the Trx is oxidized and dissociates thus removing its inhibitory effect and allowing ADK1 to stimulate apoptosis. Secondly, ASK1 activity is inhibited by binding to calcium and integrin-binding protein 1 (CIB1). An increase in the level of Ca^{2+} removes this inhibition thus allowing ASK1 to induce apoptosis.

Sprouty (SPRY)

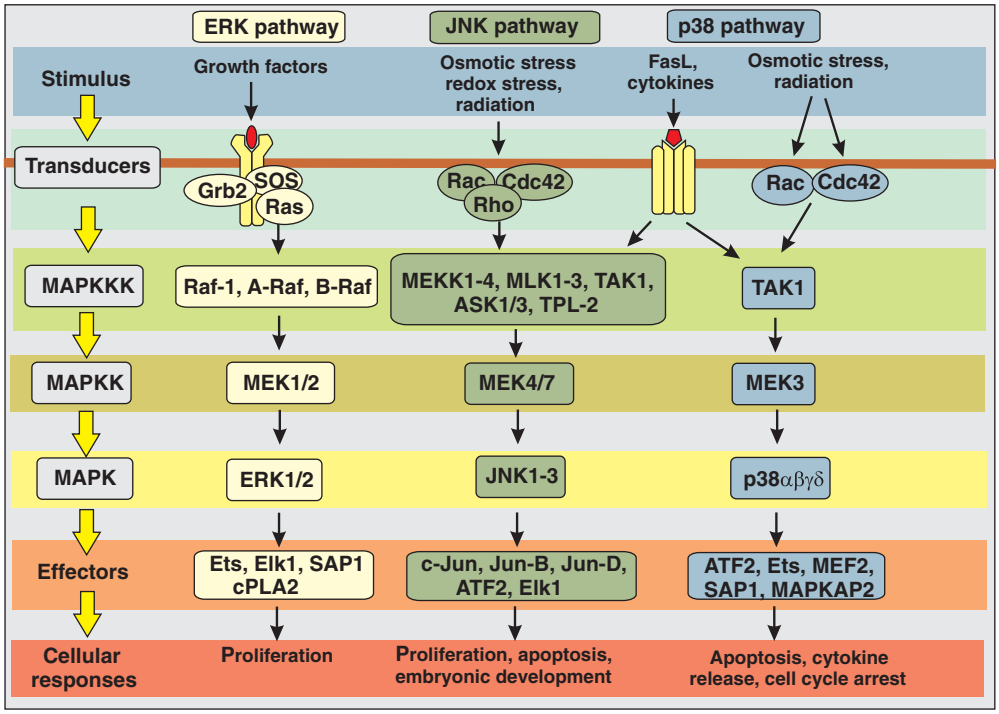
There are two families of sprouty proteins that function as inhibitors of the MAP kinase signalling pathway. The founding sprouty family has four members (SPRY1–4) while the sprouty-related, EVH1 domain-containing protein (SPRED) family contains three members (SPRED1–3) together with EVE-3, which is a splice variant of SPRED3. Members of this family have a cysteine-rich domain (CRD).

The SPRY proteins seem to function during development to control the formation of a number of tissues and organs. The level of SPRY1 is regulated by miR-21 to increase MAP kinase signalling during cardiac development.

Like the SPRY family, the SPREDs act by binding to either Ras or Raf1 to inhibit the MAP kinase signalling pathway. SPRED1 can act either as a homodimer or as a heterodimer with SPRED2.

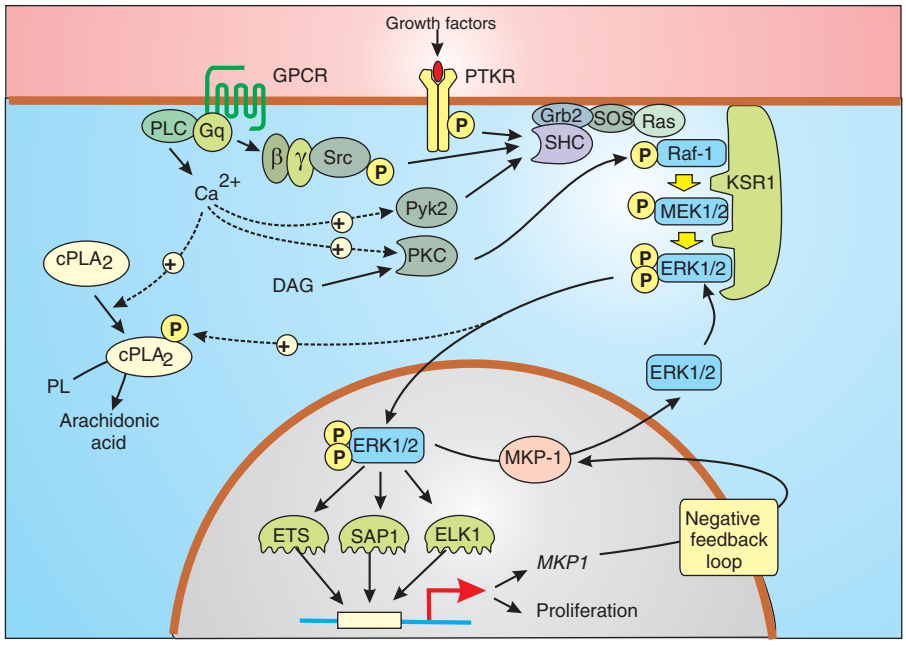
Mutations in the gene that encodes SPRED1, which will result in hyperactivation of the Ras/MAP kinase signalling

Module 2: | Figure MAPK signalling



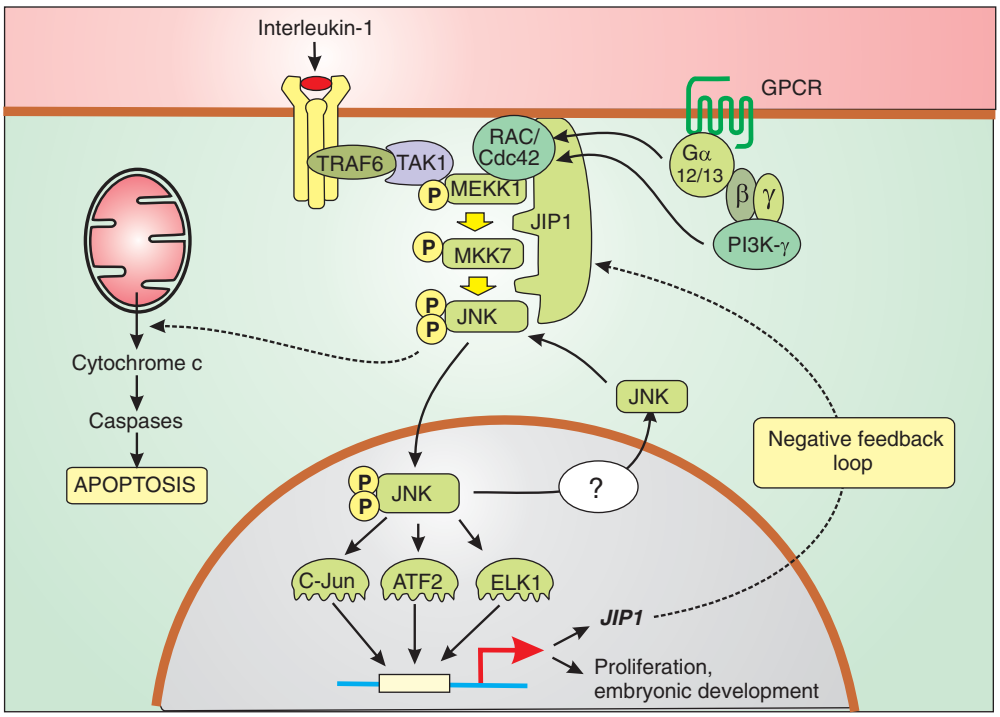
Organization of the main mitogen-activated protein kinase (MAPK) signalling pathways.
The generic pathway on the left summarizes the sequential organization of the mitogen-activated protein kinase (MAPK) signalling system. External stimuli act through a variety of transducers to stimulate the first element of the signalling pathway, which is one of the 14 different MAPK kinase kinases (MAPKKKs). These MAPKKKs then phosphorylate the next element, which is one of the seven MAPK kinases (MAPKKs). This MAPKK then phosphorylates one of the 12 MAPKs, the names of which define the different signalling pathways. The three main pathways are the ERK pathway (Module 2: Figure ERK signalling), the JNK pathway (Module 2: Figure JNK signalling) and the p38 pathway. The components that make up these different signalling pathways are summarized in the MAPK signalling toolkit (Module 2: Table MAPK signalling toolkit).

Module 2: | Figure ERK signalling



The organization and topology of the extracellular-signal-regulated kinase (ERK) pathway.
The extracellular-signal-regulated kinase (ERK) pathway, which can be made up from different components (Module 2: Figure MAPK signalling), is represented here by Raf-1, mitogen-activated protein kinase (MAPK)/ERK kinase 1/2 (MEK1/2) and ERK1/2, which can be activated by either protein tyrosine kinase-linked receptors (PTKRs) or by G protein-coupled receptors (GPCRs). (See the text for further details.)

Module 2: | Figure JNK signalling



The organization and topology of the c-Jun N-terminal kinase (JNK) pathway.
The c-Jun N-terminal kinase (JNK) pathway can be activated in many ways, including via different receptor mechanisms and by various environmental stresses such as osmotic, redox and radiation stress. These different inputs are transduced by separate mechanisms that all feed into the JNK signalling cascade. With regard to receptor activation, the JNK pathway can be activated by various cytokines such as interleukin-1 as illustrated here. The interleukin-1 receptor (IL-1R) is composed of two receptor-binding domains that interact with interleukin-1 and a non-binding accessory protein. Once activated by interleukin-1, the IL-1R recruits the adaptor protein tumour-necrosis-factor-receptor-associated factor 6 (TRAF6), which then recruits the mitogen-activated protein kinase kinase kinase (MAPKKK) called transforming growth factor β-activated kinase 1 (TAK1) responsible for initiating the phosphorylation cascade by phosphorylating MAPK/extracellular-signal-regulated kinase (ERK) kinase kinase 1 (MEKK1). The MEKK1 then phosphorylates the dual-specificity protein kinase MAPK kinase 7 (MKK7) responsible for phosphorylating the tyrosine and threonine residues on JNK. This activation cascade occurs in the vicinity of the plasma membrane, where it is organized by the scaffolding protein JNK-interacting protein 1 (JIP1). Once JNK is phosphorylated, it leaves the multimolecular activation complex and then diffuses into the nucleus, where it activates transcription factors responsible for controlling processes such as proliferation, apoptosis and embryonic development.

pathway, causes neurofibromatosis type 1-like syndrome (NFLS).

p38 pathway

The p38 pathway is one of the major signalling cassettes of the mitogen-activated protein kinase (MAPK) signalling pathway. It functions in the control of apoptosis and the release of cytokines by macrophages and neutrophils. The pathway takes its name from the family of p38 kinases, which are the MAPKs that interact with the final effectors (Module 2: Figure MAPK signalling).

The p38 pathway can be activated either by different receptor mechanisms or by various environmental stresses such as osmotic, redox or radiation stress. For example, one of the targets of the p38 pathway that is activated by UV irradiation is one of the Cdc25 enzymes that control cell cycle progression. Phosphorylation of Ser-323 on Cdc25B results in the binding of 14-3-3 protein, which then prevents this enzyme from initiating entry into mitosis.

The Toll receptor signalling pathway provides an example of how the p38 pathway is activated by a Toll receptor for lipopolysaccharide (LPS) (Module 2: Figure Toll receptor signalling).

Mitogen-activated protein kinase (MAPK) signalling properties

The different mitogen-activated protein kinase (MAPK) signalling pathways have a number of important properties that greatly enhance their signalling efficiency.

Temporal aspects of mitogen-activated protein kinase (MAPK) signalling

The outcome of these pathways is very dependent on temporal aspects, particularly signal duration. For example, prolonged stimulation is necessary to induce cell proliferation.

Fidelity

Another important property is the fidelity of these signalling pathways. The three major signalling pathways share many common features: they often share signalling components, they are often activated by similar inputs [especially in the case of the c-Jun N-terminal kinase (JNK) and p38 pathways], and they can also regulate similar

cellular processes. A question therefore arises as to how the fidelity of these signalling pathways is achieved in order to reduce cross-talk and to ensure that they carry out their particular functions. It seems that much of this fidelity is achieved by using molecular scaffolds to hold together all the components of each signalling pathway in a multimolecular complex. In this way, information can be passed from one component to the next without interference from other signalling pathways (Module 6: Figure signalling hierarchies). Examples of such scaffolds are the scaffolding proteins kinase suppressor of Ras 1 (KSR1) (Module 2: Figure ERK signalling) and JNK-interacting protein 1 (JIP1) (Module 2: Figure JNK signalling).

Other determinants of fidelity are the docking sites that enable the different mitogen-activated protein kinases (MAPKs) to bind to their specific downstream effectors.

Phenotypic remodelling of the mitogen-activated protein kinase (MAPK) signalling pathway

The mitogen-activated protein kinase (MAPK) signalling pathway operates autoregulatory loops in that the different signalling pathways can regulate the expression of their own signalling components. For example, the extracellular-signal-regulated kinase (ERK) pathway can regulate the expression of MAPK phosphatase 1 (MKP1) (Module 2: Figure ERK signalling), whereas the c-Jun N-terminal kinase (JNK) pathway induces the expression of JNK-interacting protein 1 (JIP1) (Module 2: Figure JNK signalling). This induction of MKP1 and JIP1 effectively set up negative-feedback loops that limit the activity of the MAPK signalling pathway, and is an example of signalsome stability.

Phenotypic remodelling of the MAPK signalsome may result in the abnormal cell proliferation observed in polycystic kidney disease (Module 12: Figure polycystins and polycystic kidney disease).

Nuclear factor κB (NF-κB) signalling pathway

The transcription factor nuclear factor κB (NF-κB) is activated by a large number of external stimuli such as the tumour necrosis factors (TNFs), interleukin-1 (IL-1) and the pathogen-associated molecular patterns (PAMPs), which are responsible for controlling processes such as inflammation, cell proliferation and apoptosis. NF-κB belongs to the group of transcription factors that lie latent in the cytoplasm and then translocate into the nucleus upon activation (mechanism 2 in Module 4: Figure transcription factor activation). This diversity of downstream effector processes indicates that there must be separate signalling pathways, and this is evident from the nuclear factor κB (NF-κB) signalling toolkit, which contains multiple isoforms both of the transcription factors (the NF-κB/Rel family) and of the activation components. This complexity is carried through to the nuclear factor κB (NF-κB) signalling pathway, where there are many variations on the basic theme of NF-κB activation (i.e. Mechanism 2 in Module 4: Figure transcription factor activation).

There are two remarkable aspects of the NF-κB signalling pathway. Firstly, it can control a very large number of genes that are often activated as large cohorts in specific cells by different stimuli. Secondly, it is used by a number of different signalling systems with subtle variations in the mechanism and the components that are used to convey information into the nucleus. The tumour necrosis factor α (TNFα) signalling pathway and the Toll receptor signalling pathway will be described to illustrate the main features of the NF-κB signalling pathway. The receptor activator of nuclear factor κB (NF-κB) ligand (RANKL), which is a transmembrane protein that belongs to the TNF family of cytokines, activates the RANKL receptor (RANK) that uses the NF-κB signalling pathway to control osteoclastogenesis (Module 8: Figure osteoclast differentiation).

Nuclear factor κB (NF-κB) signalling toolkit

The NF-κB signalling toolkit is composed of four main classes of signalling components (Module 2: Table NF-κB signalling toolkit).

Nuclear factor κB (NF-κB)/Rel family

The nuclear factor κB (NF-κB)/Rel family consists of five members (Module 2: Table NF-κB signalling toolkit). There is some confusion concerning the nomenclature of this family. The notations shown at the beginning of the table (p50, p52, p65, RelB and c-Rel) will be used here. All members of the family share an N-terminal Rel homology domain (RHD), which binds to DNA (Module 2: Figure NF-κB, IκB and IKK structure). The RHD is also used to associate NF-κB with the inhibitor of nuclear factor κB (NF-κB) (IκB) proteins. The NF-κB/Rel family of transcription factors normally function as heterodimers, and the p50/p65 complex was the first to be discovered. Some of the family members can form homodimers, such as p50/p50 and p52/p52, and these act as repressors of NF-κB-sensitive genes.

Inhibitor of nuclear factor κB (NF-κB) (IκB)

The inhibitor of nuclear factor κB (NF-κB) (IκB) family are characterized by an ankyrin repeat domain (Module 2: Figure NF-κB, IκB and IKK structure), which functions in its interaction with NF-κB to form the inactive complex that resides in the cytoplasm. The IκB inhibits transcription by masking the nuclear localization signal (NLS) on NF-κB, which thus prevent it from entering the nucleus. The NF-κB/IκB complex remains inactive within the cytoplasm until the IκB is removed following activation of the nuclear factor κB (NF-κB) signalling pathway. Much of the specificity within the NF-κB signalling pathway depends on these IκB isoforms being able to bind to different dimers of the NF-κB/Rel family.

Inhibitor of nuclear factor κB (IκB) kinases (IKKs)

The inhibitor of nuclear factor κB (IκB) kinases (IKKs) function as heterodimers and are responsible for phosphorylating IκB to mark it for subsequent degradation by the proteasome. The kinase domain is located in the N-terminus, whereas the C-terminus has leucine zipper

Module 2: | Table NF-κB signalling toolkit
The nuclear factor κB (NF-κB) signalling toolkit

Nuclear factor κB (NF-κB) transcription system	Comments
NF-κB/Rel family of transcription factors	
p50 (NF-κB1)	p50 has a p105 precursor protein
p52 (NF-κB2)	p52 has a p100 precursor protein
p65 (NF-κB3, also known as RelA)	
RelB	
c-Rel	
Inhibitor of NF-κB (IκB) family	
IκBα	Bind to p65 and c-Rel
IκBβ	
IκBγ	
IκBε	
IκBζ	Binds preferentially to p50
Bcl3	B cell lymphoma 3 binds to p50/p50 and p53/p53 homodimers.
Inhibitor of NF-κB (IκB) kinases (IKKs)	
IKKα	
IKKβ	
NEMO (IKKγ)	A regulatory subunit responsible for the interaction with upstream kinases on the receptor complex (Module 2: Figure NF-κB activation)
Associated proteins	
Cdc37	
Hsp90	

and helix–loop–helix domains (Module 2: Figure NF-κB, IκB and IKK structure). These two protein-association domains enable the enzyme to associate with a large multi-subunit complex in the cytoplasm. Another important component of this complex is nuclear factor κB (NF-κB) essential modulator (NEMO) (IKKγ), which is a regulatory/structural subunit responsible for the interaction with upstream kinases on the receptor as part of the tumour necrosis factor α (TNFα) signalling pathway (Module 2: Figure NF-κB activation). In the case of NF-κB activation in T-cells, the IKK is stimulated by protein kinase θ (PKCθ) using the scaffolding proteins CARMA1, Bcl10 and MALT1 (Module 9: Figure TCR signalling).

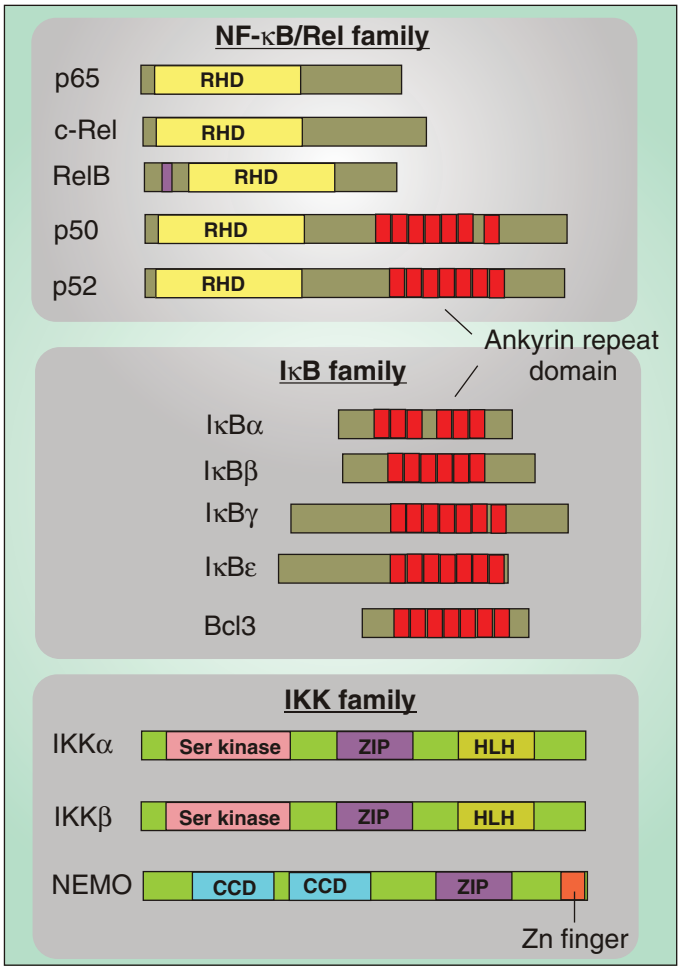
The tumour suppressor CYLD binds to NEMO and inhibits the subsequent phosphorylation of IκB as part of the TNFα signalling pathway.

Tumour necrosis factor α (TNFα) signalling pathway

The basic operation of the nuclear factor κB (NF-κB)/Rel family of transcription factors is that they are activated in the cytoplasm and then translocate into the nucleus to activate transcription (mechanism 2 in Module 4: Figure transcription factor activation). There are a number of variations in the way that this translocation process is initiated, depending on the nature of the incoming signals and the receptors that are being activated. In addition to the activation of NF-κB, these non-enzyme-containing receptors can activate other signalling pathways (Module 1: Figure cytokines). In this section, attention will focus on the NF-κB signalling pathway, which is used by the tumour necrosis factor α (TNFα). It is considered to be one of the 'classical' mechanisms as illustrated by the following sequence of events (Module 2: Figure NF-κB activation):

1. Occupation of the TNF receptor (TNF-R) by TNF induces receptor oligomerization to form a complex that attracts the adaptors TRADD and the TNFR-associated factor 2 (TRAF2), which is an adaptor that belongs to the TNF-receptor-associated factor (TRAF) family.
2. TRAF2 is a RING domain E3 ubiquitin ligase that associates with the heterotrimeric ubiquitin-conjugating (E2) complex that contains Ubc13 and Uev1A. This is a K63 ubiquitinating complex that adds ubiquitin chains to TRAF2 and this then helps to recruit the receptor-interacting protein 1 (RIP1), which is also ubiquitinated. These ubiquitin chains provide an important scaffolding role in the assembly of additional elements of the signal transduction pathway. For example TRAF2 interacts with apoptosis signal-regulating kinase 1 (ASK1) that then relays information to both the JNK signalling and p38 signalling pathways
3. The developing receptor complex attracts additional components such as the transforming growth factor β activated kinase-1 (TAK1), the TAK1-binding (TAB) proteins 1 to 3 (TAB1-3) and the multisubunit cytoplasmic complex containing the inhibitor of NF-κB (IκB) kinase (IKK) α/IKKβ dimer and the regulatory NF-κB essential modifier (NEMO) subunit, which is a central player in this translocation sequence.
4. Once this complex is complete, the TAK1 phosphorylates and activates IKKβ.
5. The IKKβ then phosphorylates IκBα on two sites (Ser-32 and Ser-36).
6. The phosphorylated IκBα is then susceptible to ubiquitination by the Skp1/cullin/F-box (SCF) ubiquitin ligase.
7. The polyubiquitinated IκBα is then sent to the proteasome, where it is degraded, resulting in the liberation of the NF-κB heterodimer p50/p65.
8. The NF-κB is imported into the nucleus, where it binds to the κB promoter elements to activate expression of many different genes (Module 4: Figure NF-κB activation and function). One of these genes is hypoxia-inducible factor 1α (HIF-1α), which thus serves to link the innate immune response to the hypoxic response.
9. Transcription ceases when NF-κB is exported from the nucleus. One of the genes activated by NF-κB is IκBα, which thus sets up a negative-feedback loop, because it binds to the NF-κB that is exported from the nucleus to reconstitute the inactive cytoplasmic complex.
10. The ubiquitin signalling pathway contributes to the recovery of this signalling cascade after TNFα is withdrawn by removing the ubiquitin scaffolds that hold together the transducing complex. The deubiquitinating enzymes A20 and CYLD are particularly active in removing the ubiquitin chains. The reversible ubiquitination of signal transducing components is thus an essential part of the processing of information by this TNF signalling pathway.

Module 2: | Figure NF-κB, IκB and IKK structure



The structure of components of the nuclear factor κB (NF-κB) signalling toolkit.
The nuclear factor κB (NF-κB)/Rel family all share a Rel homology region (RHD). The mauve box in the N-terminal region of RelB is a putative leucine zipper region. The p50 and p52 isoforms also have ankyrin repeat domains that resemble those found in the inhibitor of NF-κB (IκB) family. The IκB kinase (IKK) family has both kinase (IKKα and IKKβ) and regulatory components [NF-κB essential modulator (NEMO)]. ZIP, leucine zipper; HLH, helix-loop-helix domain; CCD, coiled-coil domain. Redrawn from *Handbook of Cell Signalling*, Vol. 3 (R.A. Bradshaw and E.A. Dennis, eds), Westwick, J.K., Schwamborn, K. and Mercurio, F., NFκB: a key integrator of cell signalling, pp. 107-114. Copyright (2003), with permission from Elsevier; see Westwick et al. 2003.

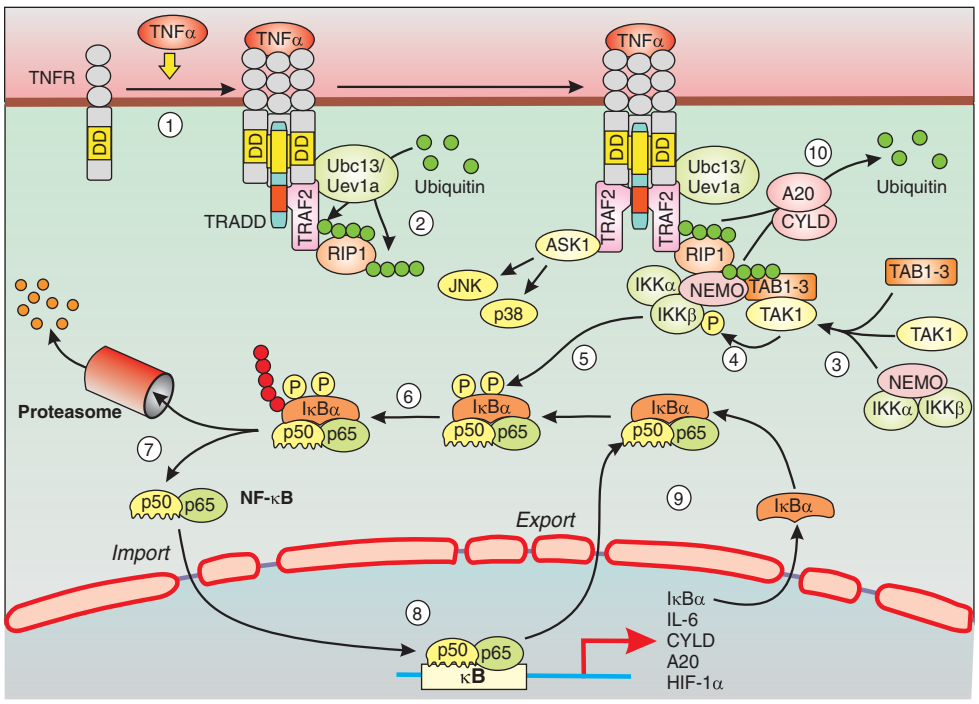
Tumour necrosis factor (TNF)-receptor-associated factor (TRAF) family

The tumour necrosis factor (TNF)-receptor-associated factor (TRAF) family has seven members, which function in the signalling pathways of various TNF receptor superfamily and Toll/interleukin-1 receptor mechanisms. TRAF2 functions as an adaptor for the tumour necrosis factor receptor (Module 2: Figure NF-κB activation). In the case of osteoclasts, it is TRAF6 that couples the receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) receptor (RANK) to downstream signalling pathways (Module 8: Figure osteoclast differentiation).

Toll receptor signalling pathway

The Toll receptor signalling pathway plays a central role in inflammatory responses (Module 11: Figure inflammation). This pathway is activated by two types of stimuli: cytokines, represented by interleukin-1 (IL-1), and stimuli derived from pathogens that are known as pathogen-associated molecular patterns (PAMPs) (Module 11: Figure formation and action of PAMPs). These PAMPs act on Toll-like receptors (TLRs) and the IL-1 receptor (IL-1R) and these form a TLR/IL-1R family that act through a similar Toll receptor signalling pathway (Module 1: Figure cytokines). The primary function of this signalling pathway is to stimulate the transcriptional processes that result in the expression of a wide range of inflammatory cytokines and immunoregulators (Module 2: Figure Toll receptor signalling). As such, the Toll receptor signalling pathway has to transmit information from the TLRs and the IL-1R on the cell surface to the transcriptional factors such as nuclear factor κB (NF-κB) and activating protein 1 (AP-1) (c-Jun/Fos) that act within the nucleus. This information transfer system will be illustrated by reference to the response of the receptor TLR4 to lipopolysaccharide (LPS) (Module 11: Figure formation and action of PAMPs). As

Module 2: | Figure NF-κB activation

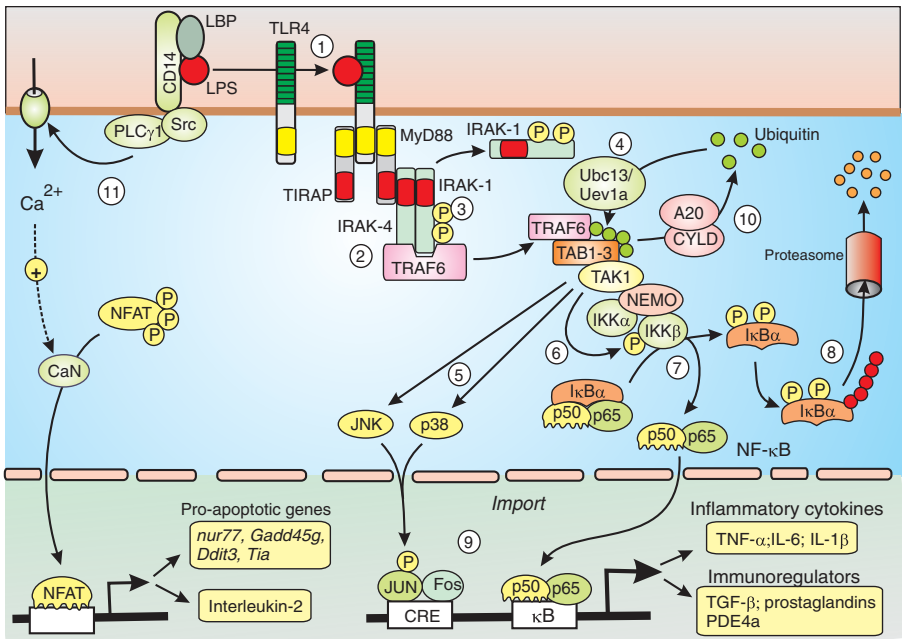


The 'classical'nuclear factor κB (NF-κB) signalling pathway activated by the tumour necrosis factor receptor (TNFR).
The p50 and p65 isoforms of the nuclear factor κB (NF-κB)/Rel family form the NF-κB dimer that is activated in the tumour necrosis factor α (TNFα) signalling pathway. The activated TNF receptor (TNFR) recruits a signalling complex to the membrane (Steps 2-4), which contains the inhibitor of NF-κB (IκB) kinase (IKK) α/IKKβ dimer that is responsible for phosphorylating the IκBα subunit that retains p50/p65 in the cytoplasm (Step 5). When the IκBα is phosphorylated, it is ubiquitinated and degraded by the proteasome (Steps 6 and 7). The p50/p65 homodimer is imported into the nucleus (Step 8), where it activates a large number of genes. One of these genes codes for IκBα, which sets up a negative-feedback loop by exporting p50/p65 from the nucleus (Step 9). Adapted from *Trends Biochem. Sci.*, Vol. 30, Viatour, P., Merville, M.-P., Bours, V. and Chariot, A., Phosphorylation of NF-κB and IκB proteins: implications in cancer and inflammation, pp. 43-52. Copyright (2004), with permission from Elsevier; see Viatour et al. 2005.

for many other signalling pathways, information is transferred through both protein–protein interactions and protein phosphorylation reactions. The ubiquitin signalling system also has an important role in orchestrating this Toll receptor signalling pathway as shown in the following sequence (Module 2: Figure Toll receptor signalling):

1. The TLR4 is a transmembrane protein that has leucine-rich repeats in its ectodomain, while the cytoplasmic domain has a Toll/interleukin 1 (IL-1) receptor (TIR) domain. The lipopolysaccharide (LPS) that initiates this signalling pathway binds first to an extracellular LPS-binding protein (LBP) and to CD14, which is a glycosylphosphatidylinositol-anchored membrane glycoprotein, and this complex carries the LPS to the Toll-like receptor 4 (TLR4).
2. The TIR domain on the TLR4 receptor forms homophilic interaction with the TIR domain of the adaptor proteins TIR adaptor protein (TIRAP) and MyD88. The latter is specific for certain TLRs, such as TLR4, but not others. The other end of these adaptors have a death domain that draws in the IL-1 receptor-associated kinases 1 and 4 (IRAK-1 and IRAK-4), which undergoes an autophosporylation reaction that enables them to bind to another adaptor called TRAF6, which belongs to the tumour necrosis factor (TNF)-receptor-associated factor (TRAF) family that has a critical role to play in the next series of reactions.
3. The IRAK-1 and TRAF6 dissociate from the receptor and move in to the cytoplasm.
4. The TRAF 6 is a RING domain E3 ubiquitin ligase that associates with the heterotrimeric ubiquitin-conjugating (E2) complex that contains Ubc13 and Uev1A. This is a K63 ubiquitinating complex that results in the autoubiquitination of TRAF6. The ubiquitinated TRAF6 then binds the transforming growth factor β activated kinase-1 (TAK1) and the TAK1-binding (TAB) proteins 1 to 3 (TAB1-3). The multisubunit cytoplasmic complex containing the inhibitor of NF-κB (IκB) kinase (IKK) α/IKKβ dimer and the regulatory NF-κB essential modifier (NEMO) subunit are also drawn into the complex. The resulting activation of TAK1 is then responsible for relaying information out to other components of the signalling pathways.
5. The TAK1 activates both the c-Jun N-terminal kinase (JNK) pathway and the p38 pathway (Module 2: Figure MAPK signalling). The JNK and p38 function to phosphorylate transcription factors such as AP-1, which binds to the cyclic AMP response element (CRE) site.

Module 2: | Figure Toll receptor signalling



The Toll receptor signalling pathway.
The lipopolysaccharide (LPS) that initiates this signalling pathway binds first to an extracellular LPS-binding protein (LBP) and to CD14, which is a glycosylphosphatidylinositol-anchored membrane glycoprotein, and this complex carries the LPS to the Toll-like receptor 4 (TLR4). The activated TLR4 then recruits a signalling complex to relay information to both the p38 and nuclear factor κB (NF-κB) signalling pathways to induce the transcription of a number of inflammatory cytokines and immunoregulators as described in the text.

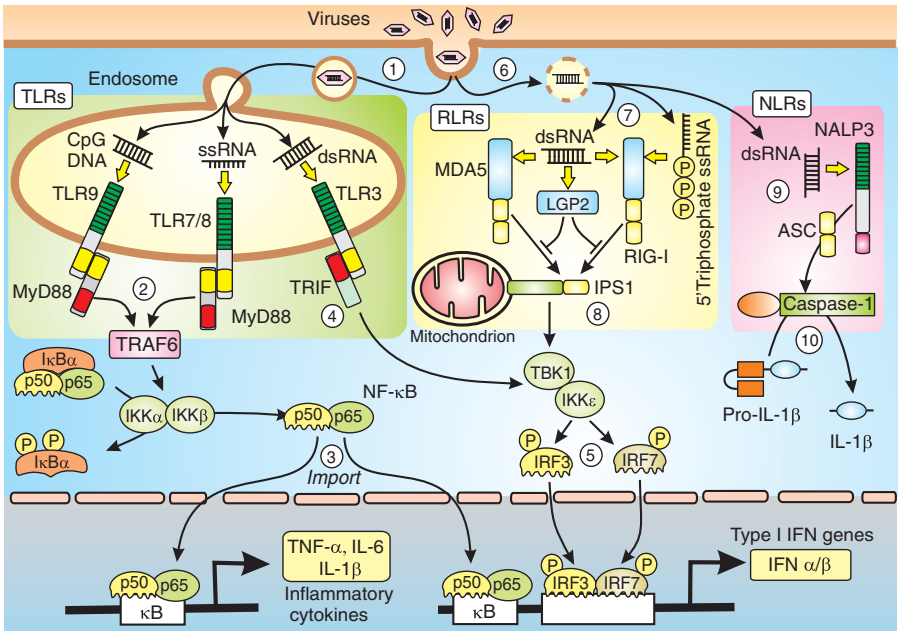
6. The other action of TAK1 is to phosphorylate the IKKβ component of the inhibitor of NF-κB (IκB) kinase (IKK) α/IKKβ dimer.
7. The phosphorylated IKKβ then acts to phosphorylate IκBα to liberate the NF-κB.
8. The phosphorylated IκBα is then susceptible to ubiquitination by the Skp1/cullin/F-box (SCF) ubiquitin ligase. The polyubiquitinated IκBα is then sent to the proteasome, where it is degraded,
9. The NF-κB enters the nucleus, binds to the κB site and activates the genes that code for the inflammatory cytokines and immunoregulators that contribute to inflammatory responses (Module 11: Figure inflammation).
10. An important aspect of the recovery of this signalling cascade after LPS is withdrawn, is the removal of the ubiquitin scaffolds that hold together the transducing complex. The deubiquitinating enzymes A20 and CYLD are particularly active in removing the ubiquitin chains. This ubiquitin signalling system thus plays an essential role in the processing of information by this Toll receptor signalling pathway.
11. In addition to functioning as a coreceptor to transfer LPS to TLR4, CD14 may also be capable of activating a Ca^{2+} signalling pathway. The CD14 activates Src that is coupled to phospholipase Cγ1 (PLCγ1), which is then capable of triggering an influx of external Ca^{2+} through an unknown mechanism. The increase in Ca^{2+} stimulates calcineurin (CaN) to dephosphorylate the transcription factor NFAT that enters the nucleus to activate expression of genes such as interleukin-2 and various pro-apoptotic genes (*nur77, Gadd45g, Ddit3 and Tia1*) (Module 2: Figure Toll receptor signalling). This Ca^{2+} pathway has been described in dendritic cells, but appears to be absent in macrophages.

This signalling pathway is particularly evident on resident macrophages (Module 11: Figure macrophage signalling) and mast cells (Module 11: Figure mast cell signalling) that respond to invading pathogens. Mutations in MyD88 have been linked to diffuse large B cell lymphoma (DLBCL).

Virus recognition and antiviral responses

The recognition and initiation of antiviral responses, which is a part of the innate immune system, depend upon the activation of a number of signalling pathways such as the Toll receptor signalling pathway. When viruses enter cells they are broken down into fragments such as double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), 5' triphosphate ssRNA or CpG DNA, which are located either in the cytoplasm or within the endosomal compartment (Module 2: Figure virus recognition). These fragments are examples of the pathogen-associated molecular patterns (PAMPs) that are responsible for triggering a variety of inflammatory responses (Module 11: Figure formation and action of PAMPs). The following sequence of reactions describes how three main groups of receptors detect these viral PAMPs to initiate a number of antiviral signalling pathways (Module 2: Figure virus recognition):

Module 2: | Figure virus recognition



Viral recognition and antiviral responses.
When viruses enter cells they are degraded into short fragment of double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), 5' triphosphate ssRNA or CpG DNA. There are three main groups of sensors that detect these fragments: Toll-like receptors (TLRs, see green box), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs, see yellow box) and nucleotide oligomerization domain (NOD)-like receptors (NLRs, see pink box). These different receptors then activate different signalling systems to induce transcription of the inflammatory cytokines and interferon as described in the text. The information used to construct this figure was taken from McCartney and Colonna (2009) and Takeuchi and Akira (2009).

Toll-like receptors (TLRs)

1. Those viral PAMPs, which are directed into the endosome, interact with Toll-like receptors (TLR3, TLR7/8 and TLR9) that are located in the endosomal membrane (see green box on the left of Module 2: Figure virus recognition).
2. The TLR9, which responds to CpG DNA, and the TLR7/8 that bind ssRNA both recruit the adaptor protein MyD88. The latter then acts through TRAF6, which is one of the tumour necrosis factor (TNF)-receptor-associated factor (TRAF) family, to stimulate the IKKαβ. The latter then activates the transcription factor NF-κB through the Toll receptor signalling pathway described in more detail earlier (Module 2: Figure Toll receptor signalling).
3. The NF-κB is imported into the nucleus where it functions to induce the transcription of both the inflammatory cytokines and the genes for type I interferon (IFN).
4. When activated by dsRNA, the TLR3 receptor interacts with the TIR-domain-containing adaptor protein inducing IFN-β (TRIF). The latter then activates the two IKK-related proteins TBK1 [TRAF-family member-associated NF-κB activator (TANK)-binding protein] and IKKε, which is also known as inducible IKK (iIKK).
5. The activated TBK1/IKKε complex then phosphorylates the interferon-regulatory factors (IFRs) IRF3 and IRF7. When phosphorylated, these two IRFs form homodimers that are then imported into the nucleus where they contribute to the transcription of the type I interferons (IFN-α and IFN-β).

Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs)

6. Those viral PAMPs that are directed into the cytoplasm interact with cytoplasmic receptors such as the RIG-I-like receptors (RLRs) (see yellow box in the middle of Module 2: Figure virus recognition).
7. The two main RLRs are retinoic acid-inducible gene I (RIG-I) itself and melanoma-associated gene 5 (MDA5). These two receptors have a characteristic DExD/H box helicase domain that is responsible for binding double-stranded RNA (dsRNA). RIG-I can also respond to 5' triphosphate ssRNA. These two receptors also have a pair of N-terminal caspase recruitment domains (CARD), which are the functional transducing components that signal to downstream elements. The LGP2 (laboratory of genetics and physiology-2), which also has a helicase domain, can also bind dsRNA, but it lacks the CARD domains and thus fails to transduce cellular signals but may act as a negative regulator of MDA5 and RIG-I.
8. The CARD domains on MDA5 and RIG-I interact with the IFN-β promoter stimulator-1 (IPS-1), which also contains an N-terminal CARD domain and is attached to the outer mitochondrial membrane. IPS1 is also known as MAVS (mitochondrial antiviral signalling), VISA (virus-inducing signalling adaptor) or CARDIF (CARD adaptor inducing IFN-β). The IPS1 protein then stimulates the TBK1/IKKε

complex that then phosphorylates the interferon-regulatory factors (IFRs) IRF3 and IRF7 as described above (see step 5).

Nucleotide oligomerization domain (NOD) protein-like receptors (NLRs)

9. The double-stranded RNA (dsRNA) in the cytoplasm can also interact with the nucleotide oligomerization domain (NOD) protein-like receptors (NLRs) (see pink box on the right of Module 2: Figure viral recognition). One of the NLRs is the Nacht domain-, Leucine-rich repeat-, and PYD-containing protein 3 (NALP3), which is also known as cryopyrin that responds to dsRNA to activate caspase-1. The interaction between NALP3 and caspase-1 is facilitated by an adaptor called apoptosis-associated speck-like protein containing a CARD (ASC). The interaction between these three proteins takes place in a macromolecular complex known as the inflammasome.
10. The activated caspase-1 contributes to the development of an inflammatory response by cleaving pro-IL-1β to form the inflammatory cytokine interleukin 1β (IL-1β).

Phospholipase D (PLD) signalling pathway

The phospholipase D (PLD) signalling pathway functions by generating phosphatidic acid (PA), which acts to regulate a wide range of cellular processes. Phospholipase D (PLD) activation depends upon a number of mechanisms, and these may vary depending on where the signalling mechanism is located within the cell. The primary messenger produced by this signalling pathway is PA, and phosphatidic acid (PA) action is carried out through a number of downstream effectors. Phosphatidic acid (PA) metabolism occurs through different pathways that generate further signalling molecules such as diacylglycerol (DAG) and lysophosphatidic acid (LPA).

Phospholipase D (PLD) activation

Mammals have two phospholipase D (PLD) genes (*PLD1* and *PLD2*), both of which have splice variants. Most attention has focused on PLD1 as a signal transducer because it has a low basal activity that increases markedly in response to external stimuli. On the other hand, PLD2 has a high basal activity and its role in signalling is uncertain. While most of the PLD2 is located on the plasma membrane, PLD1 is found predominantly on intracellular membranes (e.g. Golgi, endoplasmic reticulum and endosomes), but has also been located at the plasma membrane, particularly at caveolae. The structure of the enzyme contains motifs responsible for its membrane location and catalytic activity (Module 2: Figure PLD isoforms). The activity of PLD1 increases following stimulation of both protein tyrosine kinase-linked receptors (PTKRs) and G protein-coupled receptors (GPCRs) (Module 2: Figure PLD signalling). One of the problems with trying to understand the signalling function of this pathway is to determine just how these different receptors act to stimulate PLD1. The fact that the activation of PLD1 seems to depend on the prior activation of other signalling pathways such as the diacylglycerol (DAG)/protein kinase C (PKC) signalling cassette or the PtdIns 3-kinase signalling cassette (Module 2: Figure PLD signalling) suggests that the PLD signalling pathway is not an autonomous signalling system, but should be considered more as a downstream effector of these other signalling pathways.

A specific *N*-arachidonylphosphatidylethanolamine (NAPE) phospholipase D (PLD) (NAPE PLD) functions to generate anandamide, which is one of the endocannabinoids (Module 1: Figure anandamide).

The Arf signalling pathway plays an important role in the activation of PLD (Module 2: Figure Arf signalling).

Phosphatidic acid (PA) action

The primary messenger of the phospholipase D (PLD) signalling pathway is the lipid phosphatidic acid (PA), which has a number of actions within the cell (Module 2: Figure PLD signalling). The signalling function of PA is mainly directed towards the regulation of various enzymes such as stimulation of the target of rapamycin (TOR) and sphingosine kinase or inhibition of protein phosphatase 1. It also plays an important role in regulating phagocytosis (Module 4: Figure phagosome maturation).

In addition to such signalling functions, a local accumulation of PA may also alter the physical properties of the membrane by creating curvatures to facilitate the formation of vesicles for intracellular trafficking as occurs during the COPII-mediated transport from the ER to the Golgi (Module 4: Figure COPII-coated vesicles) and the COPI-mediated transport from the Golgi to the ER (Module 4: Figure COPI-coated vesicles).

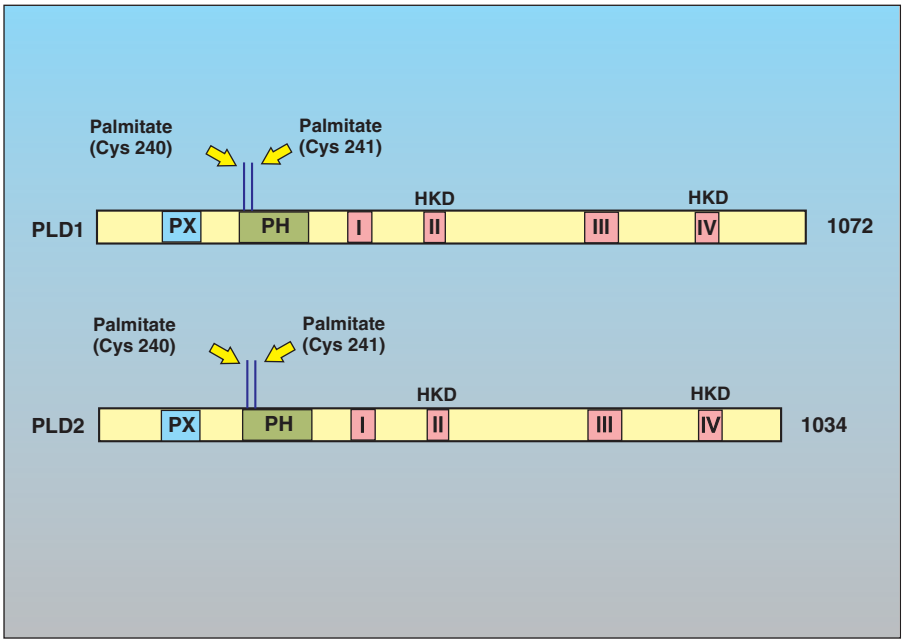
Phosphatidic acid (PA) metabolism

Two separate enzymes carry out the metabolism of phosphatidic acid (PA). It can be dephosphorylated to diacylglycerol (DAG) by a PA phosphohydrolase or it can be partially deacylated by a phospholipase A_2 (PLA_2) to form lysophosphatidic acid (LPA) (Module 2: Figure PLD signalling). The LPA released from the cell is a potent agonist on receptors of the endothelial differentiation gene (EDG) family (Module 2: Figure sphingomyelin signalling).

Sphingomyelin signalling pathway

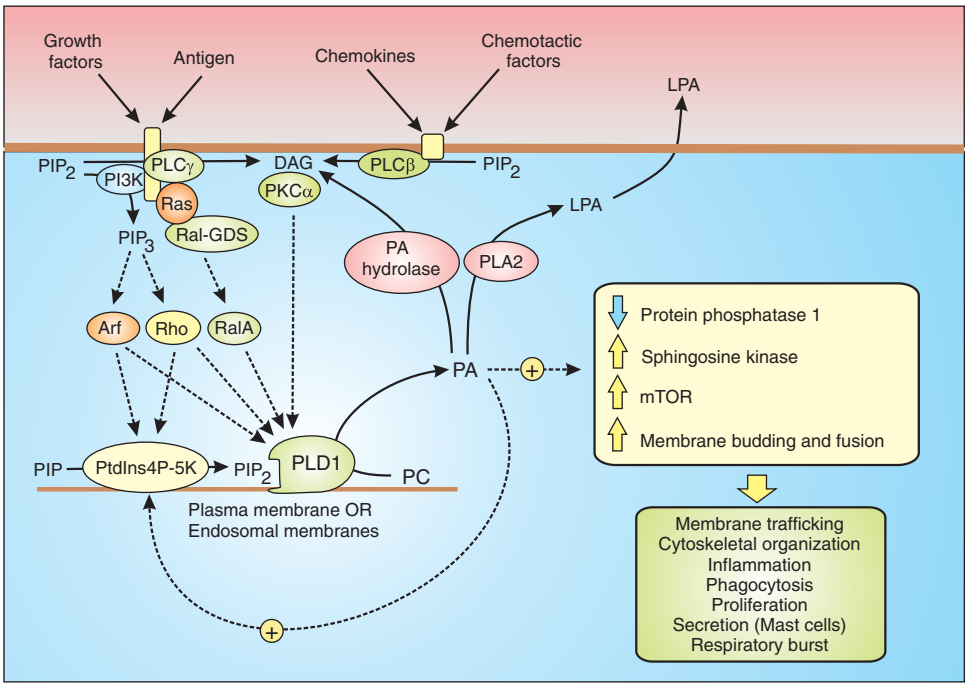
The sphingomyelin signalling pathway that has been implicated in the control of a whole host of cellular processes through the generation and function of ceramide and sphingosine 1-phosphate (S1P), which are the main messengers operating in this signalling pathway. The action of sphingosine 1-phosphate (S1P) is complicated because, in addition to acting internally, S1P is released from cells to function as an external ligand acting on cell-surface receptors. This sphingomyelinase signalling pathway also produces ceramide, another messenger that plays a significant part in processes such as cell proliferation, apoptosis and the response of the cell to stress and injury. One of the difficulties in understanding this pathway is its pleiotropic effect on cells with responses that are often diametrically

Module 2: | Figure PLD isoforms



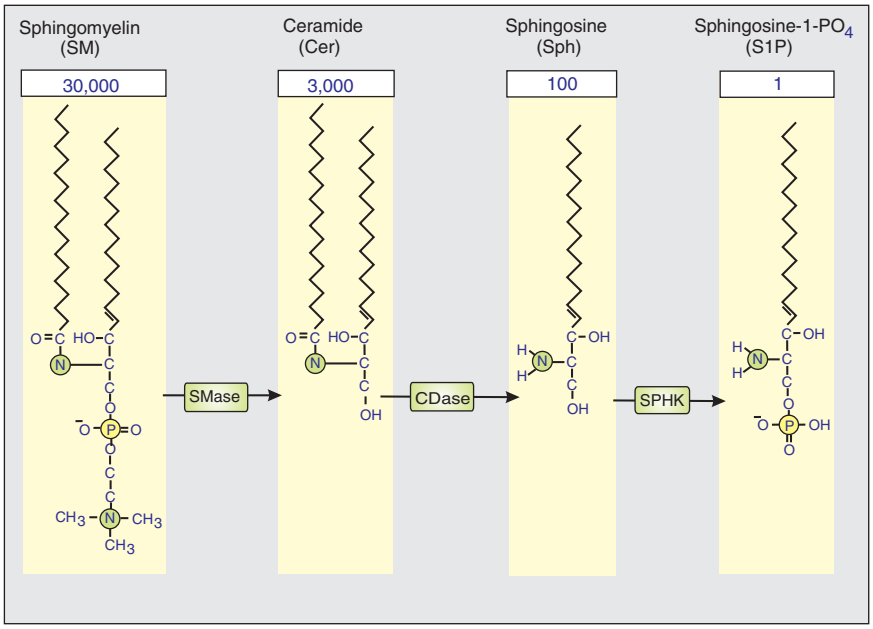
The domain structure of phospholipase D (PLD).
The two isoforms of phospholipase D (PLD) have similar domain structures. Palmitoylation of Cys-240 and Cys-241 within the pleckstrin homology (PH) domain play a critical role in localizing the enzyme to membranes. There are four conserved sequences (I-IV). Domains II and IV contain HXKXXXXD, the so-called HKD motif, which come together to form the catalytic domain. The C-terminal region has a phox homology (PX) domain and a PH domain responsible for the binding of PtdIns4,5P_2 during activation of the enzyme (Module 2: Figure PLD signalling).

Module 2: | Figure PLD signalling



The phospholipase D (PLD) signalling pathway.
This signalling pathway depends upon the enzyme phospholipase D1 (PLD1), which associates primarily with intracellular membranes (e.g. Golgi and endosomes), but is also found in the plasma membrane. The activation of PLD1 is linked to many receptors through a number of mechanisms that may vary depending on the location and which of its many functions PLD is carrying out. One activator is protein kinase Cα (PKCα), through a mechanism that is independent of its catalytic activity. Other major regulators are various small G proteins. Arf and Rho may be activated by the lipid messenger PtdIns3,4,5-trisphosphate (PIP_3) formed by the PtdIns 3-kinase signalling cassette, whereas Ral A may be activated via Ras and the GTP/GDP exchange factor Ral-GDS. In addition, PLD1 has an absolute requirement for PtdIns4,5P_2 (PIP_2), which may be part of a positive-feedback loop because the PtdIns4P 5-kinase (PtdIns4P-5K) is activated by phosphatidic acid (PA). The action of PA is terminated either by a PA phosphohydrolase, which removes phosphate to leave behind diacylglycerol (DAG), or by a phospholipase A_2 (PLA_2) to produce lysophosphatidic acid (LPA).

Module 2: | Figure sphingolipid metabolism



Sphingolipid metabolism.
The sphingomyelin signalling pathway depends on the conversion of sphingomyelin (SM) into a series of bioactive lipids capable of activating various signalling mechanisms as described in Module 2: Figure sphingomyelin signalling. The numbers represent the relative cellular levels of these sphingolipids. SMase, sphingomyelinase; CDase, ceramidase; SPHK, sphingosine kinase. Information for this Figure was taken from Hannun and Obeid (2008).

opposed, such as proliferation and apoptosis. A clue to this complexity lies in the fact that the pathway can spawn a number of messengers, and the action of these is very dependent on the current state of the cell, especially with regard to what other signalling pathways are active.

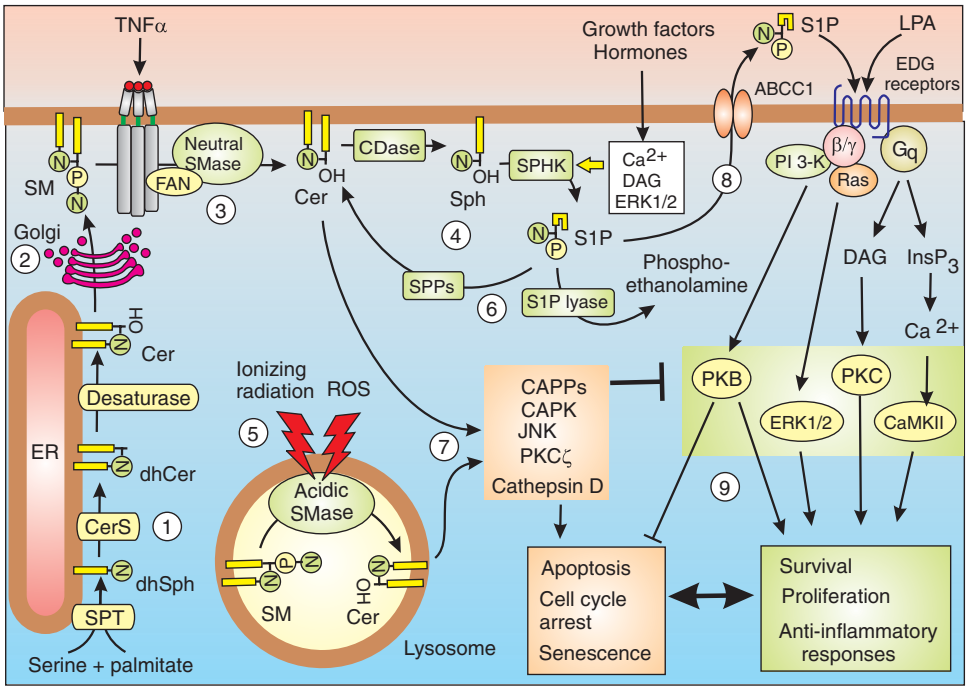
Generation and function of ceramide and sphingosine 1-phosphate (S1P)

The generation of ceramide and sphingosine 1-phosphate (S1P), which are the two main bioactive 'messenger' lipids of the shingomyelin signalling pathway, begins with the hydrolysis of sphingomyelin (SM) by various sphingomyelinases (SMases) to form ceramide (Module 2: Figure sphingolipid metabolism). A ceramidase (CDase) then cleaves off one of the fatty acid chains to form sphingosine (Sph), which is then phosphorylated by sphingosine kinase (SPHK). As illustrated by the figures in Module 2: Figure sphingolipid metabolism, the relative cellular levels of these sphingolipids vary enormously. The precursor SM is present at the highest level and this then declines during the different metabolic transformations.

An important aspect of this sphingomyelin signalling pathway is the cellular location and function of these different metabolic steps (Module 2: Figure sphingomyelin signalling):

1. Sphingomyelin synthesis is carried out first in the endoplasmic reticulum (ER), where ceramide is formed, and is completed by the Golgi. The first step is carried out by serine palmitoyl transferase (SPT) that combines serine and palmitate to form dihydrosphingosine (dhSph), which is then converted into dihydroceramide (dhCer) by dihydroceramide synthase (CerS). Finally, a desaturase converts dhCer into ceramide (Cer). Mutations in serine palmitoyl transferase (SPT) are responsible for type I hereditary sensory and autonomic neuropathies (HSAN).
2. The ceramide formed at the ER is then transported to the Golgi by a ceramide transfer protein (CERT). A sphingomyelin synthase (SMS) located in the Golgi converts ceramide into sphingomyelin (SM), which is transferred to the membrane through a vesicle transport mechanism.
3. The hydrolysis of SM in the plasma membrane is activated by receptors sensitive to stimuli such as tumour necrosis factor α (TNFα), interleukin 1, CD28 and Fas. The coupling between receptors and the neutral sphingomyelinases (SMases) has been worked out in some detail for the TNFα receptor. A factor associated with neutral SMase (FAN) functions to couple the enzyme to the neutral SMase activation domain (NSD) of the TNFα receptor. These components of the sphingomyelinase signalling pathway are often highly concentrated in lipid rafts and caveolae, which represent the site where some of the sphingomyelinases function to hydrolyse sphingomyelin.
4. The activation of neutral SMase at the plasma membrane provides one of the major sources of ceramide (Cer), which can then be metabolized to other signalling sphingolipids (Module 2: Figure sphingolipid metabolism). A ceramidase (CDase) hydrolyses Cer to sphingosine (Sph), which can then be phosphorylated by sphingosine kinase (SPHK) to form sphingosine 1-phosphate (S1P). Since the latter is soluble, it leaves

Module 2: | Figure sphingomyelin signalling



The sphingomyelin signalling pathway.
A number of stimuli can activate the neutral or acidic sphingomyelinases (SMase) to hydrolyse sphingomyelin (SM) to ceramide (Cer), which is then converted into sphingosine (Sph) by ceramidase (CDase). The sphingosine is converted into sphingosine 1-phosphate (S1P) by a sphingosine kinase (SPHK), which is sensitive to other signalling pathways using messengers such as Ca^{2+}, diacylglycerol (DAG), cyclic AMP (cAMP) and extracellular-signal-regulated kinase 1/2 (ERK1/2). Ceramide can activate a number of targets, and some of these can activate apoptosis. On the other hand, S1P can promote survival and proliferation by passing out of the cell, where it functions as an external ligand to activate endothelial differentiation gene (EDG) receptors.

the membrane and is free to diffuse both into and out of the cell (see later). The SPHK is activated by a number of signalling pathways using messengers such as Ca^{2+}, DAG, cyclic AMP and ERK1/2 that are produced by signalling pathways activated by growth factors and hormones. Ceramide can also be phosphorylated by a ceramide kinase to form ceramide 1-phosphate, for which there is no apparent function.

5. A variety of stress stimuli, such as ionizing radiation, UV irradiation and reactive oxygen species (ROS) act by stimulating the acidic sphingomyelinases (SMases) localized in lysosomes to hydrolyse sphingomyelin (SM) to form ceramide (Cer) (Module 2: Figure sphingomyelin signalling).
6. S1P is metabolized via two separate pathways: it can be converted back into sphingosine by a sphingosine 1-phosphate (S1P) phosphatase (SPP) or it can be cleaved by an S1P lyase to give palmitaldehyde and phosphoethanolamine. The lyase has a 20-amino-acid transmembrane domain that positions the enzymes in cellular membranes.

The sphingomyelinase signalling pathway is thus sensitive to external signals at a number of discrete steps. The production of ceramide is accelerated by receptors sensitive to cytokines such as TNFα or to various stress signals such as ionizing radiation and reactive oxygen species (ROS). The formation of S1P can also be enhanced by growth factor and hormone receptors that elevate second messengers such as Ca^{2+}, DAG, ERK1/2 or cyclic AMP that act by stimulating SPHK. In the absence of these signals, the main messenger will be ceramide, but this will switch over to S1P if the SPHK is activated. This interrelationship between ceramide and S1P has led to a ceramide/S1P rheostat model, where a balance between these two messengers is thought to determine cell fate. In general, ceramide seems to tip the balance in favour of cell cycle arrest, senescence and apoptosis, whereas S1P promotes survival and proliferation (Module 2: Figure sphingomyelin signalling). The different outcomes controlled by these two messengers may depend on their ability to activate separate signalling cascades.

7. The ceramide formed at the plasma membrane or in the lysosomes acts on a number of targets such as ceramide-activated protein kinase (CAPK), protein kinase Cζ (PKCζ), cathepsin D and ceramide-activated protein phosphatases (CAPP), such as PP1 and PP2A. Most of these ceramide-sensitive targets act to promote cell cycle arrest and apoptosis either directly or indirectly. For example, the cathepsin D converts Bid into tBid to promote apoptosis (see Step 3 in Module 11: Figure apoptosis). On the other hand, the CAPPs such as PP2A can dephosphorylate various components that are used by the EDG receptors to drive cell survival and proliferation. For example the dephosphorylation

of protein kinase B (PKB) not only prevents it from inhibiting apoptosis but it will also reduce its ability to enhance proliferation.

8. One of the actions of the soluble sphingosine 1-phosphate (S1P) is to diffuse out of the cell passing through the ABCC1 transporter, which is a family member of the ATP-binding cassette (ABC) transporters (Module 3: Table ABC transporters). The extracellular S1P then activates EDG receptors, which belong to the family of G protein-coupled receptors (GPCRs) (Module 1: Table G protein-coupled receptors) that are capable of relaying information down a number of signalling pathways.
9. These signalling pathways control cellular processes, such as cell survival, proliferation and anti-inflammatory responses, which are opposite to those controlled by ceramide.

This sphingomyelin signalling pathway has been implicated in a number of cellular processes:

- S1P has been proposed to release the Ca^{2+} necessary to activate the formation of PtdIns3P on endosomes during phagosome maturation (Module 4: Figure phagosome maturation).
- S1P appears to contribute to the CD28 co-stimulatory pathway during T cell activation (Module 9: Figure T cell signalling map).
- S1P contributes to the communication between endothelial cells and pericytes during angiogenesis (see Step 6 in Module 9: Figure angiogenesis signalling).

Sphingosine 1-phosphate (S1P)

The key enzyme in the formation of sphingosine 1-phosphate (S1P) is sphingosine kinase (SPHK). The messenger function of S1P is complicated by the fact that it may have both intra- and extra-cellular actions.

S1P can exert its extracellular action either in an autocrine mode (activate the same cell from which it is released) (see Step 8 in Module 2: Figure sphingomyelin signalling) or a paracrine mode (diffuse away to activate neighbouring cells). This extracellular action is mediated by endothelial differentiation gene (EDG) receptors, so-called because they were first described in human umbilical vein endothelial cells induced to differentiate by phorbol esters. There is a family of these EDG receptors that are all G protein-coupled receptors (GPCRs) capable of activating most of the conventional signalling pathways (Module 1: Table G protein-coupled receptors). The EDG 1, 3, 5, 6 and 8 receptors mediate the action of S1P, whereas the EDG 2, 4 and 7 receptors appear to respond to lysophosphatidic acid (LPA), a related lysolipid. The EDG receptors that function to induce a mobilization of internal Ca^{2+} act by stimulating the formation of inositol 1,4,5-trisphosphate ($InsP_3$). One function of the EDG receptors is to regulate cell motility and directional migration. An example of the latter is preosteoclast chemotaxis where S1P directs preosteoclasts form the bone marrow back into the blood (Module 8: Figure preosteoclast chemotaxis).

The intracellular action of S1P is not as well defined, mainly because it is often difficult to separate an intracellular action from that induced by its extracellular action. An intracellular action of S1P has been proposed for phagosome maturation, where it releases the Ca^{2+} necessary to activate the formation of PtdIns3P on endosomes (Module 4: Figure phagosome maturation). One of the problems with trying to establish such an intracellular action for S1P is that it could also mobilize internal Ca^{2+} by acting through the EDG receptors to generate $InsP_3$. However, there are indications that S1P can act directly to release Ca^{2+} from an internal store. Unlike $InsP_3$, however, release occurs without the appearance of elementary events. One of the difficulties with this hypothesis is that the channel on the endoplasmic reticulum (ER) that is opened by S1P remains to be identified. An earlier proposal that S1P might act through Scamper has not been substantiated. Another suggestion is that S1P functions as the Ca^{2+} influx factor (CIF) that is proposed to link store depletion to Ca^{2+} entry. Another proposed action of S1P is to activate the extracellular-signal-regulated kinase (ERK) cascade to promote proliferation. Overexpression of the SPHK can induce tumour formation. Therefore the precise role of S1P and its relationship to ER signalling require further clarification.

Ceramide

This is an enigmatic messenger in that it has been linked to both proliferation and apoptosis, depending very much on the background activity of other messenger systems. In keeping with its different functions, ceramide can activate a number of signalling components such as the ceramide-activated protein kinase (CAPK), protein kinase Cζ (PKCζ) and ceramide-activated protein phosphatases (CAPP), such as PP1 and PP2A. Some of these ceramide targets enable it to interact with other signalling systems such as the c-Jun N-terminal kinase (JNK) and mitochondrial systems. With regard to the latter, ceramide may activate mitochondrial reactive oxygen species (ROS) formation through ceramide-activated protein kinase (CAPK). It is important to stress that apoptosis can occur without the need for ceramide. However, there is overwhelming evidence that the formation of ceramide can strongly tip the balance in favour of apoptosis. It contributes to this activation of the cell death programme by activating the intrinsic pathway at the level of the mitochondria (Module 11: Figure apoptosis). One way of interacting with the mitochondria depends on its activation of the JNK pathway, which is known to induce apoptosis (Module 2: Figure JNK signalling). Ceramide can also activate cathepsin D, which converts Bid into tBid, which is one of the pro-apoptotic members of the Bcl-2 superfamily.

Sphingomyelinases (SMases)

Sphingomyelinases (SMases) exist in different isoforms that can be distinguished by their pH optima: acidic, neutral or alkaline. The last is found in bile and plays a role in digestion. Sphingomyelin signalling seems to depend on the acidic and neutral isoforms (Module 2: Figure sphingomyelin signalling). The neutral SMase functions to generate

ceramide in response to the activation of receptors sensitive to stimuli such as tumour necrosis factor α (TNFα) and interleukin 1 (see Step 3 in Module 2: Figure sphingomyelin signalling). The acidic form was originally identified in lysosomes where it generates ceramide in response to stress stimuli (see Step 5). There also are indications that the acidic SMase may act on sphingomyelin located in the outer leaflet. The acidic SMase is a component of lipid rafts and caveolae, which are microdomains of the plasma membrane containing high levels of the precursor sphingomyelin. It is this acidic isoform that is defective in patients with Niemann-Pick disease.

Ceramide-activated protein kinase (CAPK)

Ceramide-activated protein kinase (CAPK) is a membrane-bound proline-directed protein kinase that acts by phosphorylating Raf-1, thereby enabling ceramide to plug into the mitogen-activated protein kinase (MAPK) cascade and could account for those cases where ceramide promotes both inflammation and proliferation.

Sphingosine kinase (SPHK)

Sphingosine kinase (SPHK) appears as two isoforms (SPHK1 and SPHK2), which have different tissue distributions. SPHK1 is found at high levels in the lung and spleen, whereas SPHK2 is expressed mainly in liver and heart. Both have five highly conserved domains, with the ATP-binding site and catalytic site located in the C2 domain.

The enzyme is both membrane-bound and free in the cytosol, and there is some uncertainty as to how it is activated by cell-surface receptors. It seems likely that it responds to various downstream signals emanating from these receptors such as Ca^{2+}/calmodulin, diacylglycerol (DAG)/protein kinase C (PKC), cyclic AMP/protein kinase A (PKA) or extracellular-signal-regulated kinase 1/2 (ERK1/2) (see Step 4 in Module 2: Figure sphingomyelin signalling). Another activator is oxidized low-density lipoprotein (LDL), which may play a role in cell proliferation. Consistent with the activation by various messenger pathways is the fact that SPHK contains consensus sequences for Ca^{2+}/calmodulin binding and phosphorylation sites for PKA, casein kinase II and PKC.

Sphingosine 1-phosphate (S1P) phosphatase (SPP)

Mammals have two sphingosine 1-phosphate (S1P) phosphatases (SPPs). Both SPP1 and SPP2 have eight to ten transmembrane domains that locate the enzymes within the endoplasmic reticulum (ER). SPP-1 is located mainly in the placenta and kidneys, whereas SPP2 is found in the brain, heart, colon, kidney, small intestine and lung. These enzymes convert S1P back into ceramide and can diminish survival and promote apoptosis (see Step 6 in Module 2: Figure sphingomyelin signalling).

Ceramide-activated protein phosphatase (CAPP)

Ceramide-activated protein phosphatase (CAPP) is a member of the protein phosphatase 2A (PP2A) family of serine/threonine phosphatases that consist of three subunits: A and B are regulatory, whereas C is the catalytic subunit. In the case of CAPP, ceramide acts to stimulate phosphatase activity by binding to one of the regulatory B subunits. The ability of ceramide to inhibit growth is probably mediated through PP2A.

Scamper

A sphingolipid Ca^{2+}-release-mediating protein of the endoplasmic reticulum (ER) (Scamper) was originally proposed to be the Ca^{2+} channel on the ER that responds to signals from the sphingomyelin signalling pathway, but more recent studies have shown that it has a single membrane-spanning segment that seems to remodel the actin cytoskeleton.

Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway

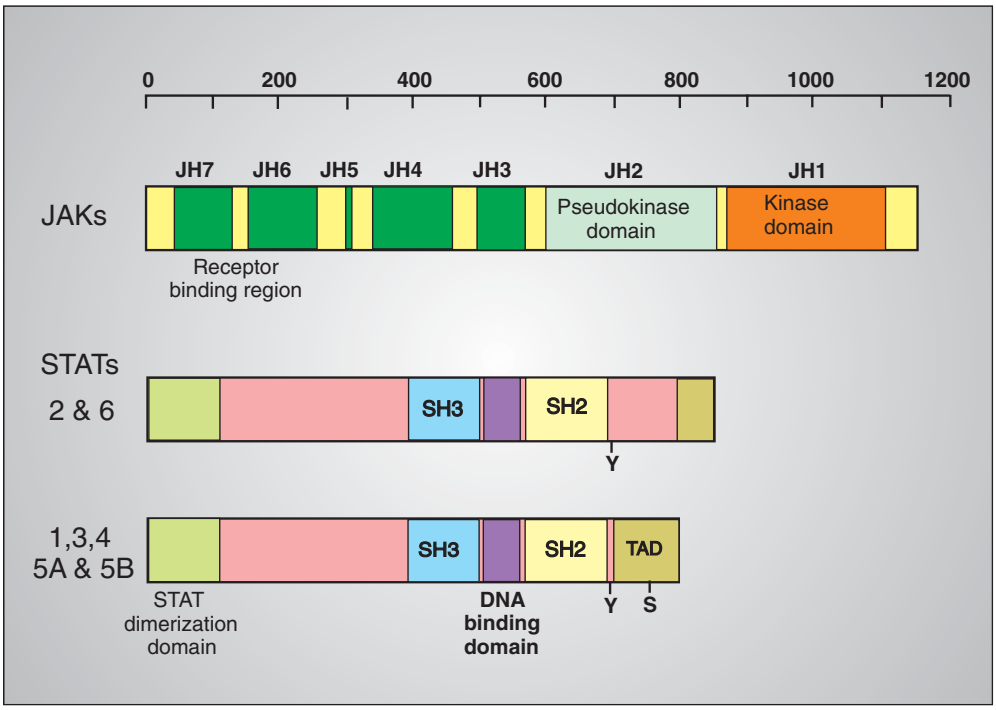
The Janus kinase (JAK) and the signal transducer and activator of transcription (STAT) function in the JAK/STAT signalling pathway provides a mechanism for rapidly activating gene transcription in response to a large number (>35) of external ligands. This signalling pathway is mainly activated by cytokines such as interferon, but is also used by receptor tyrosine kinases [epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR)], non-receptor tyrosine kinases and G protein-coupled receptors (GPCRs). The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) structure reveals the major features of these two components and how they are linked during the signal transducer and activator of transcription (STAT) activation cascade. Cell-surface receptors act by phosphorylating the STATs, which are latent transcription factors. Once phosphorylated, these STATs leave the membrane and then dimerize before migrating into the nucleus where they bind to specific DNA-binding elements to activate transcription. There is considerable evidence for a Janus kinase (JAK)/signal transducer and activator of transcription (STAT) function in growth and development.

Mutations in STAT3 have been linked to hyper-IgE syndrome (HIES).

Janus kinase (JAK)/signal transducer and activator of transcription (STAT) structure

The two major components of the signalling pathway are the Janus kinases (JAKs) and their substrates the signal transducers and activators of transcription (STATs) (Module 2: Figure JAK and STAT structure). Of the four mammalian JAKs, three are expressed fairly ubiquitously, whereas JAK3 is restricted to natural killer (NK) cells and thymocytes, with some expression in vascular smooth muscle cells and endothelium. The main structural component of the JAKs is the kinase domain that functions to phosphorylate the STATs at a key tyrosine in the region of residue 700 during the signal transducer and activator of transcription (STAT) activation cascade. The STATs have a number of functional domains whose three-dimensional structure reveals how the STAT dimers are formed and how they bind to DNA (Module 2: Figure STAT1/DNA complex).

Module 2: | Figure JAK and STAT structure



Domain structure of the JAKs and STATs.
There are four mammalian Janus kinases (JAKs): JAK1, JAK2, JAK3 and Tyk2. They all have a similar domain structure, which has seven JAK homology (JH) domains. JH1 is the kinase domain, whereas JH2 is a pseudokinase domain. Some of the other JH regions seem to contribute to the binding of JAKs to various cell-surface receptors, where they function to activate the signal transducers and activators of transcription (STATs). There are seven mammalian STAT genes. STATs 2 and 6 have 850 amino acids, whereas the others are somewhat shorter. The tyrosine residue near residue 700 is phosphorylated during the activation domain and functions as a binding site for the Src homology 2 (SH2) sites on other STATs during the dimerization process (Module 2: Figure JAK/STAT function). The DNA-binding domain is located between the SH3 (Src homology 3) and SH2 domains. The C-terminus has a transcriptional activation domain (TAD), which, on the shorter-length STATs, contains a serine residue that can modulate transcriptional activity when phosphorylated.

Signal transducer and activator of transcription (STAT) activation cascade

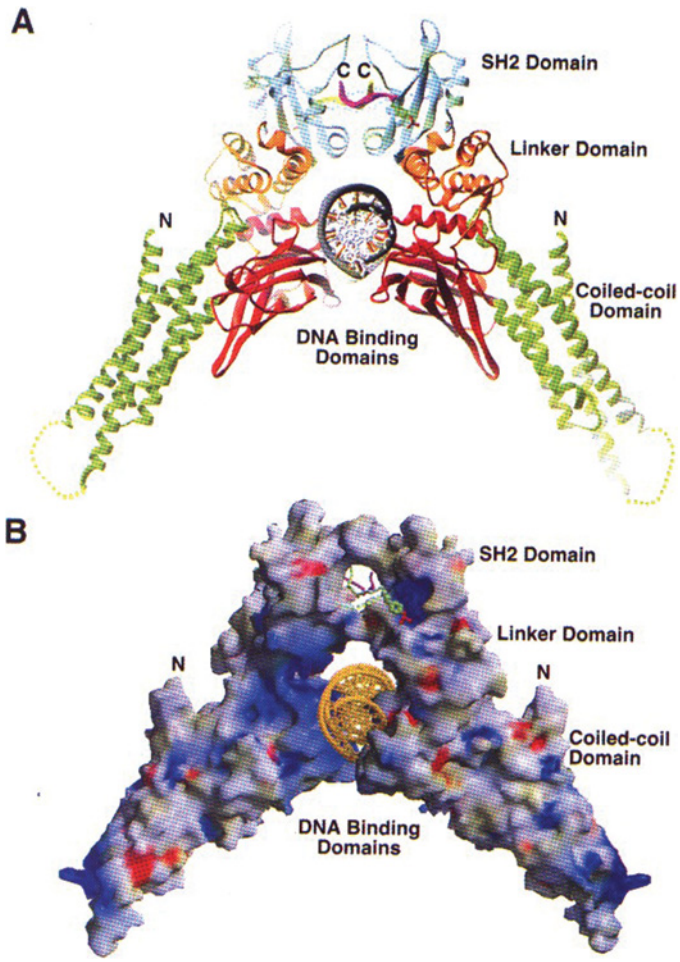
The signal transducers and activators of transcription (STATs) are a family of latent cytoplasmic receptors that are activated by a phosphorylation cascade that can be induced by many different receptors (e.g. cytokine receptors, tyrosine kinase-linked receptors, non-receptor tyrosine kinases and G protein-linked receptors). The versatility of this signalling mechanism is greatly enhanced by the way the Janus kinases (JAKs) and STATs can be mixed and matched to generate an enormous number of combinations as illustrated by the different cytokine receptors (Module 2: Figure JAK/STAT heterogeneity).

A typical activation cascade for cytokine receptors, which illustrates the essential role of the JAKs in a sequence of tyrosine phosphorylations that culminates in the phosphorylation and activation of the STATs, is outlined in the following sequence (Module 2: Figure JAK/STAT function):

1. Agonists induce dimerization by binding to the extracellular domains of the receptor subunits.
2. The JAKs, which are associated with the cytoplasmic domain of these receptors, phosphorylate each other.
3. The activated JAKs then phosphorylate tyrosine residues on the receptors to provide docking sites for the Src homology 2 (SH2) domains on the STATs.
4. Once the STATs have docked, they are then phosphorylated by the JAKs on the tyrosine residue located in the C-terminal region (Module 2: Figure JAK and STAT structure).
5. The phosphorylated STATs, which are dimerized through an intermolecular SH2–phosphotyrosine interaction, leave the receptor.
6. These dimers are imported into the nucleus using importin-α (Imp-α) and attach to promoter regions through their DNA-binding domains (Module 2: Figure STAT1/DNA complex).
7. STATs activate the transcription of a number of target genes. One group of these genes code for the suppressor of cytokine signalling proteins (SOCS), which thus function as part of a negative-feedback loop to limit the action of the signalling pathway (Module 2: Figure JAK/STAT function).
8. STAT activity is terminated by a nuclear protein tyrosine phosphatase (N-PTP) that removes the tyrosyl phosphate groups. The inactive STAT is then exported from the nucleus by chromosome region maintenance 1 (CRM1), thus completing the cycle.

For some of the other receptors, these tyrosine phosphorylations are carried out by kinases other than the JAKs. After the STATs are phosphorylated, they leave

Module 2: | Figure STAT1/DNA complex



Organization of a STAT1/DNA complex.

The ribbon diagram shown in (A) illustrates how the two DNA-binding domains of the two subunits attach the dimer to the DNA helix. The linker domain (orange) attaches to the SH2 (Src homology 2) domains (light blue) that hold the molecule together through the intermolecular SH2-phosphotyrosine interaction. The molecular surface representation shown in (B) has the same orientation as in (A). The local electrostatic potential over the cell surface is represented by the colouring, with blue representing positive and red negative potentials. (Reproduced from Cell, Vol. 93, Chen, X., Vinkemeier, U., Zhao, Y., Jeruzalmi, D., Darnell, Jr, J.E. and Kuriyan, J., Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA, pp. 827-839. Copyright (1998), with permission from Elsevier; see Chen et al. 1998.

the receptor and then dimerize before translocating into the nucleus. This activation cascade can occur quickly, with the activated STATs appearing in the nucleus within minutes. Transcriptional activity can be modulated by phosphorylation of the serine residue in the transcriptional activation domain (TAD) of STAT 1, 3, 4, 5A and 5B (Module 2: Figure JAK and STAT structure). In the case of STAT 1 and STAT4, phosphorylation enhances transcriptional activity, whereas the binding of STAT5a to DNA is greatly prolonged.

Suppressor of cytokine signalling proteins (SOCS)

The suppressor of cytokine signalling proteins (SOCSs) are induced during the activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway (Module 2: Figure JAK/STAT function). The SOCS act by inhibiting the Janus kinases (JAKs) and thus operate a negative-feedback loop to limit the action of cytokines.

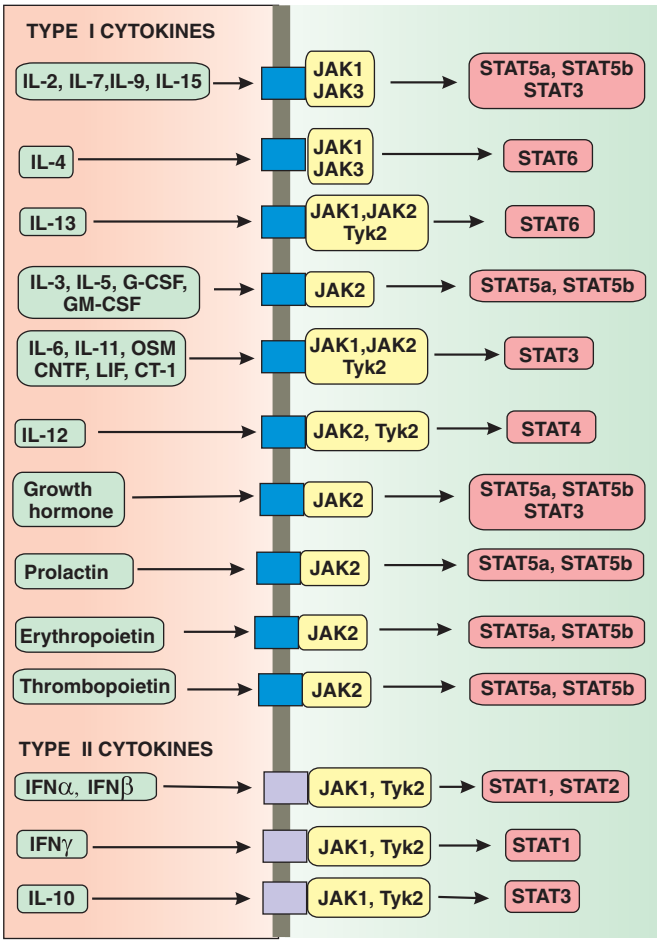
There is a marked increase in expression of the SOCS-3 isoform in hypothalamic neurons following the action of leptin during the control of food intake and body weight (Module 7: control of food intake).

Janus kinase (JAK)/signal transducer and activator of transcription (STAT) function in growth and development

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway has a primary role in the regulation of growth and development, particularly of haematopoietic cells. For example, it functions in the interleukin-2 signalling pathway responsible for driving the final signalling steps in lymphocyte activation (Module 9: Figure T cell signalling map).

A mutation in the γ_c component of cytokine receptors results in X-linked severe combined immune deficiency (X-SCID). Since JAK3 associates with γ_c, it seemed likely that X-SCID may result from an alteration in the JAK/STAT signalling pathway. This was confirmed when

Module 2: | Figure JAK/STAT heterogeneity



Heterogeneity of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) combinations used by different cytokine receptors. The cytokines acting on cell-surface receptors (blue and mauve boxes) activate different combinations of Janus kinases (JAKs) and signal transducers and activators of transcription (STATs). CNTF, ciliary neurotrophic factor; GCSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; LIF, leukaemia inhibitory factor; OSM, oncostatin M. The organization of some of the type I cytokine receptors is shown in Module 1: Figure type I cytokine receptors.

SCID was found in patients carrying mutations in JAK3. SCID resulted from a dramatic reduction in the number of T cells, highlighting the important role for this signalling pathway in lymphocyte development.

One of the functions of STAT3 is to stimulate the transcription of genes that code for anti-apoptotic factors such as Bcl-2 and the inhibitor of apoptosis (IAP) family of proteins.

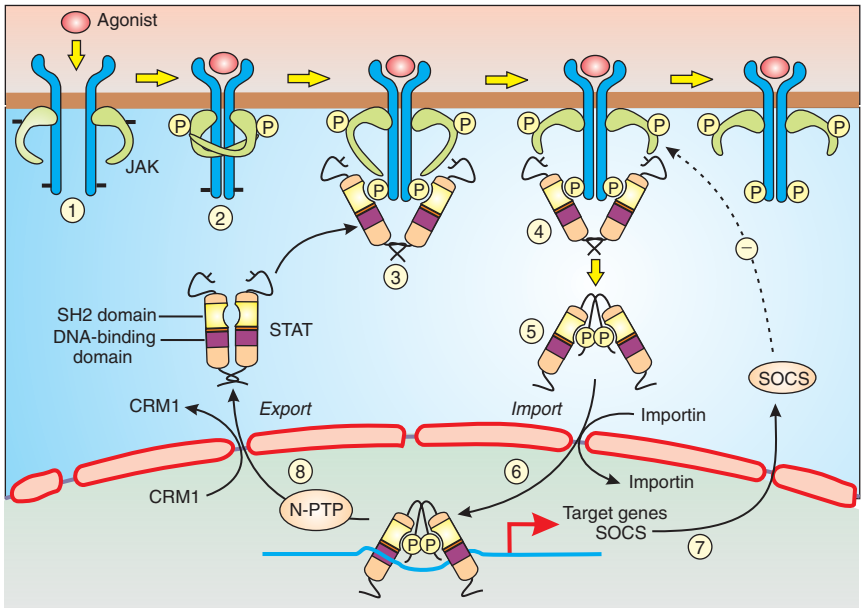
JAKs have also been implicated in certain forms of malignant transformation such as Sezary's syndrome, v-Abl-transformed cells and in some leukaemias.

Smad signalling pathway

This signalling pathway takes its name from the Smads, which are a collection of intracellular signalling molecules that act collectively to transfer information from cell-surface receptors into the nucleus. As such, some of the Smads function as transcription factors, whereas others either facilitate or inhibit this transcriptional activity. These Smads mediate the action of the transforming growth factor β (TGF-β) superfamily, which are cytokines that regulate many cellular functions such as proliferation, apoptosis, extracellular matrix formation and angiogenesis. In addition, they play a critical role in controlling events during early development and cell differentiation. There is an extensive Smad signalling toolkit, and the many components can be mixed and matched to assemble a large variety of Smad signalling pathways. The domain structure of the Smad family illustrates their multifunctional roles in the Smad signalling mechanism that functions to transfer information from activated receptors on the cell surface to gene targets in the nucleus. This signalling mechanism has two main components. Firstly, there is a process of transforming growth factor β (TGF-β) receptor activation, which is responsible for activating the Smads. Secondly, there is the Smad activation of transcription.

There are a number of mechanisms for the modulation of Smad signalling. Transforming growth factor β (TGF-β) inhibition of cell proliferation is one of the major functions of the Smad signalling pathway. It plays an important role in the differentiation of intestinal cells. Alterations in the

Module 2: | Figure JAK/STAT function



The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) activation cascade.
A schematic summary of the main sequence of events responsible for the activation of signal transducers and activators of transcription (STATs). Agonist activation of cell-surface receptors induces dimerization of receptor subunits, resulting in the stimulation of resident Janus kinases (JAKs). The JAKs then carry out a sequential series of three phosphorylation reactions to activate the STATs, which then enter the nucleus to induce transcription. The binding between STATs and DNA is shown in Module 2: Figure STAT1/DNA complex .

signalling pathways controlled by the TGF-β superfamily have been associated with many cancers of epithelial and lymphoid origins, which fail to respond to the normal anti-proliferative effects of TGF-β. The TGF-β receptor is one of the tumour suppressors that are switched off in many cancers and particularly in colorectal cancer (CRC).

An increase in the expression of TGF-β1 may play a critical role in the transition from stable hypertrophy to congestive heart failure (CHF).

Smad signalling toolkit

The Smad signalling pathway is made up from a large number of components (Module 2: Table Smad signalling toolkit). Specific cell types express different combinations of these components so that there is considerable variability in the nature of Smad signalling pathways. However, the overall organization is fairly similar and is best exemplified by the action of transforming growth factor β (TGF-β) itself. The different components of the toolkit have been grouped together on the basis of their function in the signalling pathway (Module 2: Table Smad signalling toolkit):

Ligand traps

These are a large group of proteins that control access of the ligands to their cell-surface receptors. These ligand traps can have an important inhibitory action since they can prevent the ligands from reaching their cell-surface receptors. For example, follistatin inhibits the action of activin on gonadotrophs (Module 10: Figure gonadotroph regulation).

Ligands

The Smad signalling pathway is activated by a number of closely related ligands. The flagship ligand is transforming growth factor β (TGF-β), but there are a number of other ligands such as activin and nodal that also act through the Smads (Module 2: Table Smad signalling toolkit). Nodal has a role to play in establishing left-right asymmetry in the developing embryo.

Accessory receptors

These are cell-surface receptors that function as co-receptors in that they promote the binding of the ligands to their signalling receptors. For example, the accessory receptor endoglin is essential for the action of activin receptor-like kinase 1 (ALK1) during the activation of angiogenesis.

TGF-β signalling receptors

There are two groups of signalling receptors (Type I and II) (Module 2: Table Smad signalling toolkit). These receptors contain a single membrane-spanning region that separates the extracellular ligand-binding domain from the cytosolic region that contains a serine/threonine kinase domain. The Type I receptor also contains a glycine/serine-rich (GS) domain, which is phosphorylated by the Type II receptor during the signal transduction process (as occurs during the response to TGF-β Module 2: Figure TGF-βR activation). Ligands act to complex these receptors so that the Type II can then activate the Type I, which in turn activates the Smads (Module 2: Figure Smad signalling).

Module 2: | Table Smad signalling toolkit
Components of the Smad signalling toolkit.

Component	Comment
Ligand traps	
LAP	Latency-associated polypeptide that binds TGF-β
Decorin	Binds TGF-β
α_2-Macroglobulin	Binds TGF-β
Noggin	Binds to BMPs (Module 8: Figure epidermal stem cell)
Follistatin	Binds to activins and BMPs. It inhibits activin action on gonadotrophs (Module 10: Figure gonadotroph regulation)
Chordin/SOG	Binds to BMPs
DAN/Cerberus	Binds to BMPs
Ligands	
TGF-β1	Transforming growth factor β (Module 1: Figure enzyme-linked receptors)
TGF-β2	
TGF-β3	
Activin	One of its functions is to increase the transcription of FSH in gonadotrophs (Module 10: Figure gonadotroph regulation)
Myostatin	Growth and differentiation factor 8 (GDF8) (see Module 8: Figure satellite cell activation)
Nodal	
BMP	Bone morphogenetic factor functions in the control of epidermal stem cells (Module 8: Figure epidermal stem cell), proliferation of SNO cells (Module 8: Figure HSC regulation) and differentiation of white fat cells (Module 8: Figure white fat cell differentiation)
Inhibin	Inhibits activin action in gonadotrophs (Module 10: Figure gonadotroph regulation)
Accessory receptors	
Betaglycan	Facilitates the binding of TGF-β to the Type II receptors. Mediates the action of inhibins in gonadotrophs (Module 10: Figure gonadotroph regulation)
Cripto	
Endoglin	This accessory receptor is essential for the action of ALK1 during the activation of angiogenesis
Signalling receptors	
Type I receptors	
ALK1	Activin receptor-like kinase 1 is also known as ACVRL1 (activin receptor-like 1) expressed mainly in endothelial cells
ALK2	Activin receptor-like kinase 2 is also known as activin A receptor type 1 (ACVR1), usually responds to BMP
ALK3	Activin receptor-like 3
ALK4	Activin receptor-like 4
ALK5	Activin receptor-like kinase 5 is also known as TGFβ type I (TβRI)
ALK6	Activin receptor-like 6
ALK7	Activin receptor-like 7
ACVRI	Activin receptor type I (ACVRI)
Type II receptors	
ACVRIIA	Activin receptor type IIA binds to activin and interacts with activin receptor type I (ACVRI)
ACVRIIB	
BMPRII	
TβRII	
Receptor-regulated Smads (R-Smads)	
Smad1	
Smad2	
Smad3	
Smad5	
Smad8	
Co-mediator Smad (Co-Smad)	
Smad4	A germ-line mutation in Smad4 has been linked to juvenile polyposis syndromes (JPSs).
Inhibitory Smads (I-Smad)	
Smad6	
Smad7	
Smad regulatory factors	
SARA	Smad anchor for receptor activation
Smurf1	Smad ubiquitin-regulatory factor 1. Smirf1 also regulates the degradation of Rho during neutrophil chemotaxis (Module 11: Figure neutrophil chemotactic signalling)
Smurf2	Smad ubiquitin-regulatory factor 2

The activin receptor-like kinase 1 (ALK1), which responds to TGF-β, is strongly expressed in endothelial cells where it functions in the formation of blood vessels. Loss-in-function mutations in the *ACVRL1* gene have been linked to hemorrhagic telangiectasia-2 (HHT2).

Smads

The Smads are the intracellular transducers of the Smad signalling pathway. There are three Smad types. Receptor-regulated Smads (R-Smads), which are activated by the signalling receptors, carry information into the nucleus. A single co-mediator Smad (Co-Smad) acts together with the R-Smads. Inhibitory Smads (I-Smads) set up a negative-feedback loop to limit the activity of the R-Smads. These different activities are reflected in the domain structure of the Smad family.

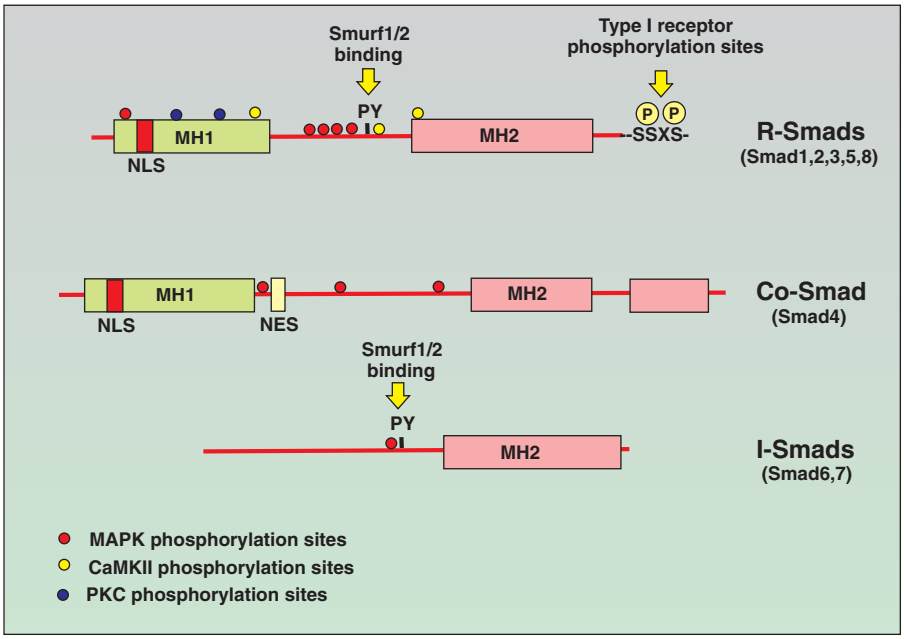
Smad regulatory factors

There are a number of proteins that contribute to the Smad signalling pathway.

Domain structure of the Smad family

The main structural feature of the Smads are the two MAD homology domains (MH1 and MH2) that function both in the protein–protein and protein–DNA interactions that occur during the process of signal transduction (Module 2: Figure Smad domain structure). The regulatory Smads also have a C-terminal SSXS motif, which is critical for the transduction process because two of the serine residues are phosphorylated by the type I receptors as part of the Smad signalling mechanism. There also are numerous other sites that are phosphorylated by various kinases from other signalling pathways. The linker region between the MH1 and MH2 domains contains a phosphotyrosine (PY) motif, which is an interaction site for the binding of the Smad ubiquitin-regulatory factors 1 and 2 (Smurf1/2), which is an ubiquitin ligase that controls the selective proteolysis

Module 2: | Figure Smad domain structure



The domain structure of the three Smad family members.
The receptor-regulated Smads (R-Smads) have a MAD homology domain 1 (MH1) in the N-terminal region and an MH2 in the C-terminal region. These two domains play a critical role in carrying out the protein-protein and protein-DNA interactions. A nuclear localization signal (NLS) is located within MH1 and functions in the transport of the R-Smads into the nucleus. The SSXS motif at the C-terminus contains the two serine residues that are phosphorylated by the Type I receptors during the process of signal transduction (Module 2: Figure TGF-βR activation).

of the Smads. The single co-mediator Smad (Co-Smad, i.e. Smad4) resembles the R-Smads in some aspects. It also has MH1 and MH2 domains, but here the latter is split. In addition to the NLS, it also has a nuclear export signal (NES). Smad4 lacks the C-terminal phosphorylation motif, but it does contain a number of phosphorylation sites. The two inhibitory Smads (I-Smads) lack an MH1 domain, but they have the Smurf1/2-binding PY motif.

Smad signalling mechanism

The Smad signalling mechanism can be divided into two parts. Firstly, there is the process of transforming growth factor β (TGF-β) receptor activation, which concerns the way in which ligands such as TGF-β interact with the signalling receptors to trigger Smad activation (Module 2: Figure TGF-βR activation). The critical aspects of this activation process are the phosphorylation reactions that occur within the receptor complex. The Type II receptors are constitutively active and phosphorylate the Type I receptors. These activated Type I receptors then act to phosphorylate the Smads.

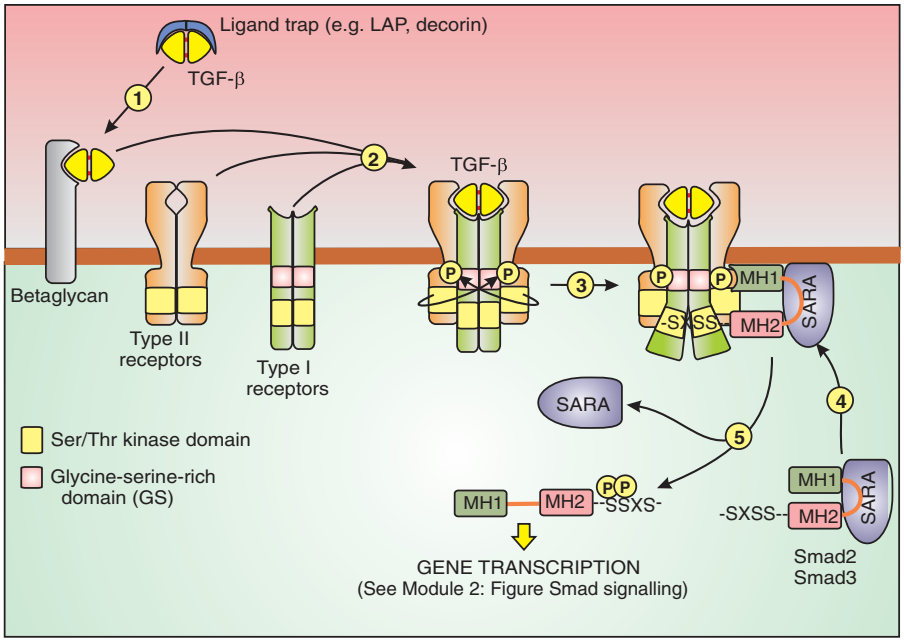
The second part is the Smad activation of transcription, during which the phosphorylated receptor-regulated Smads (R-Smads), together with their partner Smad4, translocate into the nucleus to induce gene transcription (Module 2: Figure Smad signalling).

Transforming growth factor β (TGF-β) receptor activation

Activation of the transforming growth factor β (TGF-β) receptor depends upon a series of reactions as illustrated by the following steps shown in Module 2: Figure TGF-βR activation.

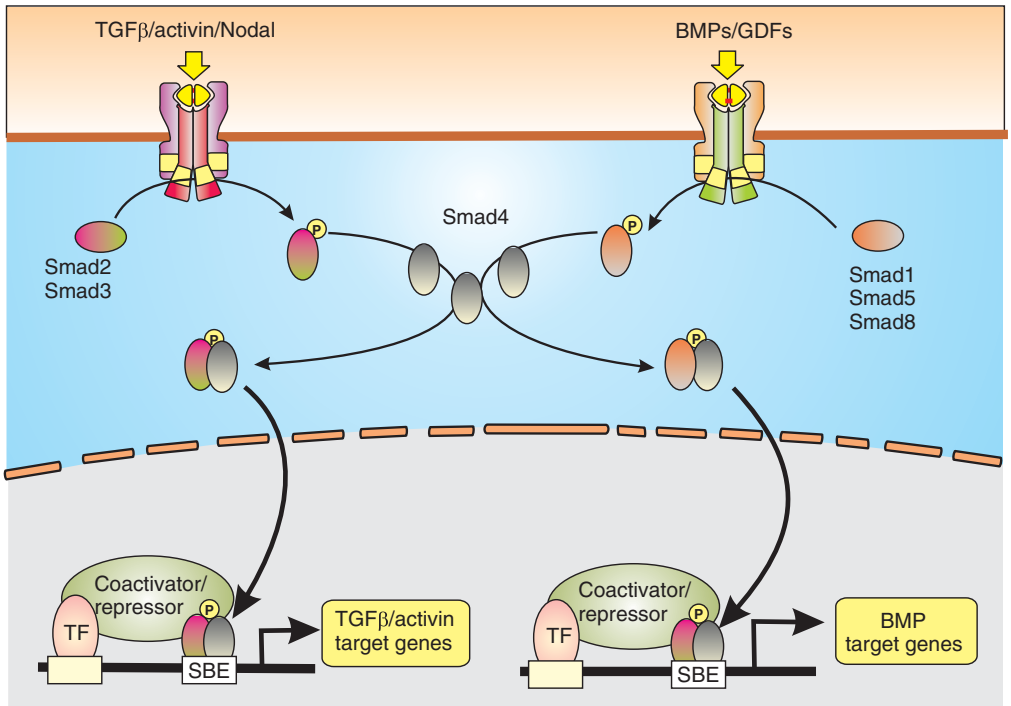
1. The ligand, in this case TGF-β, is often held in a latent ligand complex by being bound to one of the ligand trap proteins such as latency-associated polypeptide (LAP) or decorin. When it dissociates from the ligand trap, it can be taken up by one of the accessory receptors such as betaglycan.
2. The TGF-β then associates with the two TGF-β receptor components to assemble an agonist/receptor complex. In the absence of ligand, the Type I and II receptor components exist as homodimers which are then brought together by TGF-β.
3. When the two receptor types have been complexed by TGF-β, the serine/threonine kinase domain on the Type II receptors phosphorylates the serine residues on the glycine/serine-rich (GS) region of the Type I receptors.
4. These phosphorylated GS regions on the Type I receptors provide a docking site for the MAD homology domain 1 (MH1) domain of Smad2 or Smad3. This recruitment of the Smads to the membrane is facilitated by Smad anchor for receptor activation (SARA). Once attached to the receptor, the SSXS motif is brought into contact with the serine/threonine kinase domain, and two of the serine residues are phosphorylated.
5. Once Smad2/3 have been phosphorylated, their affinity for both the receptor complex and for SARA is reduced, and the two proteins pass into the cytoplasm. The activated Smads then translocate into the nucleus where

Module 2: | Figure TGF-βR activation



Activation of the transforming growth factor β (TGF-β) receptor superfamily.
The transforming growth factor β (TGF-β) receptor superfamily uses the Smad signalling pathway to transmit information into the cell. The main features of this pathway can be illustrated by considering the action of TGF-β, which activates the TGF-β receptor complex through a sequential series of reactions as described in the text.

Module 2: | Figure Smad signalling



The Smad signalling pathway links cell-surface transforming growth factor β (TGF-β) receptors to gene transcription.
There are two main types of Smad signalling. In the one shown on the left, receptors activated by ligands such as transforming growth factor β (TGF-β), activin and Nodal phosphorylate either Smad2 or Smad3. The phosphorylated Smads then heterodimerize with the co-mediator Smad (Co-Smad) Smad4 to form a dimer that then translocates into the nucleus. Once in the nucleus, the Smad dimer then binds to a specific Smad-binding element (SBE), which has a GTCT motif that is recognized by the MAD homology domain 1 (MH1).

they activate transcription (Module 2: Figure Smad signalling).

Smad activation of transcription

The receptor-regulated Smads (R-Smads) function as transcription factors responsible for activating a large number of target genes. Once they have been activated by the cell-surface receptors, the activated Smads translocate into the nucleus (Module 2: Figure Smad signalling). Some of the ligands, such as transforming growth factor β (TGF-β), activin and Nodal activate receptors that are coupled to Smad2 and Smad3, whereas bone morphogenetic protein (BMP) acts through Smad1, Smad5 or Smad8. Once these Smads have been phosphorylated on their C-terminal SSXS motif (Module 2: Figure TGF-βR activation) they leave the receptor, where they combine with Smad4 to form a dimer that then translocates into the nucleus (Module 2: Figure Smad signalling). Once the dimer enters the nucleus, it recognizes and binds to a specific DNA motif.

The transcriptional activity of the Smads is facilitated by associating with other site-specific transcription factors (TFs). Their activity is also modulated by coactivators and repressors. For example, Smad4 binds directly to the coactivator p300, which has an important role in activating transcription. The pathway on the right, which carries out the action of receptors that bind ligands such as bone morphogenetic protein (BMP), use Smad1, 5 or 8 to transmit information into the nucleus. As for the pathway described above, Smad4 is again used as a partner for the translocation process, and the transcriptional processes are also similar.

The Smads stimulate transcription of the collagen type I during the fibrogenesis induced in activated hepatic stellate cells in the liver (Module 7: Figure hepatic stellate cell).

Modulation of Smad signalling

There are a number of ways that the Smad signalling pathway can be modulated. One mechanism is by an increase in the expression of inhibitory Smads (I-Smads), which target the cell-surface receptors for degradation. This process is mediated by the Smad ubiquitin-regulatory factors (Smurfs), which are a family of ubiquitin E3 ligases that bind to the PY motif on the linker region of I-Smads (Module 2: Figure Smad domain structure). The E3 ligase functions in the ubiquitin-proteasome system (Module 1: Figure ubiquitin-proteasome system).

The mitogen-activated protein kinase (MAPK) signalling pathway can also modulate Smad signalling by phosphorylating sites located in the linker regions of Smad1 and 2 (Module 2: Figure Smad domain structure). This is an example of signalling cross-talk in that it provides a mechanism for the tyrosine kinase-linked receptors to antagonize the action of the transforming growth factor β (TGF-β) superfamily.

Transforming growth factor β (TGF-β) inhibition of cell proliferation

One of the major functions of transforming growth factor β (TGF-β) is to inhibit cell proliferation by altering expression of some of the key regulators of the cell cycle signalling pathway. One of its actions is to increase the transcription of the $p15^{INK4B}$ gene that codes for p15, which is one of the cyclin-dependent kinase (CDK) inhibitors that inhibits cyclin D/cyclin-dependent kinase 4 (CDK4) complex that is one of the early events of the cell cycle signalling cascade (Module 9: Figure cell cycle signalling mechanisms). Another important action is to repress c-Myc, which is one of the major transcriptional activators of genes that function in cell proliferation. Such an inhibition of Myc occurs during skin development when Smad2/3 induces the transcription of the Myc repressors Mad1 and Ovol1 to inhibit keratinocyte proliferation.

TGF-β plays an important role in regulating the proliferation of mesangial cells (Module 7: Figure mesangial cells). It also regulates the proliferation of stem cells such as the skeletal muscle satellite cells (Module 8: Figure Satellite cell activation) and the epidermal stem cells (Module 8: Figure epidermal stem cell).

Smad2 and 4 are tumour suppressors. A germline mutation in Smad4 has been linked to juvenile polyposis syndromes (JPSs).

Wnt signalling pathways

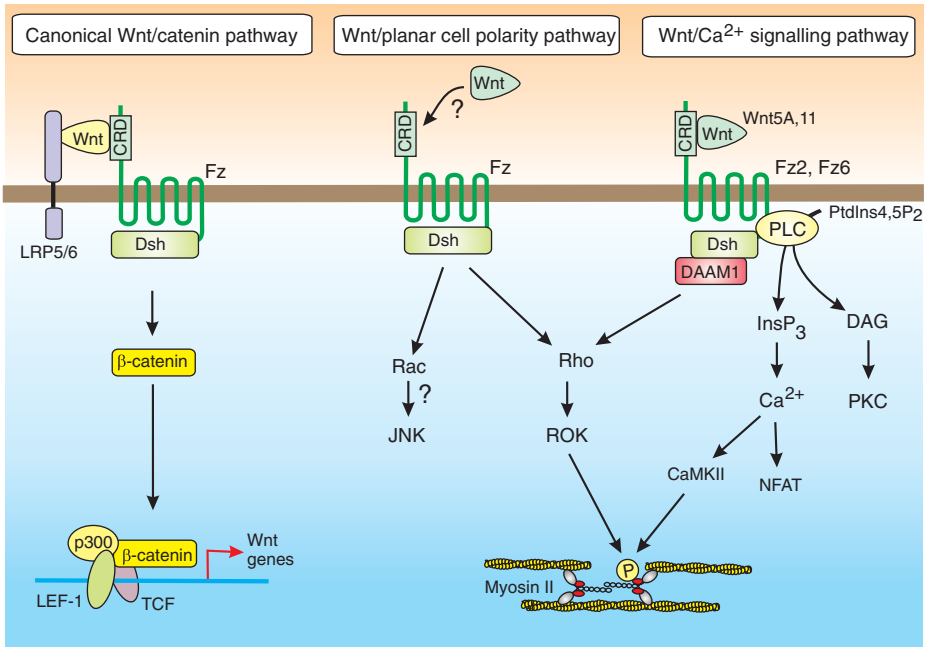
The Wnt signalling pathways play a critical role in the control of cell proliferation, cell fate specification and differentiation. These different pathways are activated by extracellular lipoprotein signalling molecules called Wnts responsible for transmitting information between cells over relatively short distances. There are three main pathways: the canonical and two non-canonical pathways (Module 2: Figure Wnt signalling pathways).

The primary function of the canonical Wnt/β-catenin pathway is to activate gene transcription to control processes during both development and in the adult organism. The functions of the non-canonical Wnt pathways are still somewhat of an enigma, since their precise functions have not been clearly identified, but there are clear indications that they may function in planar cell polarity (PCP). The Wnt/planar cell polarity (PCP) pathway, which has been characterized in *Drosophila*, appears to act through various GTP-binding proteins. A closely related Wnt/Ca^{2+} signalling pathway, which has been described in vertebrates, has a number of similar signalling components but also has additional features such as the hydrolysis of PtdIns4,5P_2 to activate signalling through InsP_3/Ca^{2+} and diacylglycerol (DAG)/protein kinase C (PKC).

Wnts

The term Wnt results from the fusion of the names of two orthologous genes, the *Drosophila* segment polarity gene Wingless (Wg) and a mouse proto-oncogene Int-1. The human genome has 19 Wnt genes, many of which appear to have distinct functions. They contain numerous cysteine residues, one of which is palmitoylated, thus making the Wnts somewhat insoluble, which may help to explain why they are short-range ligands. A large number of Wnt-binding proteins, such as secreted frizzled-related protein (SFRP) and Wnt inhibitory factor (WIF) also restrict their sphere of influence by acting as Wnt buffers.

Module 2: | Figure Wnt signalling pathways



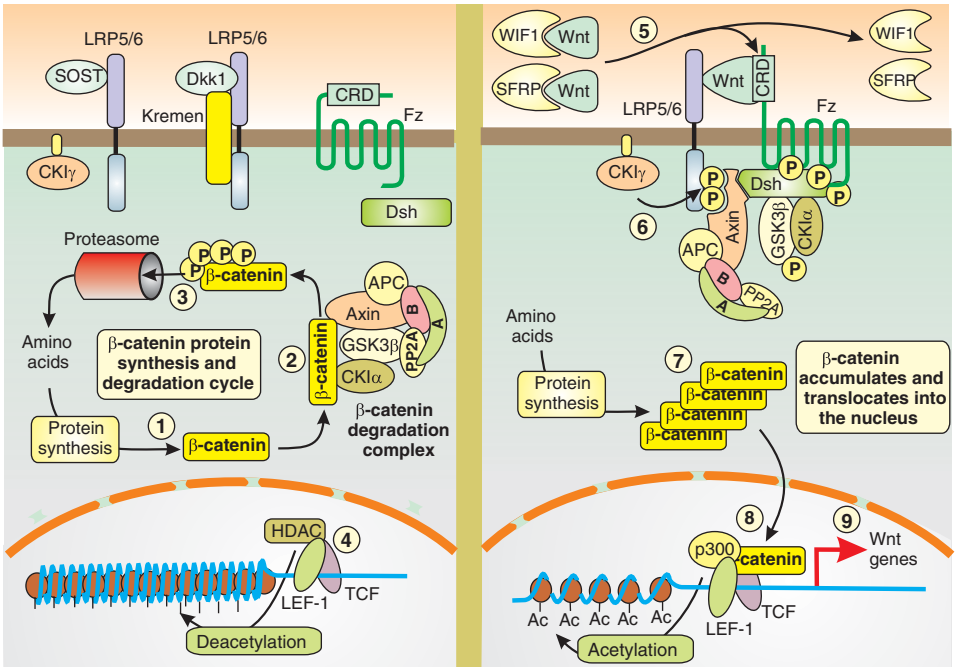
Summary of the signalling mechanisms used by the canonical and non-canonical Wnt signalling pathways.
All three signalling pathways are activated by frizzled (Fz) receptors that depend upon the dishevelled protein (Dsh) as part of the transduction mechanism to transfer information into the cell. Most is known about the canonical Wnt/β-catenin pathway (Module 2: Figure Wnt canonical pathway) with less information on the two non-canonical pathways. In the Wnt/planar cell polarity (PCP) pathway, Dsh transfers information to the small GTP-binding proteins Rho and Rac. The Wnt/Ca^{2+} signalling pathway is connected to the dishevelled-associated activator of morphogenesis 1 (DAAM1), which relays information to the Rho pathway. In addition, Dsh also relays information to phospholipase C (PLC) to produce $InsP_3$ and diacylglycerol (DAG).

Canonical Wnt/β-catenin pathway

The defining feature of the canonical Wnt/β-catenin pathway is the transcription factor β-catenin, which is responsible for regulating the transcription of Wnt target genes (Module 2: Figure Wnt canonical pathway). However, this is not the sole function of β-catenin, which also functions as a scaffolding protein in cell adhesion by providing a link between cadherin and the actin cytoskeleton. Here we consider how β-catenin functions in Wnt signalling to regulate gene transcription. There are a number of transcription factor activation mechanisms, and β-catenin belongs to those that depend on cell-surface receptors that generate cytosolic signals to activate latent transcription factors in the cytoplasm, which are then imported into the nucleus (Mechanism 2 in Module 4: Figure transcription factor activation). In the case of β-catenin, its cytosolic level is kept low because it is constantly being degraded by the proteasome as shown in the sequence of events shown in the left-hand panel in Module 2: Figure Wnt canonical pathway:

1. Formation of β-catenin by protein synthesis occurs continuously.
2. β-Catenin is drawn into a β-catenin degradation complex, which consists of the scaffolding protein axin, which binds the adenomatous polyposis coli (APC) tumour suppressor, the protein phosphatase PP2A, casein kinase Iα (CKIα), glycogen synthase kinase-3β (GSK-3β) and β-catenin. Within this multiprotein degradation complex, the GSK-3β phosphorylates β-catenin and thus targets it for destruction by the proteasome. Before β-catenin can be phosphorylated by GSK-3β it must first be primed by phosphorylating Ser-45 by casein kinase Iα (CKIα). PP2A, which consists of a scaffolding A subunit and a regulatory B subunit (Module 5: Figure PP2A holoenzyme), may inactivate the complex by dephosporylating GSK-3β.
3. The phosphorylated β-catenin is recognized by the F-box/β-TrCP/ubiquitin ligase complex, which targets it for destruction by the proteasome.
4. In the absence of β-catenin, the monomeric high-mobility group (HMG) DNA-binding proteins lymphocyte enhancer factor-1 (LEF-1) and T cell factor (TCF) inhibit the transcription of Wnt genes. An additional component of this repressor complex is histone deacetylase (HDAC), which prevents chromatin remodelling through histone deacetylation. Wnt acts to inhibit the destruction of β-catenin, which then accumulates in the cytoplasm and can enter the nucleus to promote transcription as shown in the right-hand panel in Module 2: Figure Wnt canonical pathway.
5. Wnt initiates the signalling process by binding together two cell-surface proteins: it binds to the cysteine-rich domain (CRD) of the frizzled (Fz) receptor and it also draws in the Fz co-receptor LRP5 and LRP6 (LRP5/6), which are members of the low-density lipoprotein (LDL) receptor superfamily.

Module 2: | Figure Wnt canonical pathway



The canonical Wnt/β-catenin signalling pathway.
The primary function of this signalling pathway is to regulate the activity of β-catenin, which controls transcription of the Wnt genes. The left-hand panel illustrates the resting condition where the cytosolic level of β-catenin is kept low by its continuous degradation. In response to the arrival of Wnt (as shown on the right), this degradation is inhibited and the level of β-catenin rises enabling it to induce the transcription of the Wnt genes.

6. The LRP5/6 co-receptor is then phosphorylated by membrane-bound casein kinase Iγ (CKIγ) isoform, which adds phosphates to multiple sites that have PPPSP motifs. Some of these motifs are also phosphorylated by a membrane-associated GSK-3β. These phosphorylated motifs then provide binding sites for the attachment of the scaffolding protein axin. Another key event is the binding of Dishevelled (Dsh), which is another scaffolding protein containing various signal transduction domains (e.g. DIX, PDZ and DEP). Dsh becomes hyperphosphorylated by an unknown mechanism and this contributes to its role in inhibiting the degradation complex. Axin and Dsh also bind to each other through their DIX domains. As the multiprotein complex associates with the membrane, the organization of the subunits is altered so that the activity of GSK-3β is inhibited, thus reducing the degradation of β-catenin. Loss-of-function mutations of LRP5 have been linked to osteoporosis pseudoglioma (OPPG).
7. When GSK-3β is inhibited, the newly synthesized β-catenin is stabilized and accumulates within the cytoplasm, from where it can enter the nucleus to activate transcription.
8. β-Catenin binds to LEF-1 and TCF to reduce their repressor activity to initiate the transcription of the Wnt genes. β-Catenin replaces HDAC with p300, which facilitates transcription by acetylating histones to remodel chromatin.
9. Activation of transcription of the Wnt target genes results in the activation of the developmental and proliferative effects that characterize the operation of the Wnt signalling pathway.

In summary, the Wnt signalling pathway acts by switching off the GSK-3β-dependent degradation pathway, thus enabling β-catenin to accumulate in the cytosol and to enter the nucleus to activate transcription of the Wnt target genes.

Low density lipoprotein (LDL) receptor-related protein family (LRP)

Members of the low-density lipoprotein (LDL) receptor superfamily are cell-surface proteins with multiple functions. The LDL receptor-related protein (LRP) family has multiple members (LRP1–6, LRP8, LRP10–12) with multiple functions:

- LRP11 is an endocytic receptor for apolipoprotein E (ApoE), which plays an important role in the onset of Alzheimer's disease (AD) (Module 12: Figure amyloid cascade hypothesis).
- LRP5 and LRP6 function as co-receptors in the canonical Wnt/β-catenin pathway (Module 2: Figure Wnt canonical pathway). Mutations in *LRP5* can cause either extremely high or low bone mass traits. A high bone mass syndrome (HBM) is caused by gain-of-function mutations of LRP5. On the other hand, loss-of-function LRP5 mutations are linked to osteoporosis pseudoglioma (OPPG).

Inhibitors of Wnt signalling

There are various extracellular molecules that can inhibit Wnt signalling. Some of these, such as the Wnt inhibitory factor 1 (WIF-1) and secreted frizzled-related protein (sFRP), bind to Wnt and thus prevent it from activating the Fz receptor. There is another group, such as Dickkopf (Dkk) and sclerostin (SOST) that interfere with the activity of the lipoprotein receptor-related protein (LRP) co-receptor LRP5/6. These inhibitors play an important role in preventing the proliferation of stem cells until they are required for tissue repair as occurs for the epidermal stem cells used for the hair follicle cycle (Module 8: Figure epidermal stem cell).

Wnt inhibitory factor 1 (WIF-1)

The Wnt inhibitory factor 1 (WIF-1) is a soluble extracellular factor that binds to Wnt and prevents it from interacting with the frizzled receptor (Module 2: Figure Wnt canonical pathway).

Secreted frizzled-related protein (sFRP)

The secreted frizzled-related protein (sFRP) is a soluble inhibitor that binds to Wnt and prevents it from interacting with the frizzled receptor (Module 2: Figure Wnt canonical pathway).

Dickkopf (Dkk)

Dickkopf (Dkk) binds to Kremen and the lipoprotein receptor-related protein (LRP) co-receptor LRP5/6 and this causes the complex to internalize and will thus inactivate Wnt signalling.

Sclerostin (SOST)

Sclerostin (SOST) inhibits Wnt signalling by binding to the lipoprotein receptor-related protein (LRP) co-receptor LRP5/6 (Module 2: Figure Wnt canonical pathway). SOST is produced by the osteoclasts to inhibit the proliferation and differentiation of the osteoblasts (Module 8: Figure bone cell differentiation).

Van Buchem disease is caused by a mutation in SOST that results in excessive activation of the Wnt signalling pathway.

Function of canonical Wnt signalling

The canonical Wnt pathway has an important role to play in regulating processes such as development and proliferation:

- It functions in dorsoventral specification, as has been shown for amphibia and zebrafish (Module 8: Figure dorsoventral specification).
- It functions in the control of stem cell proliferation (Module 8: Figure stem cell function). For example, Wnt signalling stimulates the proliferation of epidermal stem cells during the hair follicle cycle (Module 8: Figure epidermal stem cell).
- It controls the processes of osteoblastogenesis, which is responsible for the development of the bone-forming osteoblasts (Module 8: Figure bone cell differentiation).
- It controls the differentiation of intestinal cells and alterations in its signalling components are a major cause of colorectal cancer (CRC) (Module 12: Figure colon cancer).
- The canonical Wnt pathway inhibits the differentiation of white fat cells (Module 8: Figure white fat cell differentiation) and the differentiation of brown fat cells (Module 8: Figure brown fat cell differentiation).

The adenomatous polyposis coli (APC) protein, which contributes to the cytoplasmic complex that degrades β-catenin in the cytoplasm, is a potent tumour suppressor that is frequently mutated in cancer cells and particularly in those that develop within the intestine such as colorectal cancer (CRC). Germline mutations of APC are responsible for familial adenomatous polyposis (FAP). Mutation in the *LRP5* gene, which binds to the Wnt antagonist Dickkopf1 (DKK1), causes osteoporosis pseudoglioma (OPPG) syndrome.

Wnt/planar cell polarity (PCP) pathway

The Wnt/planar cell polarity (PCP) pathway has been characterized in insects, where it functions to establish planar cell polarity (PCP) during development (Module 2: Figure Wnt signalling pathways). Just how this pathway functions is still unclear, but there are indications that it relays information through the Rac signalling mechanisms and the Rho signalling mechanism, both of which function in the remodelling of actin. The Rho pathway acts through the Rho kinase (ROK) to activate contraction of the actin/myosin system (Module 2: Figure Rho signalling). These effects on actin remodelling and contraction may play an important role in planar cell polarity.

In vertebrates, there are similar PCP processes such as neural tube closure, the orientation of hair cell stereociliary bundles in the ear, mammalian hair follicle orientation and convergent extension (CE), during which there are large-scale movements of cells that occur during gastrulation. Components of the insect planar cell polarity pathway have also been described in vertebrates, where it includes signalling through Ca^{2+} and has thus been referred to as the Wnt/Ca^{2+} signalling pathway.

Wnt/Ca^{2+} signalling pathway

The Wnt/Ca^{2+} signalling system (Module 2: Figure Wnt signalling pathways) has been implicated in a number of planar cell polarity (PCP) processes in vertebrates. There are a number of similarities between this signalling pathway and the PCP pathway in *Drosophila*. They both can activate Rho and contraction of the actin/myosin system. The main difference is that the vertebrate system has an additional signalling component in that it can activate Ca^{2+} signalling. The activation of this Wnt/Ca^{2+} signalling pathway depends upon the frizzled receptor plugging into the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette (Module 2: Figure $InsP_3$ and DAG formation). There are reports that the Ca^{2+} signalling events associated with this pathway may act to inhibit the canonical Wnt/catenin pathway during dorsal/ventral axis specification (Module 8: Figure dorsoventral specification).

Mutations of the Fz4 receptor, which activates this Wnt/Ca^{2+} pathway, have been linked to familial exudative vitreoretinopathy (FEVR).

Casein kinase I (CKI)

The casein kinase I (CKI) family has seven members (CKIα, α2, δ, ε, γ1, γ2 and γ3), which can have very different functions in cells.

Casein kinase Iα and α2

CKIα functions as a priming kinase for glycogen synthase kinase-3β (GSK-3β) during the operation of the Wnt signalling pathway (Module 2: Figure Wnt canonical pathway).

Casein kinase Iδ

Casein kinase Iδ (CKIδ), which is 97% homologous with casein kinase Iε (CKIε), contributes to the operation of the circadian clock by phosphorylating PER1 and PER2 to control the nuclear entry and stability of these clock proteins (Module 6: Figure circadian clock molecular mechanism).

Casein kinase Iε

CKIε participates in circadian rhythmicity by phosphorylating the PER and CRY proteins to regulate their nuclear entry and stability during the operation of the PER regulatory loop (Module 6: Figure circadian clock molecular mechanism). Mutation of CKIε, which results in a decrease in the ability of this kinase to phosphorylate the PER proteins of the circadian clock, is responsible for familial advanced sleep phase syndrome (FASPS).

Casein kinase Iγ1, γ2 and γ3

CKIγ is an unusual member of the family in that it has a palmitoylation site at its C-terminus and this fatty acid anchor attaches it to the plasma membrane. This membrane location is critical for one of its main functions, which is to phosphorylate the frizzled lipoprotein receptor-related protein (LRP) co-receptor LRP5/6 during activation of the Wnt signalling pathway (Module 2: Figure Wnt canonical pathway).

Casein kinase II (CK2)

Casein kinase II (CK2) is a serine/threonine protein kinase. It functions as a heterotetramer consisting of two 44 kDa catalytic α-subunits and two regulatory β-subunits. It has a unique ability to use GTP as well as ATP as a phosphate donor. CK2 is also known as phosvitin kinase, glycogen synthase kinase 5, troponin T kinase and casein kinase G, which reflects the fact that CK2 can phosphorylate many different substrates and thus contributes to many control mechanisms. This multifunctional kinase has been implicated in many cellular processes and seems to be particularly active in controlling cell proliferation and has also been implicated in cell transformation and tumorigenesis. Many of its actions depend on its ability to phosphorylate transcription factors such as Myc, p53, Rb and activating protein 1 (AP-1). Although CK2 is constitutively active, it can also be activated by certain growth factors (insulin, IGF-I and EGF).

Hedgehog signalling pathway

The Hedgehog signalling pathway in mammals closely resembles that originally discovered and characterized in *Drosophila*. The mammalian Hedgehog signalling toolkit has many of the components found in insects. In comparison with the latter, however, much less is known about the mechanism of Hedgehog activation of transcription in mammals. There are multiple Hedgehog signalling functions that operate during both development and adult life.

Hedgehog signalling toolkit

The Hedgehog signalling pathway has a number of components (Module 2: Table Hedgehog signalling toolkit) whose names resemble those given to similar components in *Drosophila*, where this signalling system was originally identified and characterized. While many of the mammalian components have similar functions to those found in insects, it is clear that there are important differences. Since there are mammalian components that are not found in insects, the mechanism of Hedgehog activation of transcription has to be worked out separately for vertebrates.

Hedgehog activation of transcription

Hedgehog mediates its effects by activating gene transcription. Hedgehog uses mechanism 2 of the different transcription factor activation mechanisms found in cells (Module 4: Figure transcription factor activation). There are three Hedgehog transcription factors (GLI 1–3), which are held in an inactive state within the cytoplasm in resting cells (Module 2: Figure Hedgehog signalling pathway). This inactive state is maintained by the Hedgehog receptor patched (PTC), which inhibits the seven-membrane-spanning protein smoothened (SMO) that acts as the Hedgehog transducer. In the absence of a signal from SMO, the GLI transcription factors are maintained in a latent state by interacting with a large number of cytoplasmic factors (Module 2: Table Hedgehog signalling toolkit). The precise function of all these factors is still being worked out. Hedgehog arriving at the cell surface induces a train of events that activate these transcription factors so that they translocate into the nucleus to induce gene transcription (Module 2: Figure Hedgehog signalling pathway). First of all, Hedgehog binds to its receptor PTC and this removes the inhibitory effect of PTC on SMO. The latter is then able to activate GLI by removing it from the inhibitory constraints of the cytoplasmic factors so that it is now free to translocate into the nucleus to activate transcription. Some of the genes that are activated are components of the Hedgehog signalling pathway and thus set up both positive and negative feedback loops. Many of the other genes contribute to Hedgehog signalling functions.

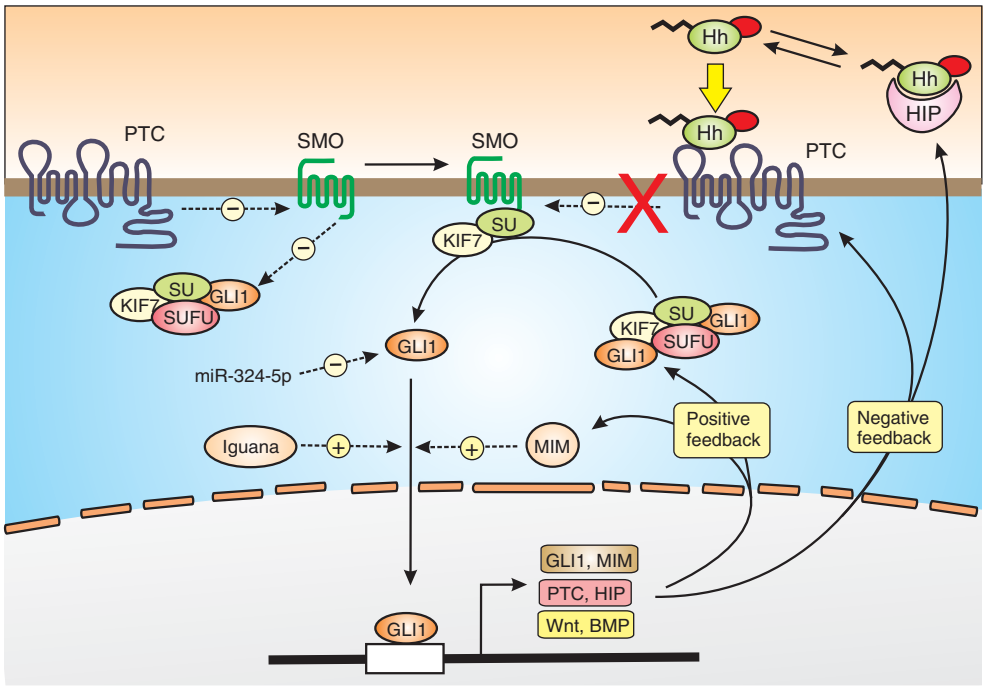
Expression of the GLI1 transcription factor is suppressed by miR-324-5p. Mutations in miR-324-5p result in the development of medulloblastomas.

Module 2: | **Table Hedgehog signalling toolkit**
The Hedgehog signalling toolkit.

Component	Comment
Hedgehog (Hh) ligands	
Shh	Sonic Hedgehog
Ihh	Indian Hedgehog
Dhh	Desert Hedgehog
Hedgehog-binding proteins	
HIP	Hh-interacting protein
Megalin	A member of the low-density lipoprotein (LDL)-receptor-related family
Hedgehog receptors	
PTC1	Patched 1
PTC2	Patched 2
Transducer	
SMO	Smoothened; a seven-membrane-spanning protein
Cytoplasmic regulators	
KIF7	Functions to repress Sh responses
FU	The fused serine/threonine protein kinase
SUFU	Suppressor of fused; a negative regulator of GLI transcription factors
MIM	Missing in metastasis; an actin-binding protein that potentiates transcription
Iguana	A zinc-finger protein that promotes localization of GLI1
IFT88	Ciliary protein that regulates GLI function
IFT172	Ciliary protein that regulates GLI function
FKBP8	Antagonizes Shh action
SIL	Cytoplasmic protein that acts downstream of PTC
Rab23	This regulator of vesicular traffic is a negative regulator of Hh signalling
Transcription factors	
The multifunctional GLI transcription factors function to either activate or repress transcription	
GLI1	A zinc-finger transcriptional activator
GLI2	A zinc-finger transcriptional activator/repressor
GLI3	A zinc-finger transcriptional repressor

The function of some of these toolkit components are illustrated in Module 2: Figure Hedgehog signalling pathway.

Module 2: | Figure Hedgehog signalling pathway



Activation of gene transcription by the Hedgehog signalling pathway.
Under resting conditions, the patched (PTC) receptor for Hedgehog (Hh) inhibits the activity of the seven-membrane-spanning receptor smoothened (SMO). In this inhibited state, SMO is not able to act on the complex of cytoplasmic factors that collectively regulate the transcription factor GLI1. When Hh is present, it binds to PTC, and this removes the inhibitory effect of the latter on SMO, which is now capable of activating transcription by releasing GLI from its associated cytoplasmic factors. GLI1 then translocates into the nucleus, where it activates a variety of genes that fall into three main groups. One group consists of components of the signalling pathway, such as GLI1 and missing in metastasis (MIM), which set up a positive-feedback loop. The second group consists of genes coding for PTC and Hh-interacting protein (HIP), which thus set up a negative-feedback loop. The HIP protein acts on the outside to bind Hh and thus reduce its activity. The last group of genes code for proteins not involved in Hedgehog signalling, but some do function in other signalling pathways such as Wnt (Module 2: Figure Wnt canonical pathway) and the bone morphogenetic proteins (BMPs) (Module 2: Figure Smad signalling).

Hedgehog signalling functions

The Hedgehog signalling pathway functions both during development and during adult life. Its function during development is wide-ranging in that it can control cell proliferation, cell determination and pattern formation of the developing embryo and the final processes of cell differentiation. Hedgehog signalling seems to play an important role in controlling the development of organs such as the skin, brain, digestive tract, pancreas and prostate. Mutations of components of this signalling pathway result in congenital malformations such as holoprosencephaly, which is a cranial defect that occurs when the midline structures of the brain and face fail to separate. The most dramatic phenotype of such malformation is cyclopia where only one eye is formed.

The function of Hedgehog continues in the adult organism, where it plays a major role in the formation and maintenance of the stem cell population. Since Hedgehog signalling plays such a major role in regulating cell proliferation, it is not surprising that alterations in this signalling pathway have been detected in many cancers such as skin cancer and some brain cancers.

For example, patched (PTC) is a tumour suppressor that is inactivated in basal cell carcinomas (BCCs), medulloblastomas, gliomas in the brain and prostate cancers.

Mutations of the PTC gene are responsible for Gorlin's syndrome, which is also known as nevoid basal cell carcinoma syndrome (NBCC). The Hedgehog signalling pathway may also contribute to the growth of tumours by enhancing the activity of stromal cells that provide the tumour cell microenvironment that supports cancer cell survival and growth. Cancer cells release hedgehog that then uses a paracrine mechanism to stimulate neighbouring stromal cells such as the blood vessels, fibroblasts, immune cells and epithelial cells. These stromal cells assist cancer cell growth by providing both an extracellular matrix and essential growth factors such as insulin-like growth factor (IGF) and Wnt.

Hippo signalling pathway

The hippo signalling pathway was discovered initially in *Drosophila* where it functions to control proliferation, cell growth and apoptosis. This signalling pathway is highly conserved and mammalian homologues of the *Drosophila* hippo signalling components, which connect transduction events at the plasma membrane to alterations in gene transduction, have now been identified. (Module 2: Figure hippo signalling pathway).

In the case of the mammalian pathway, it seems likely that there are cell-surface receptors that activate the core kinase cascade, but these remain to be identified. One putative receptor is CD44, which is a receptor for hyaluronic acid and can also respond to osteopontin and collagen. The downstream target for such cell-surface receptors is likely to be the tumour suppressor merlin (moesinezrin-radixin-like protein), which is coded for by the neurofibromatosis type 2 (Nf2) gene and is sometimes referred to as NF2. Merlin then acts to regulate the activity of mammalian sterile-20-like kinase type 1 and type 2 (MST1/2), which is the first protein kinase of the core kinase cascade.

In the next of part of the cascade, MST1/2 phosphorylates Large tumour suppressor-1 and -2 (Lats1/2). The activity of Lats1/2 is modulated by Mps one binding protein (MOB1). The ability of MOB1 to activate Lats1/2 is enhanced by its phosphorylation by MST1/2. The activated Lat1/2 then carries out the final step by phosphorylating the closely related transcription factors Yes-activated protein (YAP) and transcriptional coactivator with PDZ–binding motif (TAZ). Once they are phosphorylated, YAP and TAZ are inactivated and leave the nucleus (Module 2: Figure hippo signalling pathway). In the case of TAZ, export from the nucleus is carried out by protein 14-3-3. YAP can also be inactivated by a ubiquitination pathway that begins with its phosphorylation by casein kinase Iδ (CKIδ) or casein kinase Iε (CKIε) that marks it out for ubiquitin ligase by $SCF^{\beta\text{-}TRCP}$.

The closely related transcription factors YAP and TAZ, which have overlapping and distinct functions, operate together with other co-activators of which the TEA domain (TEAD) proteins are particulary important. This transcriptional complex activates a number of target genes including amphiregulin (AR), cysteine-rich protein connective tissue factor (CTGF) and Baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5, also known as survivin). BIRC5 is a member of the inhibitor of apoptosis (IAP) family.

Hippo signalling appears to have multiple functions in mammalian cells. Many of these functions seem to be related to controlling cell proliferation and cell growth. For example, it can control the size of organs such as the liver. Contact inhibition in cultured cells seems to require activation of this signalling cascade. Its role in cell proliferation is apparent in numerous examples where uncontrolled growth occurs in many cancers when various components of the pathway are deleted. Hepatocellular carcinoma (HCC) can be induced by the overexpression of YAP.

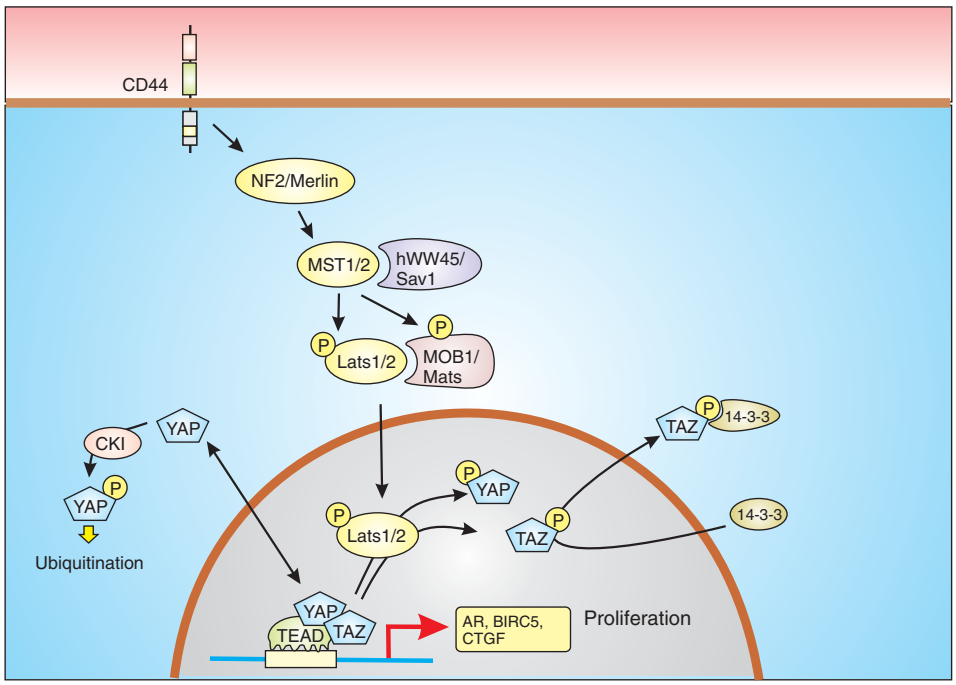
Mammalian sterile-20-like kinase type 1 (MST1)

The mammalian sterile-20-like kinase type 1 and type 2(MST1/2) function in the hippo signalling pathway where they function by phosphorylating the large tumour suppressor-1 and -2 (Lats1/2), which is a serine/threonine protein kinase that acts as a tumour suppressor (Module 2: Figure hippo signalling pathway). The mammalian homologue of the *Drosophila* protein Salvador (Sav) is the cofactor WW45 (also known as Sav1) that has an important role in the activation of MST1/2.

Large tumour suppressor (Lats)

The Large tumour suppressor-1 and -2 (Lats1/2) is a serine/threonine protein kinase that acts as a tumour suppressor by inhibiting the G1/S transition of the cell cycle (Module 8: Figure ES cell miRNAs). It functions by phosphorylating and inactivating the transcription factor Yes-associated protein (YAP), which operates in the hippo signalling pathway (Module 2: Figure hippo signalling pathway). The expression of Lats1/2 is reduced my miR372 and miR-373.

Module 2: | Figure hippo signalling pathway



Hippo signalling pathway.
The hippo signalling pathway is based on a protein phosphorylation cascade that functions to regulate the transcription of genes that play a role in regulating proliferation and cell growth.

Yes-associated protein (YAP)

The Yes-associated protein (YAP), which is the mammalian homologue of the *Drosophila* protein Yorkie (Yki), is a multifunction transcription factor that operates in the hippo signalling pathway (Module 2: Figure hippo signalling pathway). YAP shares almost 50% sequence identity with TAZ and they both have two central WW domains and a transactivation domain at the C-terminus. The transcriptional activity of YAP functions together with other co-activators such as the TEA domain (TEAD) family, which has four closely related members TEAD1–4, peroxisome-proliferator-activated receptor γ (PPARγ), thyroid transcription factor-1 (TTF-1), Runx2 and some of the SMADs. The transactivation with TEAD seems to be particularly important.

Transcriptional coactivator with PDZ binding motif (TAZ)

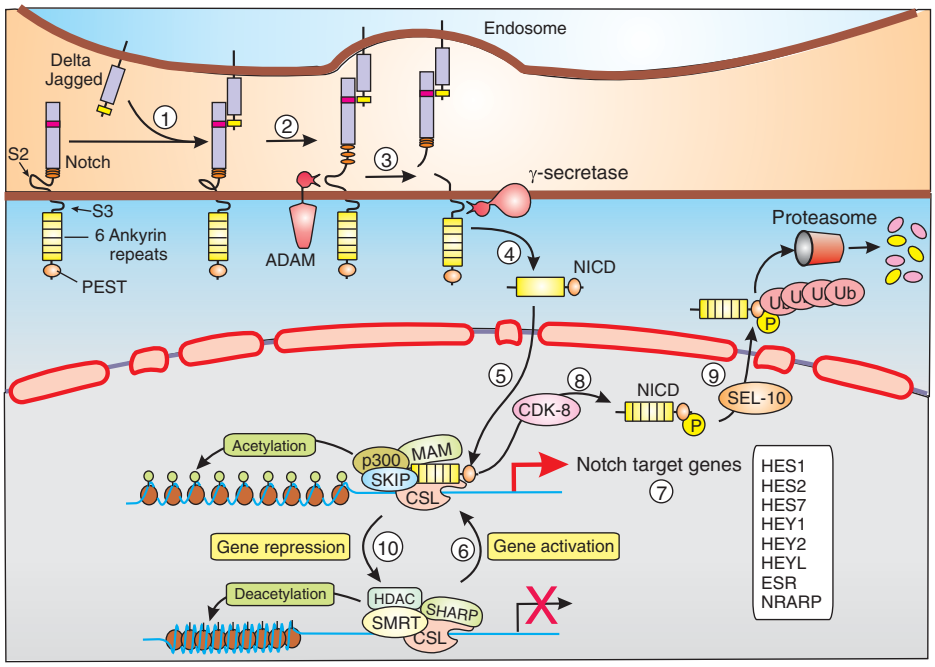
The transcriptional coactivator with PDZ-binding motif (TAZ) operates in the hippo signalling pathway (Module 2: Figure hippo signalling pathway). TAZ shares almost 50% sequence identity with YAP and they both have two central WW domains and a transactivation domain at the C-terminus. The TAZ/TEAD transcription complex may increase the expression of Zeb1, which plays a role in embryonic development by inducing the epithelial-to-mesenchymal transition (EMT).

Notch signalling pathway

The Notch signalling pathway is a highly conserved signalling system that functions in both development and adulthood. Many of its functions relate to cell-fate determination and this is particularly the case in its control of binary cell-fate decisions in stem cells. During the assymetrical divisions of stem cells, one cell retains its stem cell fate (self-renewal) while the daughter cell (progenitor cell) adopts a different state that will drive it towards proliferation and differentiation into a specific cell type (Module 8: Figure stem cell function). As these progenitor cells adopt their new cell fate, they use the Notch signalling pathway to feed information back to suppress their neighbour from adopting a similar cell fate. This is a short-range information transfer mechanism that depends upon direct contact between the cells, which is a hallmark of this signalling pathway. For example, the stimulus Jagged is an integral membrane protein located on the surface of communicating cells, whereas the Notch receptor that responds to Jagged is embedded in the surface of the receiving cell (Module 2: Figure Notch signalling).

The main feature of the transduction mechanism is deceptively simple. When Jagged binds to Notch, the Notch intracellular domain (NICD) is released into the cytoplasm and then diffuses into the nucleus where it induces the transcription of multiple Notch target genes. Despite its simplicity, this signalling system has an extensive number of interacting components (Module 2: Table Notch signalling components). Some of these function during the

Module 2: | Figure Notch signalling



Notch signalling pathway.
One of the stimuli for the Notch signalling pathway is Jagged, which is located on the surface of the communicating cell. The receptor for Jagged is Notch, which is located on the surface of the receiving cell. When Jagged interacts with Notch (Step 1), it triggers a series of steps that result in the proteolytic release of the Notch intracellular domain (NICD), which then enters the nucleus to activate transcription of Notch target genes. See the text for details of this sequence of events.

signal transduction process itself, whereas others play a role in the modulation of Notch signalling, which regulates the expression of the stimuli (Delta and Jagged) and the Notch receptor at the cell surface. The operation of the Notch signalling pathway depends upon the following steps (Module 2: Figure Notch signalling):

1. Delta or Jagged located on one cell interacts with the Notch receptor on a neighbouring cell. The N-terminal DSL (Delta, Serrate and LAG-2) domain (yellow bar) on Delta/Jagged binds to the EGF-repeats 11 and 12 (red bar) on Notch. By itself, this interaction with Notch seems to have little effect and only initiates the signal transduction sequence as a result of Delta/Jagged physically pulling on Notch.
2. The endocytosis of Delta/Jagged seems to be a critical step for receptor activation. As Delta/Jagged are drawn into the endosome, they pull on Notch, which undergoes a conformational change to expose site-2 (S2).
3. S2 is cleaved by ADAM proteases such as ADAM-10 and ADAM-17 (Module 1: Table ADAMs proteases). The external domain is shed leaving behind a short transmembrane region and the intracellular domain, which then becomes a substrate for γ-secretase.
4. The γ-secretase complex is an enzyme complex made up of presenilin, nicastrin, PEN2 and APH1 that cleaves the S3 site to release the Notch intracellular domain (NICD). The activity of γ-secretase may be inhibited by Crumbs, which is an apical polarity protein.
5. NICD released from the membrane diffuses into the nucleus and binds to CSL (CBF-1, Suppressor of Hairless, Lag-1).
6. CSL normally acts to repress Notch target genes. Repression is enhanced by CSL providing a framework to recruit co-repressors such as SMRT, SHARP, SKIP and CIR. This repression complex also recruits histone deacetylase (HDAC) that deacetylates chromatin further shutting down transcriptional activity. When NICD binds to CSL, the repression complex is disassembled and this paves the way for the assembly of an activation complex. An important part of the complex is the co-activator Mastermind (MAM) and the histone acetyl transferase p300. The latter is responsible for protein acetylation of histones to open up the chromatin to facilitate gene transcription.
7. Gene activation results in an increase in the transcription of Notch target genes. Some of these such as the hairy and enhancer of split (HES) family encode transcriptional repressors responsible for suppressing the expression of tissue specific proteins, which accounts for the ability of the Notch signalling pathway to inhibit differentiation.
8. The inactivation of target gene transcription begins when NICD is phosphorylated in the N-terminal PEST domain by kinases such as cyclin-dependent kinase-8 (CDK-8).
9. The phosphorylated NICD becomes a substrate for nuclear ubiquitin ligases such as SEL-10 and is then exported to the cytoplasm where it is degraded by the proteasome.

Module 2: | Table Notch signalling components
Notch signalling toolkit.

Components	Comments
Notch stimuli	These Notch stimuli contain an N-terminal DSL (Delta, Serrate and LAG-2) domain that interacts with the Notch receptor (Module 2: Figure Notch signalling)
Delta 1	
Delta 3	
Delta 4	
Delta-like-4 (DLL4)	Responsible for lateral inhibition during angiogenesis (Module 9: Figure angiogenesis signalling)
Jagged 1	
Jagged 2	
Receptors	
Notch 1	
Notch 2	
Notch 3	
Notch 4	
Notch modulators	
Numb	A major inhibitor of Notch. It facilitates the endocytic removal of Notch from the plasma membrane (Module 2: Figure Notch modulation)
Crumbs	May function to inhibit γ-secretase activity thereby reducing the activation of Notch
Proteases	
ADAM-10	Also known as Kuzbanian or MADM. Functions in neural and cardiac development
ADAM-17	Also known as tumour necrosis factor-α (TACE)
Furin	A protein convertase that cleaves site 1 (S1) during Notch receptor maturation
γ-secretase	An enzyme complex containing presenilin, nicastrin, PEN2 and APH1
Glycosyltransferases	
Fringe family	A family of glycosyltransferase in the Golgi that glycosylates Notch to alter its binding affinities to Delta and Jagged
Lunatic fringe	
Radical fringe	
Manic fringe	
Transcriptional regulators	
CSL	CSL [CBF-1, Su(H), Lag-1] represses Notch target genes (Module 2: Figure Notch signalling)
MAM	Mastermind
SKIP	Ski-interacting protein interacts with CSL and the ankyrin repeats of NICD
Notch target genes	
HES1	Hairy and enhancer of split 1 (HES1) represses transcription of tissue specific genes responsible for differentiation
HES2	
HES7	
HEY1	
HEY2	
HEYL	
ESR	
Ubiquitin ligases	
Itch	A Hect domain E3 ligase that functions in Notch trafficking (Module 2: Figure Notch modulation)
Deltex	A Ring finger E3 ligase that functions in Notch trafficking (Module 2: Figure Notch modulation)
Mind bomb (Mib)	Ring finger E3 ubiquitin ligases that initiate the trafficking of Delta and Jagged (Module 2: Figure Notch modulation)
Neutralized (Neur)	Ring finger E3 ubiquitin ligases that initiate the trafficking of Delta and Jagged (Module 2: Figure Notch modulation)
SEL10	A nuclear ubiquitin ligase that interacts with the phosphorylated form of NICD (see Step 8 in Module 2: Figure Notch signalling)

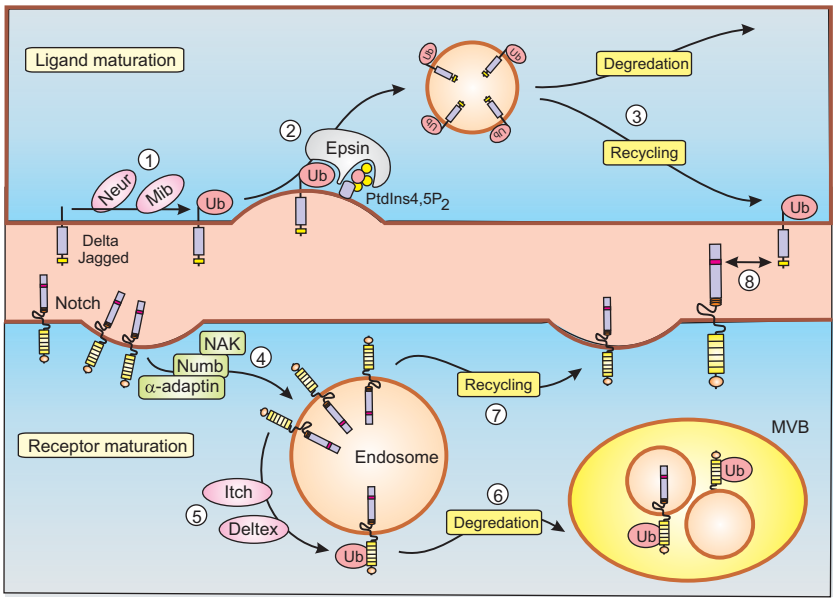
10. Once NICD is removed from the activation complex and degraded, the different co-activators fall away and are replaced by the repressors that result in gene repression. Gene repression also depends on the recruitment of histone deacetylase (HDAC) that deacetylates chromatin further shutting down transcriptional activity.

Notch signalling controls a number of developmental events and continues to have a role during adulthood particular in maintaining the stem cell population:

- Proliferation of satellite cells in skeletal muscle is enhanced by Notch signalling (Module 8: Figure Satellite cell activation).
- The self renewal of haematopoietic stem cells (HSCs) is facilitated by Notch signalling that acts by inhibiting differentiation (Module 8: Figure HSC regulation).
- Notch signalling is responsible for the process of lateral inhibition that inhibits tip cell formation during angiogenesis (see Step 2 in Module 9: Figure angiogenesis signalling).

Alterations in Notch signalling have been implicated in a number of cancers. For example, overexpression of Notch

Module 2: | Figure Notch modulation



Modulation of Notch signalling.
Trafficking through the endosomal compartment plays an important role in regulating the maturation/activity of the Notch ligands (shown at the top) and the Notch receptor (shown at the bottom). See text for further details. Mib, Mind bomb; MVB, multivesicular body; Neur, Neutralized; NAK, Numb-associated kinase; Ub, ubiquitin.

has been identified in both ovarian and medullablastomas. An up-regulation of Notch and a decrease in the inhibitor Numb has been observed in breast cancer. Mutations in Notch have been linked to T cell acute lymphoblastic leukaemia.

Modulation of Notch signalling

The primary mechanism for modulating Notch signalling is to adjust the mechanisms responsible for regulating both ligand (Delta and Jagged) and receptor (Notch) maturation (Module 2: Figure Notch modulation). In both cases, the ligands and the receptor on the cell surface are taken up into the endosomes and are then either recycled back to the cell surface or are degraded. The balance between recycling and degradation thus determines their level of membrane expression and thus sets the sensitivity of the Notch signalling pathway. The ubiquitin-proteasome system plays a critical role in regulating the trafficking of these two signalling components.

The trafficking of Delta and Jagged follows the following series of events (Module 2: Figure Notch modulation):

1. The cytoplasmic domain of Delta and Jagged are ubiquitinylated by Ring finger E3 ubiquitin ligases such as Mind bomb (Mib) and Neutralized (Neur).
2. Endocytosis of these ubiquitylated proteins is facilitated by the protein epsin that binds to ubiquitin-containing cargo proteins. In addition, epsin binds to both clathrin and to phosphatidylinositol 4,5-bisphosphate ($PtdIns4,5P_2$), which functions in the $PtdIns4,5P_2$ regulation of membrane trafficking and endocytosis.
3. The endosome then has two paths: it can move down the lysosomal path resulting in degradation of Delta and Jagged. Alternatively, it can recycle back to the plasma membrane where the ligands are then able to activate Notch.

Different players function in the regulation of Notch trafficking as shown in the following steps (Module 2: Figure Notch modulation):

4. Entry of Notch into the endosome is dependent on the protein Numb, which is a major inhibitor of Notch signalling. The Notch receptor interacts with Numb, which also binds to α-adaptin. The latter is a component of the adaptor protein-2 (AP2) complex responsible for endocytosis. A Numb-associated kinase (NAK) is also a component of this complex. The Notch receptors in the endosome have two fates.
5. Some of the Notch receptors are ubiquitinated by ubiquitin ligases such as Itch (a Hect domain E3 ligase) and Deltex (a Ring finger E3 ligase).
6. Ubiquitinylated notch receptors are transferred to the multivesicular body (MVB) en route to degradation by the lysosome.
7. Some of the Notch receptors are recycled back to the plasma membrane.
8. These Notch receptors on the cell surface are then able to interact with ligands such as Delta and Jagged to initiate the Notch signalling pathway (Module 2: Figure Notch signalling).

Delta

Delta is a transmembrane protein that functions as one of the stimuli that activate the Notch receptor (Module 2: Table Notch signalling components). The extracellular domain is made up of EGF-like repeats and there is an N-terminal DSL (Delta, Serrate and LAG-2) domain that interacts with the Notch receptor (Module 2: Figure Notch signalling). In order for Delta to function as a stimulus, it has to undergo a maturation process, which depends upon its passage through the endosome (Module 2: Figure Notch modulation). This trafficking through the endosomal compartment determines the amount of Delta in the membrane and this is an important aspect of the modulation of Notch signalling.The expression of Delta-1 is increased on the surface of myofibres during muscle damage and this activates the Notch signalling pathway to stimulate the proliferation of satellite cells (Module 8: Figure Satellite cell activation).

Jagged

Jagged is a transmembrane protein that functions as a one of the stimuli that activate the Notch receptor (Module 2: Table Notch signalling components). The extracellular domain is made up of a cysteine-rich domain, EGF-like repeats and there is an N-terminal DSL (Delta, Serrate and LAG-2) domain that interacts with the Notch receptor. In order for Jagged to function as a stimulus, it has to undergo a maturation process, which depends upon its passage through the endosome (Module 2: Figure Notch modulation). This trafficking through the endosomal compartment determines the amount of Delta in the membrane and this is an important aspect of the modulation of Notch signalling.

Notch

Notch is a transmembrane protein that functions as the receptor for the Notch signalling pathway by responding to stimuli such as Delta and Jagged (Module 2: Figure Notch signalling). The large extracellular domain of Notch is made up of a variable number (29–36) of EGF-like repeats and a Lin21/Notch repeat region. EGF-repeats 11 and 12 (red bar) on Notch provides the binding site that interacts with the N-terminal DSL (Delta, Serrate and LAG-2) domain on Delta and Jagged. The cytoplasmic domain has a series of six ankyrin repeats and a terminal PEST domain.

Notch undergoes a number of post-translational modifications as it moves through the endoplasmic reticulum (ER) and the Golgi. While in the ER, certain sites on the EGF repeats are fucosylated by the chaperone O-fut. After the addition of these fucose groups, further extension of the carbohydrate chains is carried out by the Fringe family (Module 2: Table Notch signalling components). The degree of glycosylation can markedly influence the affinity of the Notch receptor for its ligands. The last modification, which occurs in the Golgi, is the cleavage of the molecule at site 1 (S1) by the protease; the heterodimeric receptor is then inserted into the membrane.

Trafficking of Notch through the endosomal compartment determines the amount of receptor in the membrane and this is an important aspect of the modulation of Notch signalling (Module 2: Figure Notch modulation).

Endoplasmic reticulum (ER) stress signalling

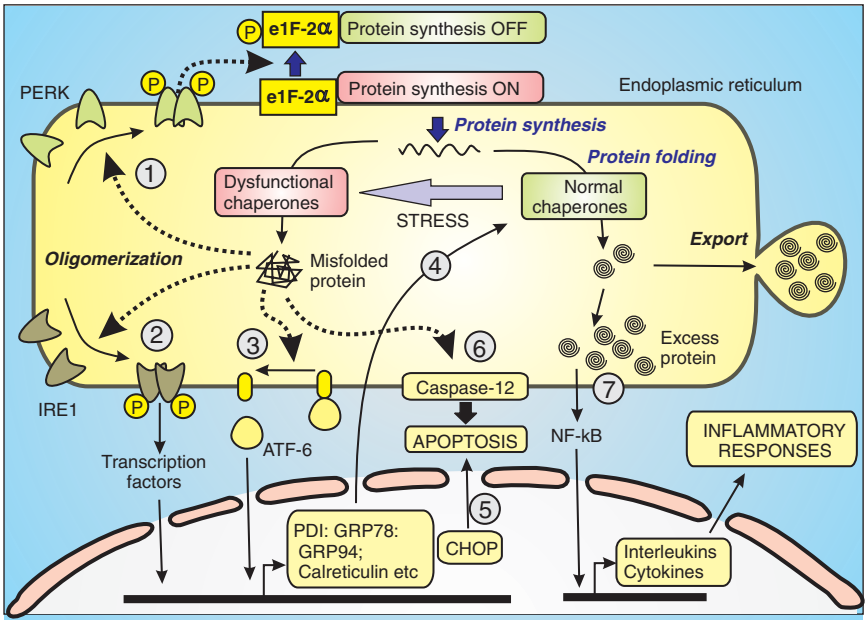
The endoplasmic reticulum (ER) has sophisticated stress signalling pathways that enable it to adapt to a whole host of stress factors mainly concerned with the way in which proteins are synthesized and packaged. Maintenance of a constant level of Ca^{2+} within the lumen of the ER is essential for the post-translational processing, folding and export of proteins. This protein processing is carried out by a number of Ca^{2+}-sensitive chaperones such as 78 kDa glucose-regulatory protein (GRP78) [also known as immunoglobulin heavy-chain-binding protein (BiP)], GRP94 (endoplasmin) and calnexin. GRP78 may function as a modulator of the $InsP_3$ receptor by regulating how this release channel is assembled (Module 3: Figure $InsP_3R$ regulation).

Any decline in the luminal level of Ca^{2+} results in the accumulation of misfolded proteins and the activation of the ER stress signalling pathways (Module 2: Figure ER stress signalling):

1. Oligomerization and autophosphorylation of PKR (protein kinase R)-like ER kinase (PERK) sets off a phosphorylation cascade that culminates in the phosphorylation and inactivation of the translation eukaryotic initiation factor eIF-2α, resulting in protein synthesis being switched off.
2. Oligomerization and autophosphorylation of IRE1 initiates one of the transcriptional signalling pathways responsible for the up-regulation of the various chaperones.
3. Another of the transcriptional pathways depends upon the activation of the ER membrane-bound activating transcription factor 6 (ATF6), which is released from the ER to enter the nucleus, where it interacts with the ER stress-response element of the C/EBP (CCAAT/enhancer-binding protein)-homologous protein 10 (CHOP) gene.
4. The various chaperones are then expressed within the ER, where they participate in protein folding.
5. One of the genes activated during the stress response is CHOP, which acts as a transcription factor and can contribute to apoptosis.
6. Caspase 12, which is associated with the ER membrane, is also activated and contributes to ER stress-induced apoptosis.
7. An excessive accumulation of proteins within the ER results in the activation of the transcription factor nuclear factor κB (NF-κB), which acts to increase the production of interferons and cytokines, so contributing to an inflammatory response.

These stress pathways are then responsible for switching off ongoing protein synthesis, for up-regulating the production of new chaperones, for inducing apoptosis and for activating inflammatory responses. The degree to which these different responses are activated depends upon the

Module 2: | Figure ER stress signalling



Endoplasmic reticulum (ER) stress signalling pathways.
An accumulation of misfolded proteins or an excessive accumulation of normal proteins activate a number of signalling pathways. Chaperones within the endoplasmic reticulum (ER) lumen are responsible for folding newly synthesized proteins into their tertiary structures prior to their export to the Golgi. A variety of stress factors, including a decline in the luminal level of Ca^{2+}, results in dysfunctional chaperones and an accumulation of misfolded proteins that can activate a number of signalling pathways.

nature of the stress. The fact that the ER can up-regulate chaperone levels results in the phenomenon of tolerance, whereby treatment of cells with low levels of stress stimuli can make cells much more tolerant to subsequent stressful stimuli.

Unfolded protein response (UPR)

An accumulation of misfolded proteins induces an unfolded protein response (UPR), which switches off ongoing protein synthesis and also activates various transcriptional cascades that result in the up-regulation of many of the key chaperones in an attempt to improve the defective protein packaging machinery (Module 2: Figure ER stress signalling). Activating transcription factor 6 (ATF6) is one of the transcription factors activated by the UPR.

Endoplasmic reticulum (ER) overload response (EOR)

An excessive build-up of proteins, as occurs during viral infections, switches on an endoplasmic reticulum (ER) overload response (EOR) that acts through the nuclear factor κB (NF-κB) signalling cascade to stimulate the release of interferons and cytokines as part of an inflammatory response.

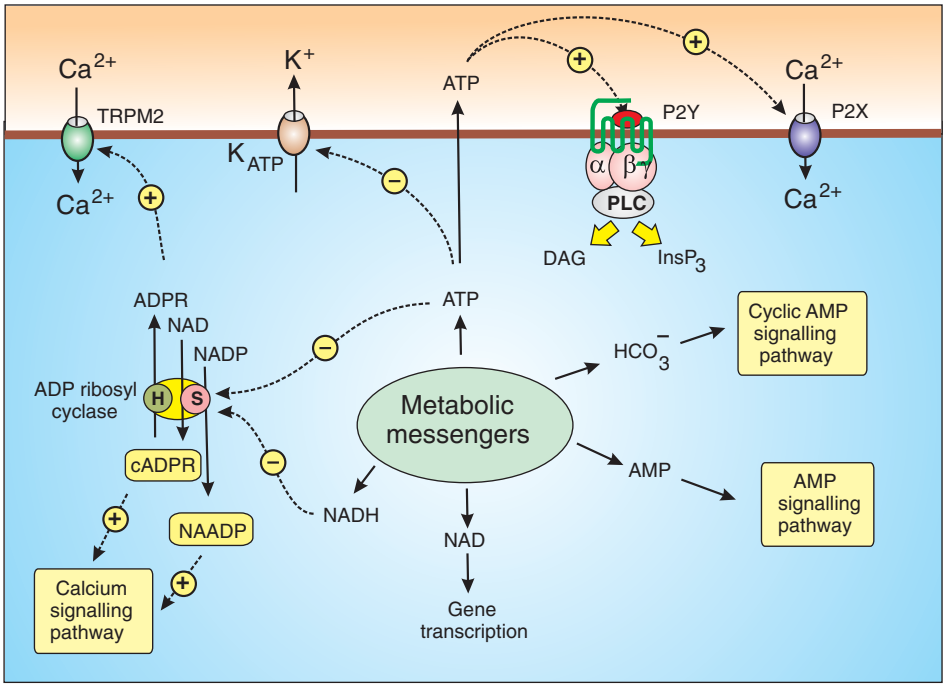
Activation of apoptosis

Endoplasmic reticulum (ER) stress signalling pathways can also contribute to apoptosis (Module 11: Figure apoptosis). For example, one of the UPR pathways depends upon the release of the transcription factor activating transcription factor 6 (ATF6), which acts to switch on C/EBP (CCAAT/enhancer-binding protein)-homologous protein 10 (CHOP). Another mechanism depends upon the ER directly activating a subset of caspases during ER stress. A critical component is caspase 12, which is associated with the ER membrane and is released by proteolytic cleavage following ER stress. Several mechanisms have been proposed for this activation process. One suggestion is that the stress sensor molecule IRE1 recruits tumour-necrosis-factor-receptor-associated factor (TRAF) which then binds to caspase 12, making it sensitive to the Ca^{2+}-responsive cysteine protease m-calpain. Another suggestion is that the hydrolysis of caspase 12 is mediated by caspase 7, which is recruited to the membrane during ER stress. An interesting aspect of this mechanism is that glucose-regulated protein 78 kDa (GRP78) appears to inhibit this activation process by forming a complex with caspase 7 and caspase 12. Once caspase 12 is released into the cytosol, it activates a specific cascade involving caspase 9 and caspase 3 in a cytochrome *c*-independent manner.

Metabolic messengers

There are a number of cellular metabolites that function as metabolic messengers to integrate the activities of cellular metabolism and cell signalling (Module 2: Figure metabolic messengers). In this context, a metabolic messenger is defined fairly widely: it includes components that are either a part of, or are derived from, cellular metabolism. Metabolism is regulated at many different levels. The most direct control depends upon feedback processes where

Module 2: | Figure metabolic messengers



Interaction between metabolic messengers and cell signalling pathways.
A number of metabolic intermediates can interact with various cell signalling pathways. ATP plays a significant role through its ability to close ATP-sensitive K^+ channels as occurs in insulin-secreting β-cells (Module 7: Figure β-cell signalling). ATP that is released from the cell functions to activate ATP-sensitive P2X channels (Module 3: Figure P2X receptor structure). ATP may also play an important role in regulating the activity of ADP-ribosyl cyclase that produces the Ca^{2+}-mobilizing messengers cyclic ADP-ribose (cADPR) and nicotinic acid-adenine dinucleotide phosphate (NAADP) (Module 2: Figure cADPR/NAADP function). AMP is an important activator of the AMP signalling pathway (Module 2: Figure AMPK control of metabolism). The breakdown product of cADPR is ADP-ribose (ADPR), which is an activator of the transient receptor potential melastatin 2 (TRPM2) channel. Bicarbonate (HCO_3^-) is an activator of soluble adenylyl cyclase and thus contributes to the cyclic AMP signalling pathway (Module 2: Figure cyclic AMP signalling).

certain substrates or products function as positive or negative regulators of the enzymes that synthesize or metabolize them. They are not signalling mechanisms in the strict sense, but are based on relatively straightforward mass action reactions that ensure that metabolism proceeds in an orderly and regulated manner. However, some of the metabolic components can be considered to be metabolic messengers because they activate or modulate clearly defined signalling pathways. There are a number of such metabolic messengers:

- Adenosine triphosphate (ATP)
- Adenosine monophosphate (AMP)
- Fatty acids
- Bicarbonate (HCO_3^-)
- NAD^+ signalling pathways

The AMP signalling pathway is of major importance with regard to metabolic signalling systems. Components of various redox signalling systems, such as the nitric oxide signalling pathway, are also related to such metabolic signalling pathways.

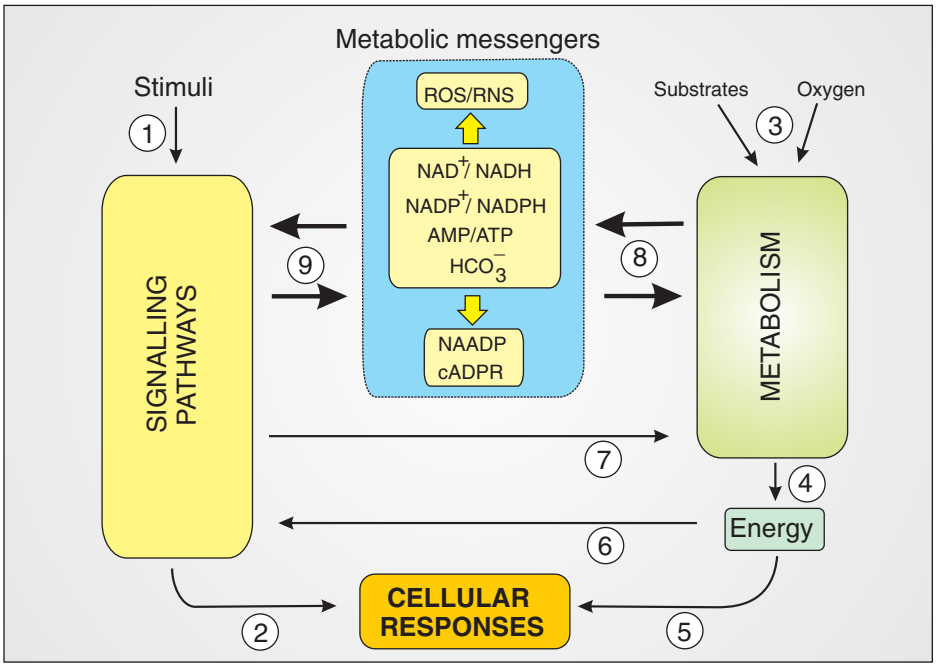
Signalling through metabolites is a highly integrated system because some of the signalling pathways activated by the metabolic messengers can feed back to regulate metabolism (Module 2: Figure metabolic signalling). An example of how a change in metabolism can affect a signalling pathway has been described in cardiac myocytes, where the addition of pyruvate to increase metabolism has a marked effect on Ca^{2+} signalling (Module 2: Figure pyruvate and Ca^{2+} signalling).

AMP signalling pathway

Adenosine monophosphate (AMP)

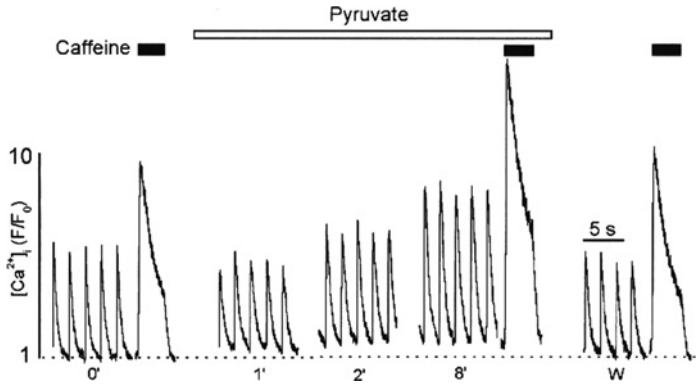
Cells have an AMP signalling pathway that is activated by an increase in the AMP/ATP ratio, which results in the activation of an AMP-activated protein kinase (AMPK). AMP thus functions as a second messenger, since it is responsible for activating the signalling pathway. The AMPK that responds to AMP has been referred to as the "fuel gauge" in that it responds to a decrease in the level of ATP. This signalling cascade is sensitive to many stimuli, such as cell stress, oxidative damage, hypoxia and glucose deprivation. Once activated, AMPK induces an up-regulation of ATP-generating systems (fatty acid oxidation, glycolysis and mitochondrial biogenesis) while simultaneously down-regulating processes that consume energy (fatty acid synthesis and gluconeogenesis). One of its important actions is to reduce protein synthesis when energy levels are low by regulating the activity of the target of rapamycin (TOR) (Module 9: Figure target of rapamycin signalling). Another of its actions is to regulate the transcription of

Module 2: | Figure metabolic signalling



Function of metabolic hormones in integrating the operation of cell metabolism and cell signalling pathways.
The signalling cascade begins with the arrival of an agonist (1) that recruits specific signalling pathways to activate a cellular response (2). Metabolism uses oxygen and substrates (3) to provide the energy (4) that not only powers the cellular responses (5), but also drives signalling pathways (6). The signalling pathways can also regulate the supply of energy by direct effects on metabolism (7). Superimposed on these more direct interactions, there are a variety of metabolic messengers that form a complex signalling network (8 and 9) that integrate the activity of both the signalling and metabolic cascades.

Module 2: | Figure pyruvate and Ca^{2+} signalling



The effect of pyruvate on Ca^{2+} signalling in cardiac myocytes.
The traces represent the Ca^{2+} spikes that are generated when isolated cardiac myocytes are electrically stimulated at 0.5 Hz. Under control conditions (0'), there are constant amplitude transients resulting from the release from the internal store. The size of this store is indicated by the amplitude of the much larger transient when caffeine (10 mM) is added. The next series of transients were taken 1, 2 and 8 min after addition of 10 mM pyruvate, which caused an increase in both the resting level and the transient amplitudes. This increase in the amplitude of the Ca^{2+} transients may have resulted from an increase in the content of the internal store, as indicated by the very large caffeine-induced transient apparent at the end of the 8-min sequence. The effect of pyruvate was reversible because the signalling system was back to its control values 4 min after washing out the pyruvate (W). A possible interpretation of this experiment is that pyruvate enhanced metabolism and the increase in ATP concentration may have enhanced the formation of cyclic ADP-ribose (cADPR) to stimulate the pump that transfers Ca^{2+} into the internal store (Module 2: Figure cADPR/NAADP function). Reproduced from Zima, A.V., Kockskämper, J., Meijia-Alvarez, R. and Blatter, L.A. (2003) Pyruvate modulates cardiac sarcoplasmic reticulum Ca^{2+} release in rats via mitochondria-dependent and -independent mechanisms. J. Physiol. 550:765-783, with permission from Blackwell Publishing; see Zima et al. 2003.

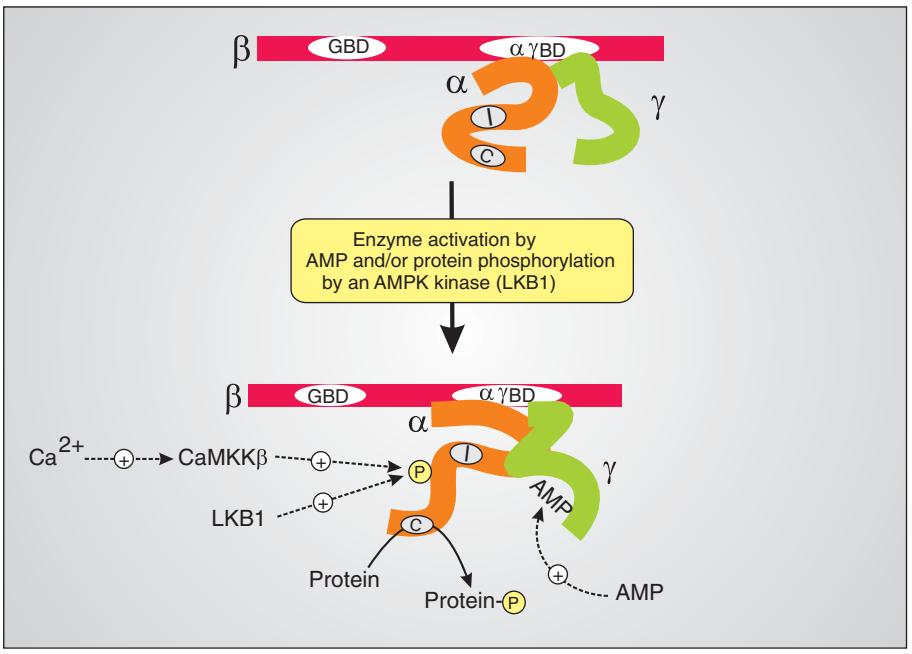
genes that function in the metabolism of glucose, fatty acids and cholesterol. The AMPK signalling pathway may also play a role in stimulating mitochondrial biosynthesis.

AMPK has also been implicated in cell growth control, where it functions to regulate the protein kinase TOR that controls protein synthesis.

AMP-activated protein kinase (AMPK)

AMP-activated protein kinase (AMPK) plays a critical role in regulating the usage of fuels such as glucose and fatty acids. However, it can have additional functions such as the regulation of insulin secretion by pancreatic β-cells, and may also play a role in controlling satiety centres in

Module 2: | Figure AMPK structure



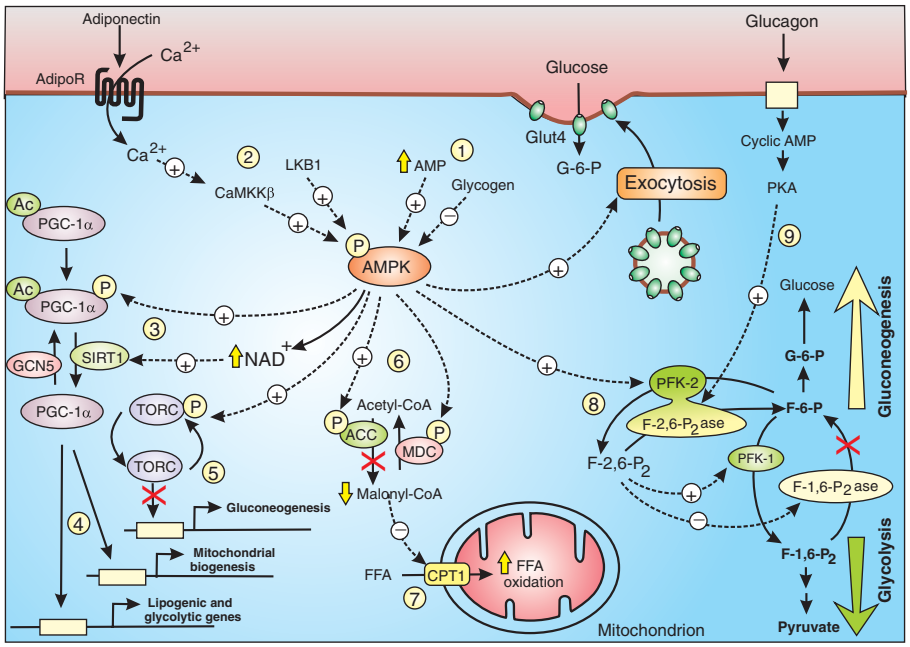
Structure and function of AMP-activated protein kinase (AMPK).
AMP-activated protein kinase (AMPK) is an αβγ heterotrimer. The β-subunit, which contains a C-terminal αγ-binding domain (αγBD), functions as a scaffold to organize the other two subunits. The α-subunit has an N-terminal catalytic domain (C), which is kept quiescent at rest by binding to an autoinhibitory domain (I). This α-subunit is activated by both AMP and by an AMPK kinase (AMPKK). The γ-subunit binds AMP and undergoes a conformation change that is transmitted to the α-subunit, causing the enzyme to open up so that its catalytic site can begin to phosphorylate its substrate proteins. Enzyme activity is also regulated by an AMPKK known as LKB1, which phosphorylates Thr-172. The β-subunit also contains a glycogen-binding domain (GBD), which enables AMPK to associate with glycogen that serves to inhibit the enzyme.

the hypothalamus. AMPK is a trimeric protein (Module 2: Figure AMPK structure) made up of multiple isoforms of a catalytic α subunit (α1 and α2) associated with β-subunits (β1 and β2) and γ-subunits (γ1–γ3). Cells express different combinations of these different isoforms.

AMPK carries out its function as a pleiotropic regulator of cell metabolism by regulating a large number of processes (Module 2: Figure AMPK control of metabolism):

1. One of the primary regulators of AMPK is AMP, which is elevated when the level of ATP falls. Glycogen can inhibit the activity of AMPK by interacting with the glycogen-binding domain (GBD) of the β subunit (Module 2: Figure AMPK structure).
2. Phosphorylation also plays a key role in regulating the activity of AMPK. There are two major AMPK kinases-LKB1 and Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ). The LKB1 is thought to be constitutively active, but there are indications that its activity might be regulated by acetylation. The activation through CaMKKβ may explain how adiponectin exerts its effects on metabolism. The adiponectin receptors (AdipoR1 and AdipoR2) are thought to act by promoting the entry of Ca^{2+} that then stimulates CaMKKβ to phosphorylate and activate AMPK (Module 2: Figure AMPK control of metabolism).
3. One of the important actions of AMPK is to regulate the activity of peroxisome-proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α), which is a key transcriptional regulator of genes involved in metabolism and particularly those that control mitochondrial biogenesis. PGC-1α activity is controlled both by its expression level and by post-translational mechanisms of which phosphorylation and acetylation are key processes. Phosphorylation of PGC-1α by AMPK acts to prime it for subsequent deacylation by the deacetylase SIRT1. AMPK also acts to increase the level of NAD^+, which enhances the the activity of SIRT1. The activity of PGC-1α is inhibited following its acetylation by the acetyltransferase GCN5.
4. One of the primary actions of PGC-1α is to stimulate the expression of genes that contribute to ATP generation, such as those that function in fatty acid oxidation, glycolysis and mitochondrial biogenesis.
5. AMPK phosphorylates TORC2 to prevent it from acting as a cofactor to activate genes responsible for gluconeogenesis. For example, in the case of liver cells, AMPK can phosphorylate transducer of regulated cyclic AMP response element-binding protein 2 (TORC2), which is then prevented from acting as a cofactor for the cyclic AMP response element-binding protein (CREB) transcription factor, which can activate genes responsible for gluconeogenesis (Module 7: Figure liver cell signalling). AMPK can also inhibit the activity of various transcription factors such as the sterol regulatory element-binding protein 1c (SREBp1c) and hepatocyte nuclear factor 4α (HNF4α), which regulate lipogenic and glycolytic genes respectively.

Module 2: | Figure AMPK control of metabolism



The pleiotropic action of AMP-activated protein kinase (AMPK) on cell metabolism.
An increase in the level of AMP during metabolic stress activates AMP-activated protein kinase (AMPK), which then has a number of actions, as outlined in the text.

6. Lipid metabolism is strongly influenced by AMPK, which acts to divert fatty acids away from lipid synthesis and directs them towards the mitochondrial oxidative pathway to produce ATP. The AMPK-dependent phosphorylation of acetyl-CoA carboxylase (ACC) reduces the conversion of acetyl-CoA into malonyl-CoA.
7. The fall in malonyl-CoA levels has two import consequences. First, fatty acid synthesis is reduced because malonyl-CoA is an important precursor for lipid synthesis. Secondly, there is an increase in mitochondrial ATP formation because malonyl-CoA normally inhibits fatty acid oxidation.
8. AMPK can influence the balance between glycolysis and gluconeogenesis by stimulating the formation of fructose 2,6-bisphosphate (F-2,6-P_2), which is a potent regulator of glycolysis through its ability to activate 6-phosphofructo-1-kinase (PFK-1) and to inhibit fructose-1,6-bisphosphate 1-phosphatase. The formation of F-2,6-P_2 is regulated by two separate signalling pathways. AMPK promotes glycolysis by stimulating the phosphofructokinase (PFK-2) of the bifunctional enzyme, which has both kinase and phosphatase activities. Cyclic AMP acting through the fructose-2,6-bisphosphate 2-phosphatase component lowers the level of F-2,6-P_2, which reduces glycolysis and promotes gluconeogenesis.
9. AMPK stimulates the translocation of the glucose transporter (GLUT4) to the plasma membrane, where it facilitates the entry of glucose in skeletal muscle and heart cells.

In summary, AMPK switches the cell away from energy-requiring processes towards energy conservation. It does this by exerting rapid effects on processes such as glucose entry and glycolysis, as well as longer-term effects, by regulating the transcription of genes for mitochondrial biogensis and glycolytic and lipogenic hormones (Module 2: Figure AMPK control of metabolism). The AMPK signalling pathway functions in many different cell types:

- In liver cells, AMPK inhibits gluconeogensis by reducing the activity of PGC-1α (Module 7: Figure liver cell signalling)
- AMPK regulates insulin biosynthesis in insulin-secreting β1 cells (Module 7: Figure β-cell signalling)
- O_2-sensing by the glomus cells in the carotid body (Module 10: Figure carotid body chemoreception)
- Control of Glut4 insertion during excitation–metabolism coupling in skeletal muscle (see step 10 in Module 7: Figure skeletal muscle E-C coupling)
- AMPK plays an important role in cell growth control by reducing protein synthesis when energy levels are low by acting through TOR (Module 9: Figure target of rapamycin signalling).
- The AMP signalling pathway plays an important role in the control of autophagy (Module 11: Figure autophagy).

The fact that AMPK plays such a central role in regulating energy metabolism has important implications for diabetes.

Glycogen storage disease in humans is caused by a mutation of the AMPK γ3-subunit.

LKB1

LKB1 is a serine/threonine protein kinase that functions to activate AMP-activated protein kinase (AMPK) by

phosphorylating Thr-172 on the α-subunit (Module 2: Figure AMPK structure). The activation of AMPK is very dependent upon LKB1. Inactivation of the latter is responsible for Peutz-Jeghers syndrome. As such, LKB1 is considered to be one of the tumour suppressors.

Adenosine triphosphate (ATP)

ATP is an important metabolic messenger in that it has a number of different actions both as an internal and an external signal (Module 2: Figure metabolic messengers). With regard to the former, one of its main functions is to regulate the activity of the ATP-sensitive K^+ (K_{ATP}) channel, which is particularly important in regulating the release of insulin (Module 7: Figure β-cell signalling). It also plays a role in regulating the activity of the ADP-ribosyl cyclase that generates the Ca^{2+}-mobilizing messengers cyclic ADP-ribose (cADPR) and nicotinic acid–dinucleotide phosphate (NAADP) (Module 2: Figure cADPR/NAADP function). ATP is also released from the cell and can activate P2X receptors that activate Ca^{2+} entry or P2Y receptors that are coupled to phospholipase C (PLC) to generate inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) (Module 2: Figure metabolic messengers).

Bicarbonate (HCO_3^-)

The CO_2 produced during cellular metabolism is rapidly converted into bicarbonate (HCO_3^-), which acts as a messenger to report the current state of metabolism. The HCO_3^- activates soluble adenylyl cyclase (Module 2: Figure cyclic AMP signalling) to generate cyclic AMP that has many signalling functions, including an effect on cellular metabolism through its activation of glycogen metabolism.

Fatty acids

Fatty acids come in two main forms: saturated and polyunsaturated. There are two main types of polyunsaturated fatty acid: omega-6 and omega-3 fatty acids. These are referred to as essential fatty acids since they are not made by the body but are components of the diet: omega-6 fatty acids are derived mainly from vegetable oils, whereas the omega-3 fatty acids are found in certain plants (linolenic acid) or from marine fish and shellfish (eicosapentanoic acid and docosahexanoic acid). Fatty acids not only provide an important energy source but can also be considered as metabolic messengers that contribute to a number of metabolic control mechanisms. For example, they control gluconeogenesis in liver cells by activating the peroxisome-proliferator-activated receptor α (PPARα) (Module 7: Figure liver cell signalling). Free fatty acids are also responsible for activating the uncoupling protein 1 (UCP1) that provides the proton leak during noradrenaline-induced heat production by brown fat cell mitochondria (Module 7: Figure brown fat cell). The omega-3 fatty acids have a number of specific signalling functions.

Arachidonic acid (AA) and other polyunsaturated fatty acids (PUFAs) such as docosahexenoic acid, linolenic and linoleic acid can activate the TREK subfamily of two-pore domain K^+ (K_{2P}) channels.

The positive-feedback loop that free fatty acids (FFAs) exert on the process of lipogenesis (i.e. Step 7 in Module 7: Figure metabolic energy network) may exacerbate the onset of obesity by enhancing fat storage.

The build-up of FFAs that occur during obesity play a critical role in inducing the insulin resistance (Module 12: Figure insulin resistance) that leads to the development of diabetes.

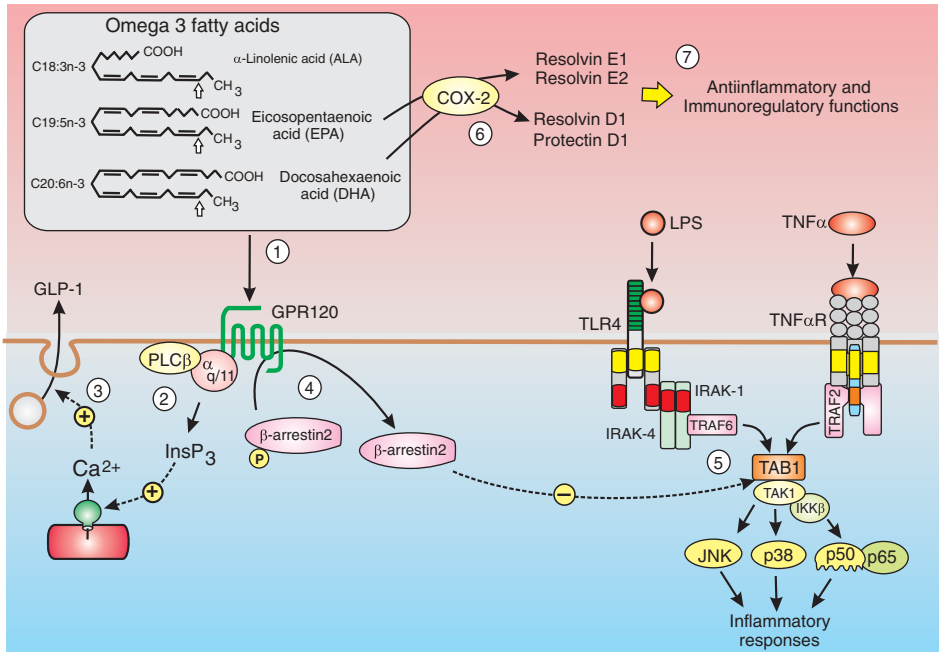
Omega 3 fatty acids

The omega-3 fatty acids such as α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are typical polyunsaturated fatty acids (PUFAs). These PUFAs are essential for normal cellular function. ALA and α-linolenic acid, which are the precursors of arachidonic acid and DHA respectively, are not synthesized *de novo* but are obtained from the diet. Arachidonic acid is the precursor of the endocannabinoids, such as anandamide and 2-arachidonoylglycerol (2-AG), which have important signalling functions (Module 1: Figure anandamide).

The first double bond of these omega-3 fatty acids is located at the third carbon atom from the methyl (CH_3) end of the molecule (see white arrow in Module 2: Figure omega-3 fatty acids). These omega-3 fatty acids have a number of specific signalling functions as outlined in Module 2: Figure omega-3 fatty acids:

1. When GPR120 was first identified, it was classed as an orphan receptor as there was no known ligand. Subsequently, the omega-3 fatty acids were identified as stimuli for this typical G-protein-coupled receptor (GPCR).
2. GPR120 is coupled through $G_{q/11}$ and phospholipase C to activate the Inositol 1,4,5-trisphosphate ($InsP_3$) and Ca^{2+} release signalling pathway.
3. One of the functions of the increase in Ca^{2+} might be to stimulate the release of glucagon-like peptide 1 (GLP-1).
4. Another function of the GPR120 receptors is to activate the multifunctional adaptor protein arrestin-2.
5. The anti-inflammatory responses of omega-3 fatty acids might be carried out by arrestin-2 inactivating the TAK1-binding protein 1 (TAB1), which can inhibit the signalling pathways used by the Toll receptor signalling pathway (Module 2: Figure Toll receptor signalling) or by the tumour necrosis factor α (TNFα) signalling pathway (Module 2: Figure NF-κB activation).
6. Apart from the intracellular signalling pathways described above, the omega-3 fatty acids are metabolized by cyclooxygenase 2 (COX2) to form the resolvins and the protectins, which are potent anti-inflammatory and immunoregulatory agents.
7. The anti-inflammatory action of the omega-3 fatty acids might have beneficial effects for coronary artery disease, cardiac disease and for the insulin resistance that results in metabolic syndrome.

Module 2: | Figure omega-3 fatty acids



Omega 3 fatty acid function.

The polyunsaturated fatty acids (PUFAs) have a number of actions. They act through GPR120 receptors to stimulate $InsP_3/Ca^{2+}$ signalling and they also activate arrestin-2 that can inhibit the inflammatory responses initiated by toll-like receptor 4 (TLR4) or by tumour necrosis factor α receptor (TNFαR). They can also be converted by cyclooxygenase 2 (COX2) into the resolvins and protectins, which have anti-inflammatory and immunoregulatory actions.

A decrease in dietary omega-3 fatty acids results in a decline in the ability of anandamide to activate long-term depression (LTD) in neurons and this might be the cause of various neuropsychiatric diseases such as depression.

NAD signalling pathways

As the name implies, the NAD signalling pathways encompass signalling systems that depend on nicotinamide–adenine dinucleotide (NAD^+) and nicotinamide–adenine dinucleotide phosphate ($NADP^+$). These two metabolic cofactors exist in the cell as NAD^+/NADH and $NADP^+$/NADPH redox couples and have a number of important functions both as messengers and as precursors of other metabolic messengers.

NAD and NADP as metabolic messengers

- NAD^+ functions to regulate a number of cellular processes including energy metabolism, gene transcription, DNA repair and perhaps ageing as well.
- NADH activates the C-terminal binding protein (CtBP), which is a transcriptional corepressor that functions during growth and development.
- NADH and NADPH regulate the transcription factors Clock/BMAL1 and NPAS2/BMAL1 that control gene expression during the operation of the circadian clock.
- NADPH appears to regulate the ADP-ribosyl cyclase that is responsible for producing both cyclic ADP-ribose (cADPR) and nicotinic acid–adenine dinucleotide phosphate (NAADP) (Module 2: Figure cADPR/NAADP function).

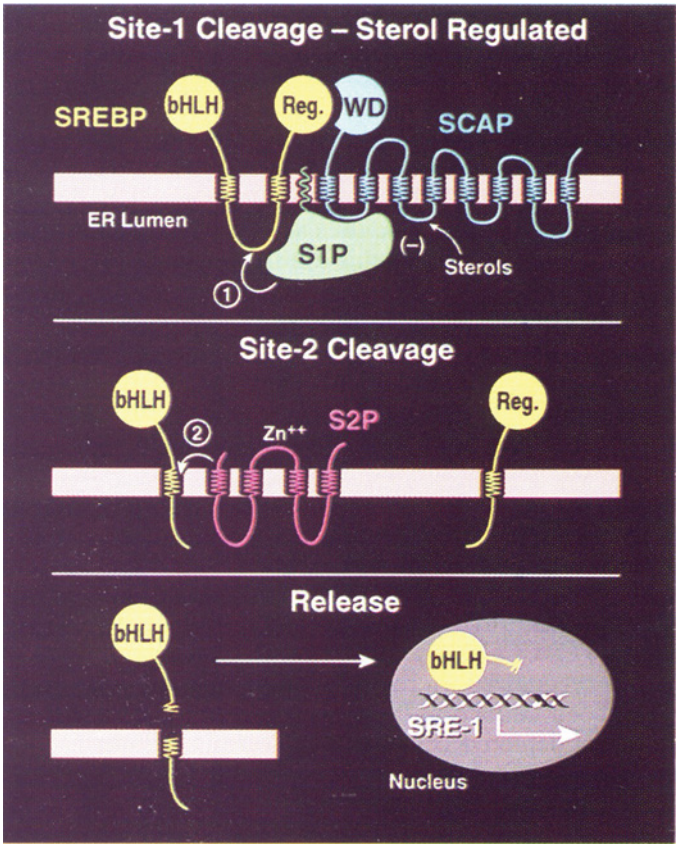
NAD and NADP as precursors of metabolic messengers

- NAD^+ is a precursor of cyclic ADP-ribose (cADPR) signalling pathway that functions in Ca^{2+} signalling.
- Metabolism of cADPR generates ADPR, which regulates transient receptor potential melastatin 2 (TRPM2), a member of the transient receptor potential (TRP) ion channel family that controls the entry of external Ca^{2+} (Module 2: Figure cADPR/NAADP function).
- $NADP^+$ is a precursor of nicotinic acid–adenine dinucleotide phosphate (NAADP) signalling pathway that functions in Ca^{2+} signalling.
- NADPH is the substrate used to generate reactive oxygen species (ROS), which function as second messengers to regulate a number of cellular proteins (Module 2: Figure plasma membrane ROS formation).

Sterol sensing and cholesterol biosynthesis

The level of cholesterol in cell membranes is regulated by a signalling system that can sense the level of sterols and then relay information to the nucleus to adjust the transcription of the genes responsible for cholesterol biosynthesis. The signalling system is based on membrane-bound transcription factors: the sterol regulatory element-binding proteins (SREBPs), which are integral membrane proteins located in the endoplasmic reticulum (ER). They have two membrane-spanning domains with the free ends projecting into the cytosol. The N-terminal region is the latent transcriptional regulator, which is cleaved by a sterol-regulated system of proteases (Module 2: Figure sterol sensing). Once released into the cytosol, these

Module 2: | Figure sterol sensing



Sterol signalling at the endoplasmic reticulum (ER) membrane.
Sterols bind to the sterol regulatory element-binding proteins (SREBP) cleavage-activating protein (SCAP) resulting in the activation of site-1 protease (S1P), which then cleaves the SREBPs at a point on the luminal loop. A site-2 protease (S2P) then hydrolyses the N-terminal bHLH (basic helix–loop–helix) fragment to release the cytosolic portion, which then diffuses into the nucleus to initiate gene transcription. Reproduced from Brown, M.S. and Goldstein, J.L. (1999) A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc. Natl. Acad. Sci. U.S.A. 96:11041–11048. Copyright (1999) National Academy of Sciences, U.S.A.; see Brown and Goldstein 1999).

transcription factors dimerize through a basic loop–helix–leucine zipper and enter the nucleus, where they bind to sterol regulatory elements on the genes that code for the enzymes of lipid biosynthesis (cholesterol, unsaturated fatty acids and triacylglycerols) and lipid-uptake mechanisms. The sterol sensor is a SREBP cleavage-activating protein (SCAP). When sterols build up in the membranes, they bind to SCAP to inhibit the proteolytic cleavage of the SREBPs. The ER thus plays a central role both in sensing the level of sterols and then providing the output signals that make the necessary adjustments to lipid synthesis to maintain a constant level of membrane cholesterol.

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