Integration of interferon-α/β signalling to p53 responses in tumour suppression and antiviral defence

Akinori Takaoka[1], Sumio Hayakawa[1], Hideyuki Yanai[1], Dagmar Stoiber[1]*, Hideo Negishi[1], Hideaki Kikuchi[1], Shigeru Sasaki[2], Kohzoh Imai[2], Tsukasa Shibue[1], Kenya Honda[1] & Tadatsugu Taniguchi[1]

[1]*Department of Immunology, Faculty of Medicine and Graduate School of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan*
[2]*First Department of Internal Medicine, Sapporo Medical University School of Medicine, South 1, West 17, Chuo-ku, Sapporo 060-8556, Japan*

* Present address: Institute of Pharmacology, University of Vienna, Waehringerstrasse 13a, A-1090 Vienna, Austria

Swift elimination of undesirable cells is an important feature in tumour suppression and immunity. The tumour suppressor p53 and interferon-α and -β (IFN-α/β) are essential for the induction of apoptosis in cancerous cells and in antiviral immune responses, respectively, but little is known about their interrelationship. Here we show that transcription of the *p53* gene is induced by IFN-α/β, accompanied by an increase in p53 protein level. IFN-α/β signalling itself does not activate p53; rather, it contributes to boosting p53 responses to stress signals. We show examples in which *p53* gene induction by IFN-α/β contributes to tumour suppression. Furthermore, we show that p53 is activated in virally infected cells to evoke an apoptotic response and that p53 is critical for antiviral defence of the host. Our study reveals a hitherto unrecognized link between p53 and IFN-α/β in tumour suppression and antiviral immunity, which may have therapeutic implications.

The tumour suppressor p53, activated in response to DNA damage, induces cell cycle arrest or apoptosis through transcriptional activation of its target genes, hence having a central role in tumour suppression[1–5]. So far, it is not known whether p53 contributes to the immune responses that lead to the eradication of pathogens such as viruses. On the other hand, IFN-α/β, both of which are essential cytokines for antiviral immunity, are sometimes referred to as 'negative growth factors', and manifest anti-oncogenic activities[6–10]. In fact, IFN-α/β are used for the treatment of some forms of human cancer but the molecular basis for the treatment is poorly understood[11–13]. Until know, little if anything has been known about the link between the p53 and IFN-α/β system.

Induction of p53 protein by IFN-α/β

When wild-type mouse embryonic fibroblasts (MEFs) were stimulated with IFN-β, a notable increase in the level of p53 was observed (Fig. 1a). A similar observation was made in cells stimulated with IFN-α (data not shown) but not with IFN-γ (Supplementary Fig. 1). The increase in p53 level with IFN-β treatment was dose-dependent, with about fourfold induction achieved at a high concentration of IFN-β (Fig. 1b). The p53 induction by IFN-β was also observed in two human hepatic cancer cell lines, HepG2 and HLE, p53 function being abrogated in the latter cells by a mutation in the DNA-binding domain[14] (Fig. 1c). We performed a pulse–chase experiment to examine whether the observed p53 protein induction is secondary to suppression of the p53 degradation pathway by IFN-β, typically the MDM2-mediated pathway[15–17]. However, no difference was observed in the half-life of p53 (40–45 min) between the IFN-treated and untreated MEFs (Fig. 1d), suggesting that p53 protein synthesis is induced by IFN-β stimulation.

Induction of the *p53* gene by IFN-α/β

Information is limited about the induction of the *p53* gene[18], and the above results prompted us to examine whether IFN-α/β induces *p53* gene transcription. Inspection of mouse and human *p53* genes has revealed sequences characteristic of the interferon-stimulated response element (ISRE) within their promoter or first-intron regions (Fig. 2a); ISRE is activated by the IFN-activated transcription factor ISGF3, a heterotrimeric complex consisting of a signal transducer and activator of transcription factor 1 (Stat1), Stat2 and interferon regulatory factor 9 (IRF-9)[19–21]. As shown in Fig. 2b (left panel), p53 messenger RNA is induced by IFN-β in wild-type MEFs by about threefold; however, this induction was not observed in MEFs from mice deficient in the *Irf9* gene (IRF-9$^{-/-}$ MEFs). Consistently, p53 protein induction was also abolished in IRF-9$^{-/-}$ MEFs (Fig. 2b, right panel). Similar mRNA induction was observed in IFN-α-stimulated wild-type MEFs (data not shown) as well as IFN-β-stimulated HepG2 cells (Supplementary Fig. 2a). These results suggest that p53 mRNA induction by IFN-β is caused by the transcriptional activation of the gene by ISGF3.

Activation of the *p53*-derived ISREs by ISGF3

Of note, *p53* gene induction by IFN-β is not as strong as that of conventional IFN-inducible genes such as 2′-5′ oligoadenylate synthetase (*OAS*). In MEFs, the induction of p53 and OAS mRNAs are about threefold and eightfold, respectively (Fig. 2b; see below). This difference may be due to the lower affinities of the *p53* ISREs (denoted as p53-ISRE1 and p53-ISRE2 in Fig. 2a) for ISGF3. To verify this point, we compared the affinities of the p53-ISREs by electrophoretic mobility shift assay (EMSA) using *OAS* ISRE as the probe for ISGF3. As shown in Fig. 2c, approximately eightfold higher concentration of the p53-ISREs relative to that of the *OAS* ISRE is required to inhibit *OAS* ISRE–ISGF3 complex formation by 50%. Similar observations were made for the two human *p53* ISREs (h-p53-ISRE1 and h-p53-ISRE2; Fig. 2a, see also Supplementary Fig. 2b).

Consistently, a transient reporter gene assay revealed that the mouse p53-ISREs are activated by IFN-β, but they need to be multimerized five times to achieve an induction level of the reporter gene similar to that achieved by the monomeric ISRE of the *OAS* gene (Fig. 2d). To examine ISGF3 binding to the *p53* ISRE sequences *in vivo*, we performed chromatin immunoprecipitation (ChIP) assay by treating wild-type MEFs with IFN-β, followed by immunoprecipitation with an anti-Stat2 antibody. As shown in Fig. 2e, DNA

bands diagnostic to p53-ISRE1 and p53-ISRE2 were detected in the cells stimulated with IFN-β. These results *in toto* indicate that IFN-β induces *p53* gene transcription through the activation of ISGF3, which binds to the *p53* ISREs.

Suppression of oncogene-induced cell transformation

The above results prompted us to examine whether IFN-β induces activation of p53. However, no evidence was observed for the serine phosphorylation of p53 in the IFN-β-stimulated MEFs (see Fig. 4a, left panel), and the target genes of p53 were not induced in these cells (Supplementary Fig. 3). This is perhaps not surprising in view of the fact that IFN-β does not evoke p53 responses in normally growing cells. Hence, the p53 induction by IFN-β may be considered as a means to boost a p53 response to stress signals, suggesting a role for IFN-β induction of p53 in tumour suppression.

To address this issue, we first examined the effect of IFN-β in cells in which p53 is under negative regulation by an oncogene. One of the best-known examples is the *E6* gene of the human papilloma viruses (HPV), the product of which targets p53 by inducing its degradation by the ubiquitin proteolytic pathway[22]. Persistent infection with oncogenic HPV types represents a major risk factor for the development of cervical cancer, in which E6 is known to have a critical role (together with another oncoprotein, E7 (ref. 23)). When HPV16 E6 is expressed in MEFs, a notable decrease in the basal p53 protein level is observed, but this is countered by IFN-β (Fig. 3a, left panel). Interestingly, the induction of p53 mRNA and protein continues to increase in these cells at least up to 9 h after stimulation (Fig. 3a). This is not due to the IFN action on the E6-mediated degradation of p53 (Supplementary Fig. 4a). The mechanism of this sustained induction is therefore currently unknown but, interestingly, a very similar observation was made with HeLa cells, which are derived from human cervical cancer and express the E6 protein (Supplementary Fig. 4b).

Primary MEFs do not become transformed when HPV E6 is expressed alone; however, they do so when an additional oncogene such as activated Ha-Ras is co-expressed. As shown in Fig. 3b, the numbers of transformed colonies in MEFs expressing E6 and Ha-Ras are markedly suppressed by IFN-β in a dose-dependent manner (about 80% inhibition at a concentration of 10^3 U ml^{-1}). In these cells, p53 mRNA is also IFN-β-inducible, accompanied by enhanced phosphorylation of p53 (Ser 18) (Supplementary Fig. 5a, b). On the other hand, the expression of the mRNAs for E6 and mTERT, a telomerase subunit known to be E6-inducible[24], remained unaffected by IFN-β (Supplementary Fig. 6). Of note,

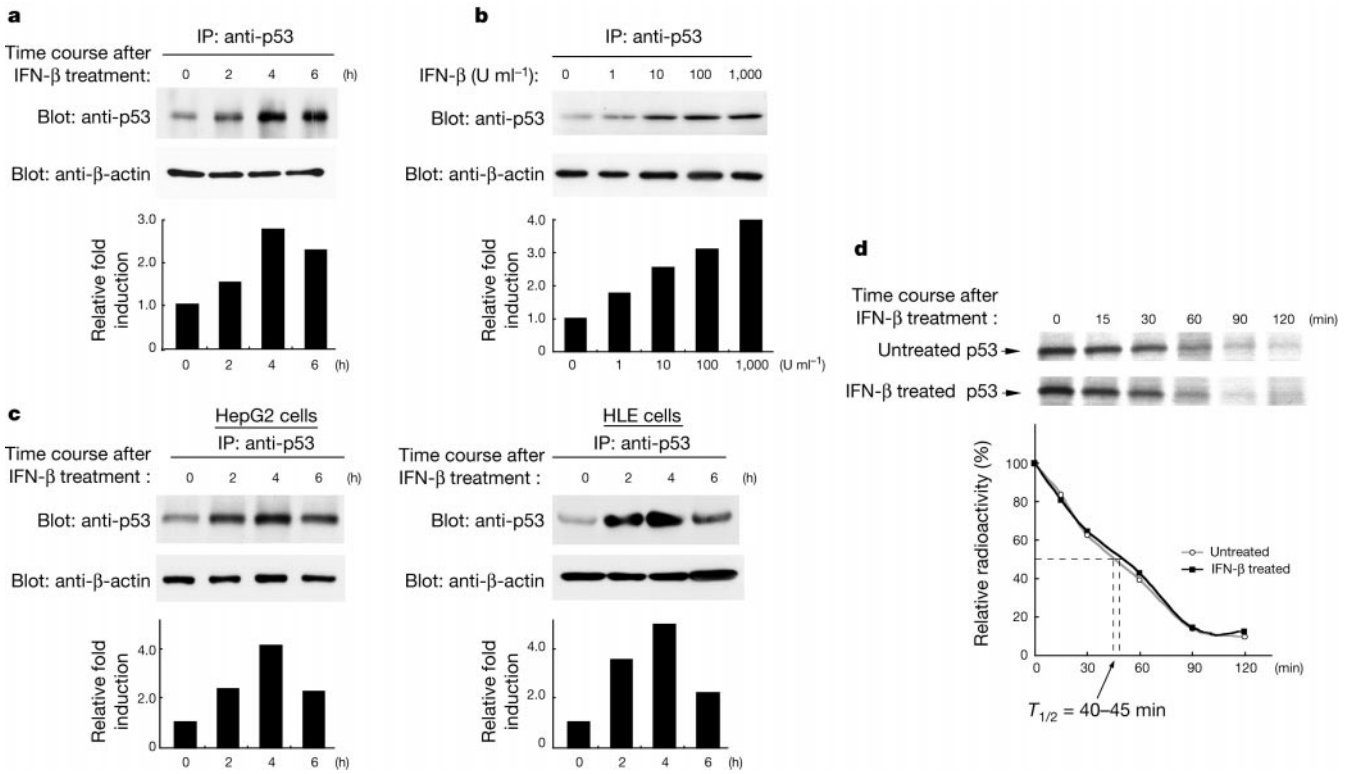

Figure 1 Induction of p53 protein by IFN-β. **a**, MEFs were treated with mouse IFN-β and cell lysates were immunoprecipitated (IP) with anti-p53 antibody. Immunoprecipitates were separated by 9% SDS–PAGE, and immunoblotting analysis (blot) was carried out with the anti-p53 antibody (anti-p53). The supernatants after anti-p53 immunoprecipitation were directly subjected to immunoblotting with the anti-β-actin antibody as the control. We chose this method, as western blotting of the whole-cell lysates gives a high background, but a consistent result was obtained with this approach as well (see Supplementary Fig. 10). The lower panel shows the quantitative display of the induction levels of p53 protein, which were standardized to the intensities of each blot by anti-β-actin, as determined using a densitometer. These and the following experiments were repeated at least three times and the results were highly reproducible. **b**, Dose-dependent induction of p53 by IFN-β. MEFs were treated with various concentrations of IFN-β, and cell lysates were subjected to immunoprecipitation and immunoblot analyses as described above. Results of the quantitative analysis for the induction levels of the p53 protein are shown in the lower panel. **c**, Induction of p53 protein in human hepatocelluar carcinoma cell lines. Analysis was performed as described in **a**, except that the cells were stimulated with human IFN-β. **d**, Effect of IFN-β on degradation of p53 protein. After pulse labelling with [^{35}S]methionine and [^{35}S]cysteine, MEFs were treated with or without IFN-β. Cell lysates were prepared at the indicated times and subjected to immunoprecipitation with the anti-p53 antibody and analysed by SDS–PAGE. The incorporated radioactivity was measured by BAS5000 (Fujix) and shown in the lower panel. IFN-β did not affect the degradation of p53 protein in human HepG2 cells (data not shown).

such suppression was not observed in the MEFs from *p53*-deficient mice ($p53^{-/-}$ MEFs[25]), which undergo transformation as induced by Ha-Ras alone (Fig. 3b). Thus, IFN-β exerts a strong anti-oncogenic activity in E6-dependent cell transformation (Fig. 3b), and this is probably mediated by sustaining p53 expression through transcriptional induction of the gene.

We also examined the effect of IFN-β on the DNA damage-induced apoptotic response of MEFs expressing the adenovirus E1A oncoprotein, as it is well known that this response is p53-dependent and critical for tumour suppression[26]. Binding of annexin V–fluorescein isothiocyanate (FITC; annexin V staining) and cell viability assay revealed that pretreatment of the MEFs with IFN-β resulted in a notable enhancement of apoptosis in response to X-ray irradiation (Fig. 3c, and data not shown). Congruent with this observation is the finding that p53 with serine phosphorylation was increased by IFN-β treatment in E1A-expressing MEFs (Supplementary Fig. 7).

Enhancement of cancer cell apoptosis by IFN

The induction of p53 by IFN-α/β suggests that IFN-treated cells are more susceptible to p53-dependent apoptosis in response to DNA-damaging agents such as chemotherapeutic agents used in

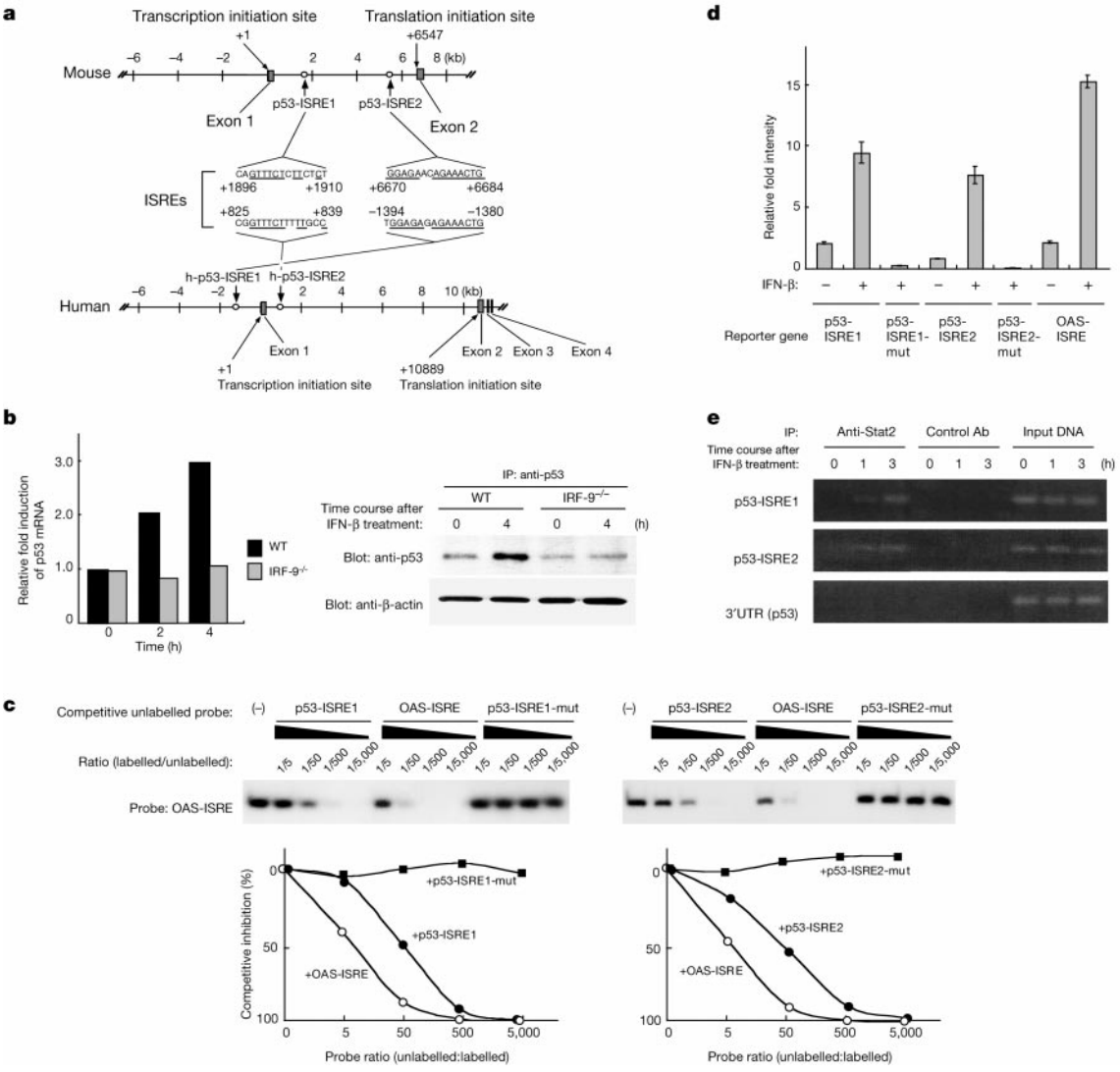

Figure 2 Transcriptional induction of *p53* gene by IFN-β. **a**, Putative ISREs in proximal promoter regions of the murine and human *p53* genes are shown. **b**, Wild-type (WT) or IRF-$9^{-/-}$ MEFs were stimulated with IFN-β for the indicated periods, and the induction of p53 mRNA levels was analysed by quantitative real-time RT–PCR (left). Four hours after IFN-β treatment, cell lysates were also extracted, and p53 protein level was evaluated by immunoprecipitation and immunoblotting analysis with the anti-p53 antibody and anti-β-actin antibody (right). **c**, Lysates from the MEFs stimulated by IFN-β were prepared and subjected to EMSA. Competition assay was carried out with an excessive amount of unlabelled p53-ISRE probes. The ratios of the amount of radiolabelled to unlabelled probes are indicated above each lane. OAS-ISRE is an ISRE probe derived from the *OAS* gene. mut-p53-ISRE1 or mut-p53-ISRE2 is a probe with mutations in the consensus sequence (see Methods). **d**, Transcriptional activation by IFN-β through the *p53*-gene-derived ISREs. After transfection with the luciferase reporter plasmids, p53-ISRE1-luc or p53-ISRE2-luc, MEFs were stimulated with IFN-β, and induction level of luciferase activity with or without IFN-β stimulation was subsequently measured. pOAS-ISRE-luc was used as a positive control for ISGF3-dependent activation. **e**, Chromatin immunoprecipitation assay results. ISGF3 binding to the endogenous ISREs in the *p53* gene was examined using MEFs stimulated with IFN-β. PCR was carried out to detect endogenous ISREs in the gene (p53-ISRE1 and p53-ISRE2) in immunoprecipitated chromatin fragments: Lanes 1, 2 and 3 show PCR amplification of target sequences in immunoprecipitated chromatin fragments with the anti-Stat2 antibody. Lanes 4, 5 and 6 show the results of PCR using immunoprecipitated samples with anti-control antibody (Ab). Input (lanes 7, 8 and 9) represents PCR amplification of the total input DNA. The results of PCR with primers that detect the 3′-UTR of the *p53* gene are shown in the bottom panel. The results were highly reproducible.

cancer therapy. 5-Fluorouracil (5-FU) is one agent whose efficacy closely correlates with the p53 status of cancer cells[27]. In view of the induction of p53 by IFN-β in human cancer cell lines HepG2 and HLE (Fig. 1c), we examined whether IFN-β enhances the apoptotic response in these cells. As shown in Fig. 3d, loss of viability of HepG2 cells was significantly enhanced by IFN-β in a dose-dependent manner at a concentration of 5-FU that itself has only a slight effect on the cell. In contrast, such an effect was not seen in HLE cells, in which p53 is functionally inactive[14]. These results can be interpreted to mean that the IFN-β-induced p53 apoptotic response comes into action in these cells; a severalfold increase in p53 level would make HepG2 cells sensitive enough to respond to sub-optimal doses of 5-FU.

It is known that HeLa cells undergo apoptosis in response to DNA damage in a p53-dependent manner[28,29]. In view of our finding that p53 is also induced by IFN-β in this cell line, we asked whether the apoptotic response is enhanced by IFN-β. As shown in Fig. 3e, significant enhancement was observed in X-ray irradiation-induced apoptosis.

Activation of p53 in virally infected cells

It has been reported that p53 function and IFN signalling are

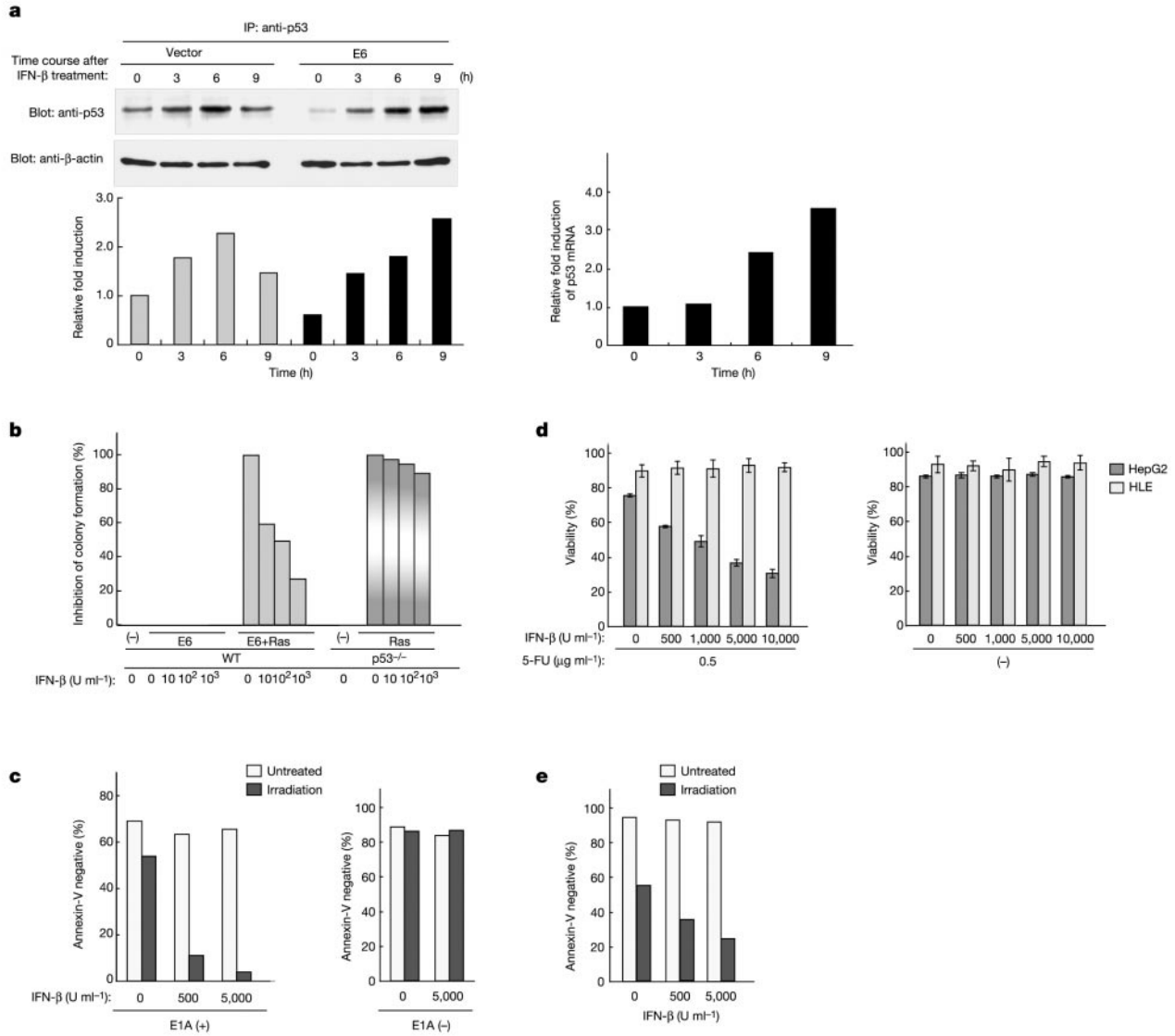

Figure 3 Suppression of oncogene-induced cell transformation and enhancement of oncogene-dependent apoptosis by IFN-β. **a**, Sustained upregulation of p53 protein level by IFN-β in MEFs expressing type 16 HPV-derived E6 oncoprotein. MEFs were transfected with pLRHL (vector) or pLRHL-16E6 (E6) via retroviral gene transfer, and after IFN-β treatment the induction level of the p53 protein or mRNA at each time point was analysed by immunoprecipitation and immunoblotting analysis (left) or quantitative real-time RT–PCR (right). **b**, Suppression of oncogene-induced colony formation by IFN-β. Wild-type MEFs, retrovirally transduced with only HPV16 E6 protein or both E6 and activated Ha-Ras, or $p53^{-/-}$ MEFs expressing activated Ha-Ras, were seeded on soft agar in 60-mm dishes in the absence or presence of IFN-β, and were incubated for three weeks. The medium with the same IFN-β concentration was changed every three days. The histogram represents the relative number of colonies (%) compared with that of colonies cultured in the absence of IFN-β. Results are a representative of two independent experiments. **c**, Enhancement of DNA-damage-induced apoptosis in E1A-expressing MEFs after pretreatment with IFN-β (left). The percentage of annexin V–FITC-bound cells was determined 40 h after X-ray irradiation. Radiation-induced apoptosis and its enhancement by IFN-β were not observed in MEFs that do not express E1A (right). **d**, HepG2 or HLE cells were incubated with 5-FU (0.5 μg ml^{-1}) alone or in combination with IFN-β. All cells were collected 72 h after the addition of 5-FU, and viable cells were counted by trypan blue exclusion staining. The proportion of cells incubated without 5-FU was defined as 100% viability. **e**, Enhancement of radiation-induced apoptosis in HeLa cells after the pretreatment with IFN-β. The percentage of annexin V–FITC-bound cells was determined 40 h after X-ray irradiation.

inhibited by products of oncogenic viruses[23,30–34]; however, it is unknown whether p53 itself has any role in the antiviral defence mechanism. Previous studies have shown that *p53*-deficient mice show no sign of abnormality in T and B cells, and that they can mount adaptive immune responses normally[35]. In view of the newly identified connection between p53 and IFN-α/β, we investigated whether p53 has a role in antiviral responses.

When MEFs were infected with vesicular stomatitis virus (VSV), marked phosphorylation of p53 was detected by an antibody against Ser 18 (Fig. 4a, left panel). This p53 phosphorylation was also observed in MEFs infected with other types of virus, such as Newcastle disease virus (NDV) and herpes simplex virus (HSV) (Fig. 4a, right panel). Furthermore, the same observation—that is, phosphorylation of Ser 15 of human p53 (ref. 36)—was made in the VSV-infected HepG2 cells (Fig. 4b).

The ATM kinase is known to be involved in this phosphorylation in response to DNA damage[37,38]. We therefore asked whether the virus-induced phosphorylation of Ser 18 is also ATM-dependent in the following experimental setting. *p53*-deficient and *p53/Atm* doubly deficient MEFs[39] were infected with a p53-expressing retrovirus, and the phosphorylation of the expressed p53 in the presence or absence of ATM was examined after infection by VSV or HSV. Perhaps surprisingly, as shown in Fig. 4c, phosphorylation of p53 at Ser 18 by VSV or HSV was hardly detectable in MEFs deficient in the *Atm* gene, suggesting an integral role of ATM in the virus-induced phosphorylation of p53. We infer that, similar to DNA damage, ATM activation is caused by changes in the chromatin structure in virus-infected cells[40].

Induction of p53 target genes by virus

It was difficult to examine critically the serine phosphorylation at other sites, because the available antibodies did not react well even with the p53 from irradiated MEFs. Therefore, we examined the induction of p53 target genes in MEFs after VSV infection, to test whether virus infection results in p53 activation. RNA blotting analysis revealed that mRNAs for *Mdm2* and *Puma* are clearly inducible by VSV: the mRNA induction begins 8 h and peaks 12 h after infection (Fig. 4d, left panel). Interestingly, mRNAs for $p21^{WAF1/Cip1}$ and *Noxa*, which are both induced in response to DNA damage (Fig. 4d, right panel; see also ref. 41) are not induced by the virus. These results suggest that p53 is indeed in its active form in VSV-infected cells, but that its activation profile of target genes is distinct from that induced by DNA-damaging agents. Although the mechanism of this differential activation of p53-inducible genes is currently unknown, it is interesting that $p21^{WAF1/Cip1}$, the induction of which acts in favour of cell survival (ref. 42 and T. S., unpublished data), is not virus-inducible—the absence of $p21^{WAF1/Cip1}$ induction would favour p53-mediated

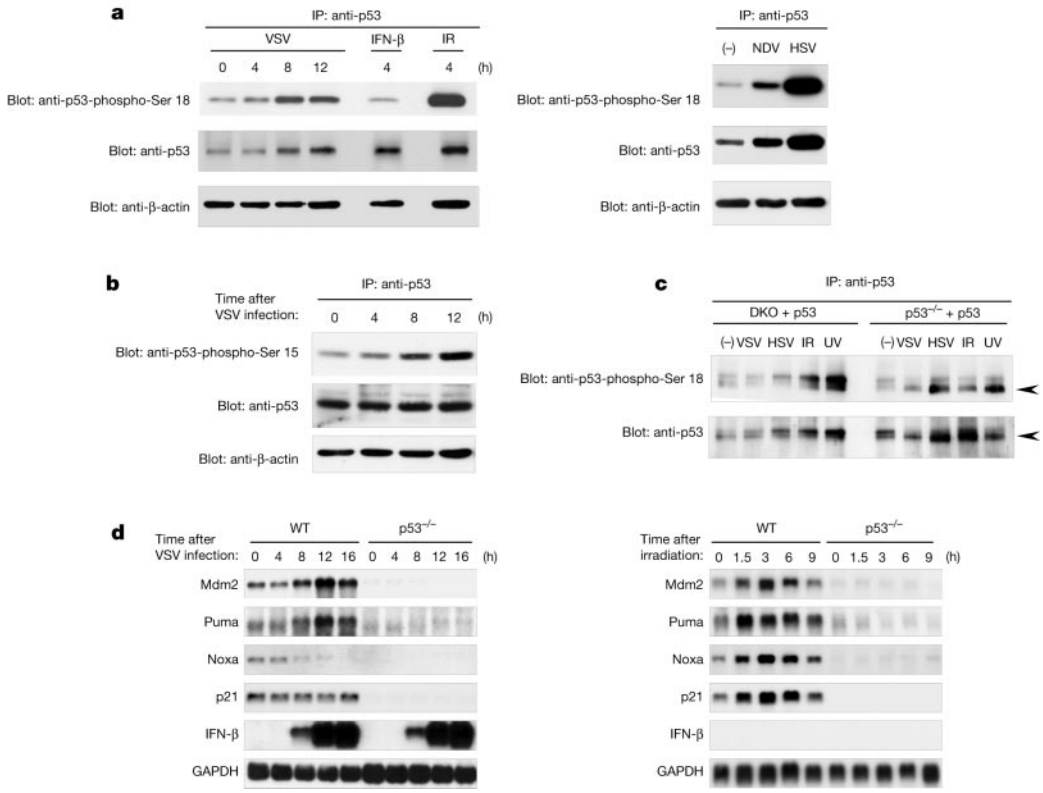

Figure 4 Activation of p53 by viruses. **a**, Induction of phosphorylation of p53 at Ser 18 by virus infection. MEFs were infected with VSV (left), NDV or HSV (right), or treated with IFN-β for 4 h, and cell lysates were analysed by immunoprecipitation with the anti-p53 antibody and immunoblotting with specific antibodies against phospho-Ser 18 or p53. Phosphorylation of Ser 18 at 4 h after X-ray irradiation (IR) is shown as a positive control. Note that p53 phosphorylation is not induced by IFN-β. **b**, VSV-induced phosphorylation of human p53 at Ser 15. Human HepG2 cells were infected by VSV and phosphorylation of Ser 15 was examined as described above for mouse p53. **c**, ATM-dependent phosphorylation of p53-Ser 18 by virus infection. $p53^{-/-}$ MEFs and MEFs doubly deficient for p53 and ATM (DKO) were infected with p53-expressing retrovirus, and the phosphorylation status of Ser 18 was examined 8 h after VSV infection or ultraviolet irradiation (UV), or 4 h after X-ray irradiation (IR), by immunoprecipitation and immunoblotting analysis as described in **a**. The arrow indicates the band corresponding to p53 protein, whereas an additional band above this arrow represents a nonspecific band that is also detected in the MEFs transfected with a control vector (data not shown). It is known that p53 phosphorylation by UV is dependent on another kinase, ATR. **d**, Differential activation of p53 target genes after viral infection or X-ray irradiation. After VSV infection for the indicated time periods, the kinetics of p53-dependent gene induction were analysed by RNA blotting in wild-type or $p53^{-/-}$ MEFs. The result of the induction of target genes of p53 after X-ray irradiation is shown in the right panel.

apoptosis. It is possible that this difference is a reflection of the status of p53 phosphorylation and/or activation of other transcription factors acting cooperatively with p53 on these target genes.

p53-dependent apoptosis in virus-infected cells

Activation of p53 and induction of its proapoptotic target genes in virally infected cells suggest that these events lead to an apoptotic response of the infected cells, an event that can be considered as altruistic suicide that limits virus replication. In fact, as shown in Fig. 5a, wild-type MEFs infected with VSV eventually underwent apoptosis as revealed by annexin V staining, but this response was strongly suppressed in $p53^{-/-}$ MEFs. In this regard, it is worth noting that the upregulation of p53 in response to VSV infection can be detected in wild-type MEFs but not in IFN-α/β receptor 1 (IFNAR1)-deficient MEFs ($IFNAR1^{-/-}$ MEFs) (Fig. 5b). It is worth highlighting two features of p53 expression and phosphorylation. First, p53 phosphorylation (at Ser 18) is induced in $IFNAR1^{-/-}$ MEFs, indicating that the IFN signal is not necessary for this event. In fact, this observation is consistent with our notion that IFN does not activate p53 but contributes to the enhancement of the p53 response by inducing the *p53* gene. Second, the p53 protein level is slightly increased by VSV infection in $IFNAR1^{-/-}$ MEFs, suggesting that VSV may stabilize the p53 protein. This may be an interesting issue to be addressed further. Regarding the contribution of IFN-induced p53 in the virus-induced apoptotic response, we also found that $IFNAR1^{-/-}$ MEFs are indeed more resistant to VSV-induced apoptosis than wild-type MEFs (Supplementary Fig. 8). Therefore, although careful interpretation of this observation will be required, it is possible that p53 induction by virus-induced IFN-α/β may at least in part enhance the apoptotic response in virally infected MEFs.

To determine whether the absence of p53 response affects the extent of virus replication, we used the following assay. Wild-type MEFs were infected with VSV as described above, and virus titres were monitored in the supernatant of the infected cells after they all died as a result of the infection. As shown in Fig. 5c, the virus yield was more than 30-fold higher in $p53^{-/-}$ MEFs than in wild-type MEFs. These results suggest that the p53-dependent apoptosis in virally infected cells contributes to limiting virus replication. In order to verify the role of p53 in antiviral response *in vivo*, we examined the susceptibility of $p53^{-/-}$ mice to VSV. Surprisingly, as shown in Fig. 5d, these mice succumbed to VSV infection. In addition, a marked increase (about 100-fold) was observed in the sera of $p53^{-/-}$ mice compared with wild-type mice (Fig. 5e). These observations lend further support to the notion that the p53 response to virus infection constitutes a critical aspect of antiviral immunity.

Discussion

The regulation of the tumour suppressor p53 has been extensively studied at the protein level; however, little is known about the transcriptional regulation of the *p53* gene. In this study, we have shown that the *p53* gene is transcriptionally induced by IFN-α/β through ISGF3 activation, demonstrating *p53* gene induction by a cytokine. Whereas IFN-α/β induces p53 mRNA and increases its protein level, p53-mediated responses such as cell cycle arrest or

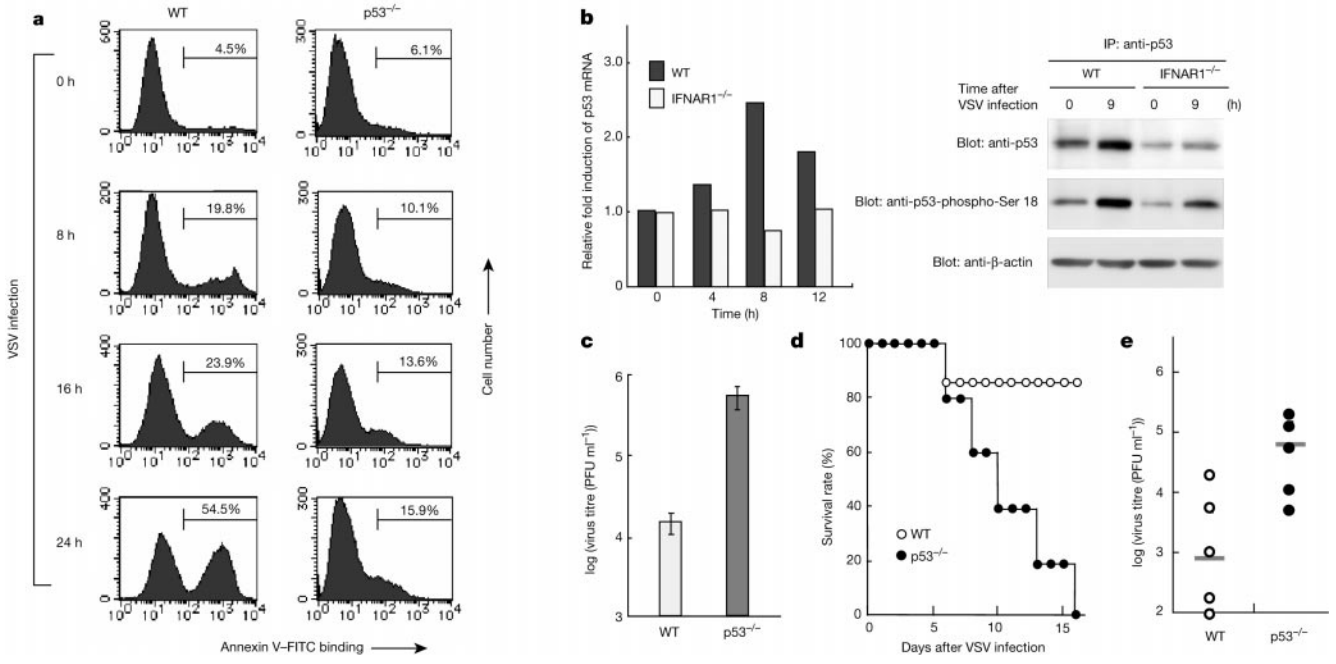

Figure 5 Role of p53 in antiviral defence. **a**, p53-dependent apoptosis in virally infected cells. Wild-type and $p53^{-/-}$ MEFs were infected by VSV, and the apoptotic response was analysed by annexin V–FITC staining. **b**, Upregulation of the levels of p53 mRNA after VSV infection in wild-type MEFs but not in $IFNAR1^{-/-}$ MEFs (left). The induction levels of p53 mRNA at the indicated times after VSV infection were analysed by quantitative real-time RT–PCR analysis, and normalized to the expression levels of the 28S rRNA. The upregulation of p53 protein level in wild-type and $IFNAR1^{-/-}$ MEFs in response to VSV infection is shown (right). Wild-type and $IFNAR1^{-/-}$ MEFs were infected with VSV, and at 9 h after infection cell lysates were immunoprecipitated (IP) with the anti-p53 antibody. Subsequently, immunoblotting analysis (blot) was carried out with the anti-p53 or anti-p53-phospho-Ser 18 antibody. **c**, Virus titres of the supernatant of the wild-type and $p53^{-/-}$ MEFs after the viability of all the cells was lost after VSV infection (10 M.O.I.). **d**, Survival rate of wild-type and $p53^{-/-}$ mice after VSV infection. Ten wild-type and nine $p53^{-/-}$ mice were intravenously infected with VSV (2×10^6 plaque-forming units (PFU)), and the survival of these mice was monitored daily. All of the $p53^{-/-}$ mice died within 16 days after infection, whereas the wild-type mice, except for one, survived. **e**, Virus titres in sera of virus-infected mice. Wild-type and $p53^{-/-}$ mice were infected with VSV as described in **d**. Sera from each mouse were collected two days after infection, and VSV titres in the sera were determined by the plaque-formation assay. The bars indicate the mean values of the virus titre. The difference in virus titre between wild-type and $p53^{-/-}$ mice was statistically significant ($P < 0.02$, Student's *t*-test).

apoptosis were not observed in cells treated with IFN-α/β alone. Although seemingly futile, this induction could therefore be viewed as a regulated 'trade-off' to provide the cell with a greater dynamic range in its response to a variety of stress signals. This notion is supported by the present findings that cells treated with IFN-β evoke more robust p53 responses than do untreated cells (Fig. 3). Notably, induction of the p53 mRNA by IFN-β is relatively weak compared with that of the mRNAs of conventional IFN-inducible genes. It is likely that the low affinity of the p53-ISREs to IFN-activated ISGF3 accounts for the weak induction (Fig. 2). In view of the fact that p53 is an extremely efficient inhibitor of cell growth, the modest induction of p53 by IFNs may help to avoid adverse p53 actions in normal growing cells.

There are numerous reports on the usefulness of IFN-α/β for the treatment of some types of human cancer, including HPV-associated cervical cancer and hepatic cancer[43–45]. However, the molecular basis of IFN action in cancer treatment still remains largely elusive. Our study suggests that one mechanism of the anti-tumour action of IFN may involve p53 induction. In this regard, it is interesting that IFNAR1$^{-/-}$ MEFs undergo spontaneous transformation *in vitro* and that the mutant mice develop papilloma of the skin at a high incidence (about eightfold relative to wild-type mice) when treated with chemical carcinogens (A.T. and N. Tanaka, unpublished data). In addition, our study suggests the possible usefulness of treating human cancers with IFN-α/β in combination with chemotherapeutic drugs that activate p53. Combined therapy with IFN-α/β and chemotherapeutic drugs such as 5-FU may permit the use of lower doses of chemotherapeutic agents, which would otherwise exert toxic side effects by p53-independent mechanisms. This will be a clinically interesting issue to address further.

Our results reveal an important role of p53 in the complex innate antiviral host defences (Fig. 5). Prompt induction of apoptosis of virus-infected cells via p53 activation will be beneficial to the host in limiting virus replication: virus production would be suppressed if the infected cells undergo rapid apoptosis before the onset of cellular lysis (Fig. 5a). Apoptotic cells would be swiftly engulfed by phagocytic cells *in vivo* and this event would also contribute to inhibiting the spread of virus (Fig. 5e). This p53 response is not absolutely dependent on but could be enhanced by IFN-α/β. Interestingly, in the absence of p53, IFN-treated cells are more resistant to apoptosis on infection with VSV or encephalomyocarditis virus (EMCV) (Supplementary Fig. 9a, b), and the total VSV virus yield becomes higher in p53$^{-/-}$ MEFs than in wild-type MEFs when these cells were treated by IFN-β followed by the infection (Supplementary Fig. 9c). On the basis of these findings, one may infer the following events: at the early phase of virus infection, virus-infected cells produce IFN-α/β and eventually undergo p53-dependent apoptosis. On the other hand, virus-induced IFN-α/β may act on the surrounding, uninfected cells to help antiviral defences by inducing cellular genes that inhibit virus replication and, in addition, by inducing p53 to prime cells for enhanced apoptosis. However, the details of how IFN-α/β and p53 cooperate in antiviral immunity need to be clarified further. Our study reveals a hitherto unrecognized cooperation between p53 and IFN-α/β, providing a new link between tumour suppression and antiviral host defence. □

Methods

Mice, cell cultures, viruses and reagents

The generation of IRF-9$^{-/-}$ and p53$^{-/-}$ mice has been described previously[25,46]. IFNAR1$^{-/-}$ mice were purchased from B&K Universal Group Ltd. MEFs were prepared following a standard procedure[47]. The above mice were all on the C57BL/6 background. ATM and p53-double-deficient MEFs[39] were provided by C. Westphal. The human hepatoma cell lines HepG2 and HLE were purchased from the Health Science Research Resources Bank. Recombinant human and mouse IFN-β were provided by Toray Co., Ltd. Unless otherwise stated, the concentration of IFN-β used in the assays was 500 U ml^{-1}. The infection of MEFs with VSV, HSV, NDV and EMCV was carried out as described previously[48], and multiplicity of infection (M.O.I.) is 1.0 unless stated otherwise. X-ray irradiation was performed at 30 Gy.

Immunoprecipitation and immunoblotting

Cell lysis, immunoprecipitation and immunoblotting were carried out as described[47]. Antibodies against the following proteins were purchased: p53 (Ab-1; Oncogene Research Products), p53 (FL-393; Santa Cruz Biotechnology) and β-actin (AC-15; Sigma-Aldrich). For the detection of mouse p53 phosphorylation on Ser 18, phospho-Ser 15-specific antiserum was provided by Y. Taya[36].

RNA analysis

RNA extraction and polymerase chain reaction with reverse transcription (RT–PCR) analysis were performed as described previously[47]. Quantitative real-time RT–PCR was performed with the Lightcycler and SYBR Green system (Roche Molecular Biochemicals), and the data were normalized by the expression level of 28S ribosomal RNA for each sample. The following oligonucleotide primers specific to mouse p53 and 28S rRNA were used: p53, 5′-ACTGCATGGACGATCTGTTG-3′ (sense) and 5′-GCCATAGTTGCCCT GGTAAG-3′ (antisense); 28S rRNA, 5′-CAGGGGAATCCGACTGTTTA-3′ (sense) and 5′-ATGACGAGGCATTTGGCTAC-3′ (antisense). For RNA blot analysis, equal amounts of total RNA (5 μg) were loaded in each lane. Probes for Mdm2, Noxa, p21$^{WAF1/Cip1}$, IFN-β and GAPDH mRNAs have been described previously[41,48]. A hybridization probe for Puma mRNA was generated by PCR amplification of a complementary DNA fragment corresponding to codons 262–843 of the *Puma* gene.

Retroviral expression

The expression of activated Ha-Ras was mediated by retroviral gene transfer, wherein the pGDV12ras was used as described previously[49]. The details of the retroviral expression vectors for the HPV16 E6 protein, E1A and wild-type p53 are mentioned in Supplementary Information. Retroviral transduction into MEFs was carried out as described previously[47,48].

Apoptosis assay

Annexin V staining with FITC-conjugated anti-annexin V (Molecular Probes) was carried out, and samples were subjected to flow cytometric analysis with FACS calibur (Becton & Dickinson).

Chromatin immunoprecipitation assay

The ChIP assay was performed according to the manufacturer's protocol (Upstate Biotechnology) with a slight modification. Briefly, MEFs were treated with or without IFN-β for 1.5 and 3.0 h before formaldehyde cross-linking. The specific antibodies used for immunoprecipitations were the anti-Stat2 antibody (Upstate Biotechnology) and a control antibody (rabbit IgG). After protein–DNA cross-links in the immunoprecipitates were reversed, the purified DNA was analysed by PCR (35 cycles; 30 s at 95 °C, 60 s at 55 °C, 60 s at 72 °C) with primers that detect sequences containing p53-ISRE1 (nucleotide number +1757 to +1901) or p53-ISRE2 (+5619 to +5780), and a pair of negative-control primers that recognize the DNA sequence (+11093 to +11250) of the 3′-untranslated region (UTR) of the *p53* gene. The PCR products were visualized on an ethidium bromide gel.

Transformation assay

Anchorage-independent colony formation was carried out as described previously[49]. Cells were seeded at 1×10^5 cells into 60-mm dishes in a suspension of 1.3% methylcellulose gel dissolved in DMEM medium supplemented with 10% FCS on top of a bed composed of 0.53% agarose in the same culture medium, and incubated with or without the indicated amounts of IFN-β. The number of colonies formed was determined three weeks after the seeding.

Measurement of virus titre

Virus titre in the cells or mice infected with VSV was determined as described previously[46].

EMSA

We analysed Celera proprietary human and murine genomic databases of the *p53* gene and identified two putative ISREs upstream of the transcriptional initiation site and in the first intron using the TRANSFAC database. For EMSA, competition assay was carried out with different concentrations of each non-radiolabelled oligonucleotide probe containing the element of p53-ISRE1 or p53-ISRE2, as well as those of mut-p53-ISRE1 and mut-p53-ISRE2, which have mutated sequences in the putative ISREs: p53-ISRE1, +1891 CAGTTTCTCTTCTCT +1915; p53-ISRE2, +5665 GGAGAACAGAAACTG +5689; mut-p53-ISRE1, CAGCCATTCCTCTCT; mut-p53-ISRE2, GGAGAGCAATGGCTG. After MEFs were treated with IFN-β for 45 min, EMSA was performed as described previously[47]. Equal amounts of proteins from whole-cell extracts were loaded onto each lane.

Assays of transcriptional activity

Oligonucleotides corresponding to region +1892 to +1913 or region +5668 to +5689 of the murine *p53* gene were synthesized. Each of the five copies of the oligonucleotide was tandemly multimerized and ligated to a fragment (region −55 to 0 of the IFN-β gene (*Ifnb*) promoter containing a TATA box and a cap site (ref. 50)). The resultant fragments were cloned into the *Sac*I and *Kpn*I sites of the pGL2-basic vector (Promega) (p53-ISRE1-luc and p53-ISRE2-luc, respectively). Similarly, mut-p53-ISRE1-luc and mut-p53-ISRE2-luc were constructed by introducing the same mutations in the ISRE sequence as described above. After transfection, MEFs were stimulated with IFN-β for 8 h and luciferase activities were analysed by the dual luciferase assay (Roche Diagnostics) including the

measurement of β-galactosidase activities that were used as an internal control for transfection efficiency. Twenty-four hours after transfection, cells were treated with IFN-β for 8 h, and cell extracts were prepared and subjected to the luciferase assay. pOAS-ISRE-luc, which contains the ISRE (GGGAAATGGAAACT) from the *OAS* gene[46], was used as a positive control. The averages and standard deviations of the values of luciferase activities from the triplicates of a representative experiment are shown.

Received 19 March; accepted 28 May 2003; doi:10.1038/nature01850.
Published online 16 July 2003.

1. Oren, M. Relationship of p53 to the control of apoptotic cell death. *Semin. Cancer Biol.* **5,** 221–227 (1994).
2. Jacks, T. & Weinberg, R. A. Cell-cycle control and its watchman. *Nature* **381,** 643–644 (1996).
3. Prives, C. & Hall, P. A. The p53 pathway. *J. Pathol.* **187,** 112–126 (1999).
4. Vogelstein, B., Lane, D. & Levine, A. J. Surfing the p53 network. *Nature* **408,** 307–310 (2000).
5. Vousden, K. H. & Lu, X. Live or let die: the cell's response to p53. *Nature Rev. Cancer* **2,** 594–604 (2002).
6. Pestka, S., Langer, J. A., Zoon, K. C. & Samuel, C. E. Interferons and their actions. *Annu. Rev. Biochem.* **56,** 727–777 (1987).
7. De Maeyer, E. & De Maeyer-Guignard, J. *Interferons and Other Regulatory Cytokines* (John Wiley and Sons, New York, 1988).
8. Vilcek, J. & Sen, G. S. in *Fields Virology* 3rd edn (eds Fields, D. M., Knipe, P. M. & Howley, P. M.) 375–399 (Lippincott-Raven, Philadelphia, 1996).
9. Biron, C. A. Interferons α and β as immune regulators—a new look. *Immunity* **14,** 661–664 (2001).
10. Gresser, I. & Belardelli, F. Endogenous type I interferons as a defense against tumors. *Cytokine Growth Factor Rev.* **13,** 111–118 (2002).
11. Strander, H. & Einhorn, S. Interferons and the tumor cell. *Biotherapy* **8,** 213–218 (1996).
12. Gutterman, J. U. Cytokine therapeutics: lessons from interferon α. *Proc. Natl Acad. Sci. USA* **91,** 1198–1205 (1994).
13. Pfeffer, L. M. *et al.* Biological properties of recombinant α-interferons: 40th anniversary of the discovery of interferons. *Cancer Res.* **58,** 2489–2499 (1998).
14. Hsu, I. C. *et al. p53* gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines. *Carcinogenesis* **14,** 987–992 (1993).
15. Haupt, Y., Maya, R., Kazaz, A. & Oren, M. Mdm2 promotes the rapid degradation of p53. *Nature* **387,** 296–299 (1997).
16. Kubbutat, M. H., Jones, S. N. & Vousden, K. H. Regulation of p53 stability by Mdm2. *Nature* **387,** 299–303 (1997).
17. Sherr, C. J. & Weber, J. D. The ARF/p53 pathway. *Curr. Opin. Genet. Dev.* **10,** 94–99 (2000).
18. Ginsberg, D., Oren, M., Yaniv, M. & Piette, J. Protein-binding elements in the promoter region of the mouse *p53* gene. *Oncogene* **5,** 1285–1290 (1990).
19. Darnell, J. E. Jr, Kerr, I. M. & Stark, G. R. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264,** 1415–1421 (1994).
20. Haque, S. J. & Williams, B. R. Identification and characterization of an interferon (IFN)-stimulated response element-IFN-stimulated gene factor 3-independent signaling pathway for IFN-α. *J. Biol. Chem.* **269,** 19523–19529 (1994).
21. Bluyssen, A. R., Durbin, J. E. & Levy, D. E. ISGF3γ p48, a specificity switch for interferon activated transcription factors. *Cytokine Growth Factor Rev.* **7,** 11–17 (1996).
22. Scheffner, M., Huibregtse, J. M., Vierstra, R. D. & Howley, P. M. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75,** 495–505 (1993).
23. zur Hausen, H. Papillomaviruses and cancer: from basic studies to clinical application. *Nature Rev. Cancer* **2,** 342–350 (2002).
24. Veldman, T., Horikawa, I., Barrett, J. C. & Schlegel, R. Transcriptional activation of the telomerase *hTERT* gene by human papillomavirus type 16 E6 oncoprotein. *J. Virol.* **75,** 4467–4472 (2001).
25. Tsukada, T. *et al.* Enhanced proliferative potential in culture of cells from p53-deficient mice. *Oncogene* **8,** 3313–3322 (1993).
26. Lowe, S. W., Ruley, H. E., Jacks, T. & Housman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74,** 957–967 (1993).
27. Bunz, F. *et al.* Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J. Clin. Invest.* **104,** 263–269 (1999).
28. Butz, K. *et al.* Functional p53 protein in human papillomavirus-positive cancer cells. *Oncogene* **10,** 927–936 (1995).
29. Mendonca, M. S. *et al.* Delayed apoptotic responses associated with radiation-induced neoplastic transformation of human hybrid cells. *Cancer Res.* **59,** 3972–3979 (1999).
30. Neil, J. C., Cameron, E. R. & Baxter, E. W. p53 and tumour viruses: catching the guardian off-guard. *Trends Microbiol.* **5,** 115–120 (1997).
31. Evan, G. & Littlewood, T. A matter of life and cell death. *Science* **281,** 1317–1322 (1998).
32. Levy, D. E. & Garcia-Sastre, A. The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. *Cytokine Growth Factor Rev.* **12,** 143–156 (2001).
33. Goodbourn, S., Didcock, L. & Randall, R. E. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. *J. Gen. Virol.* **81,** 2341–2364 (2000).
34. Katze, M. G., He, Y. & Gale, M. Jr Viruses and interferon: a fight for supremacy. *Nature Rev. Immunol.* **2,** 675–687 (2002).
35. Donehower, L. A. *et al.* Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356,** 215–221 (1992).
36. Shieh, S. Y., Ikeda, M., Taya, Y. & Prives, C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91,** 325–334 (1997).
37. Banin, S. *et al.* Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* **281,** 1674–1677 (1998).
38. Canman, C. E. *et al.* Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* **281,** 1677–1679 (1998).
39. Westphal, C. H. *et al.* Genetic interactions between ATM and p53 influence cellular proliferation and irradiation-induced cell cycle checkpoints. *Cancer Res.* **57,** 1664–1667 (1997).
40. Bakkenist, C. J. & Kastan, M. B. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421,** 499–506 (2003).
41. Oda, E. *et al.* Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288,** 1053–1058 (2000).
42. Polyak, K., Waldman, T., He, T. C., Kinzler, K. W. & Vogelstein, B. Genetic determinants of p53-induced apoptosis and growth arrest. *Genes Dev.* **10,** 1945–1952 (1996).
43. Koromilas, A. E., Li, S. & Matlashewski, G. Control of interferon signaling in human papillomavirus infection. *Cytokine Growth Factor Rev.* **12,** 157–170 (2001).
44. Wadler, S. & Schwartz, E. L. Antineoplastic activity of the combination of interferon and cytotoxic agents against experimental and human malignancies: a review. *Cancer Res.* **50,** 3473–3486 (1990).
45. Miyamoto, A. *et al.* Advanced hepatocellular carcinoma with distant metastases, successfully treated by a combination therapy of α-interferon and oral tegafur/uracil. *J. Gastroenterol. Hepatol.* **15,** 1447–1451 (2000).
46. Kimura, T. *et al.* Essential and non-redundant roles of p48 (ISGF3γ) and IRF-1 in both type I and type II interferon responses, as revealed by gene targeting studies. *Genes Cells* **1,** 115–124 (1996).
47. Takaoka, A. *et al.* Cross talk between interferon-γ and -α/β signaling components in caveolar membrane domains. *Science* **288,** 2357–2360 (2000).
48. Sato, M. *et al.* Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for *IFN-α/β* gene induction. *Immunity* **13,** 539–548 (2000).
49. Tanaka, N. *et al.* Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. *Cell* **77,** 829–839 (1994).
50. Fujita, T., Ohno, S., Yasumitsu, H. & Taniguchi, T. Delimitation and properties of DNA sequences required for the regulated expression of human *interferon-β* gene. *Cell* **41,** 489–496 (1985).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank Y. Taya for anti-p53-P-Ser 15 antibody; Y. Hirabayashi and T. Inoue for $p53^{-/-}$ mice; T. Kiyono for pLRHL-16E6; C. Westphal for ATM/p53 deficient MEFs; K. Segawa for pSVp53c17 and pLSVNc-9; T. Nakajima for adenovirus 2 E1A 12S cDNA; Toray Co., Ltd for recombinant human and mouse IFN-β; J. Vilcek and E. Barsoumian for critical reading of the manuscript; H. zur Hausen, F. Melchers, Y. Taya and N. Tanaka for advice; and T. Yokochi for technical assistance. This work was supported in part by a grant for Advanced Research on Cancer from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Grants-in-Aid from the Ministry of Education, Science and Culture (Scientific Research on Priority Areas), grants of the Virtual Research Institute of Ageing of Nippon Boehringer Ingelheim, the Welfide Medicinal Research Foundation, Kanae Foundation for Life & Socio-Medical Science, and Senri Life Science Foundation. D.S. was a postdoctoral fellow, supported by the Japan Society for the Promotion of Science.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to T.T. (tada@m.u-tokyo.ac.jp).