1   **Rab5 and Alsin regulate stress-activated cytoprotective signaling on mitochondria**

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Abstract

Mitochondrial stress response is essential for cell survival, and damaged mitochondria are a hallmark of neurodegenerative diseases. Thus, it is fundamental to understand how mitochondria relay information within the cell. Here, by investigating mitochondrial-endosomal contact sites we made the surprising observation that the small GTPase Rab5 translocates from early endosomes to mitochondria upon oxidative stress. This process is reversible and accompanied by an increase in Rab5-positive endosomes in contact with mitochondria. Interestingly, activation of Rab5 on mitochondria depends on the Rab5-GEF ALS2/Alsin, encoded by a gene mutated in amyotrophic lateral sclerosis (ALS). Alsin-deficient human induced pluripotent stem cell-derived spinal motor neurons are defective in relocating Rab5 to mitochondria and display increased susceptibility to oxidative stress. These findings define a novel pathway whereby Alsin catalyzes the assembly of the Rab5 endocytic machinery on mitochondria. Defects in stress-sensing by endosomes could be crucial for mitochondrial quality control during the onset of ALS.

Introduction

Mitochondria, the organelle for cellular metabolism and ATP production, play an essential role in a number of other cellular processes such as calcium signaling, lipid synthesis and trafficking, metabolite transport, apoptosis, and reactive oxygen species (ROS) production in the cell (Mesmin, 2016, Ott, Gogvadze et al., 2007, Rizzuto, De Stefani et al., 2012). Many of these processes necessitate communication with other cellular compartments. For example, membrane contact sites (MCS) between endoplasmic reticulum (ER) and mitochondria are important for Ca^{2+} and lipid transfer (de Brito & Scorrano, 2008), mitochondria fission (Friedman, Lackner et al., 2011), and regulation of apoptosis (Prudent, Zunino et al., 2015). Lipid droplets and peroxisomes interact with mitochondria to regulate fatty acid oxidation (Cohen, Klug et al., 2014, Pu, Ha et al., 2011). Oxidized and damaged proteins can be selectively delivered to peroxisomes and lysosomes via mitochondrial-derived vesicles (Sugiura, McLelland et al., 2014). These examples demonstrate an extensive functional interplay between organelles, either directly via MCS and/or indirectly via vesicular intermediates. However, the underlying molecular mechanisms remain poorly understood and, in particular, the functional relationship between mitochondria and the endocytic system is largely unexplored.

The endocytic pathway is responsible for maintaining cellular homeostasis by internalizing, sorting, recycling and/or degrading distinct types of cargo molecules (Huotari & Helenius, 2011). Rab GTPases serve as molecular signatures on endosomes, regulating their biogenesis and functions (Pfeffer, 2017, Zerial & McBride, 2001, Zhen & Stenmark, 2015). Ligand-receptor complexes at the plasma membrane (PM) are internalized into early endosomes (EE) marked by small GTPase Rab5, followed by either recycling to the PM via Rab4 and Rab11-positive recycling endosomes (RE), or routed to late endosomes (LE) and lysosomes for degradation. The latter process occurs via the conversion of Rab5-positive EE into Rab7-positive LE (Rink, Ghigo et al., 2005, Sonnichsen, De

57   Renzis et al., 2000, Ullrich, Reinsch et al., 1996). Rab proteins can thus dynamically associate with the

58   membranes, conferring functional plasticity to organelles. On these endosomal membranes, Rab

59   proteins recruit a plethora of effectors for membrane tethering and fusion, cargo sorting and signaling

60   (Sorkin & von Zastrow, 2009, Stenmark, 2009, Zerial & McBride, 2001). For example, EEA1 is a

61   dimeric coiled-coiled Rab5 effector protein that tethers two vesicles to allow efficient fusion between

62   Rab5-harbouring membranes (Murray, Jahnel et al., 2016, Ohya, Miaczynska et al., 2009). Other Rab5

63   effectors such as APPL1 are involved in regulating metabolic and inflammatory responses (Schenck,

64   Goto-Silva et al., 2008, Wen, Yang et al., 2010). Rab activation, and thus stabilization after

65   recruitment, on the membrane requires guanine nucleotide exchange factors (GEFs) (Blumer, Rey et

66   al., 2013). Rab5 GEFs constitute a family of VPS9 domain-containing proteins, including Rabex-5

67   (Horiuchi, Lippe et al., 1997), RME-6 (Sato, Sato et al., 2005), amyotrophic lateral sclerosis protein 2

68   (ALS2/Alsin) (Otomo, Hadano et al., 2003), and mammalian Ras and Rab interactor 1, 2, 3 (Rin1-3)

69   (Hu, Bliss et al., 2005). The rationale behind this complexity is that Rab5 must be specifically

70   regulated by different GEFs in space and time. In this respect, the function of many Rab5 GEFs

71   remains unclear.

72   Physical interactions between the endosomal machinery and mitochondria serve important

73   functions in cell homeostasis, repair and apoptosis. For example, transfer of iron (Das, Nag et al., 2016,

74   Sheftel, Zhang et al., 2007) and cholesterol (Charman, Kennedy et al., 2010) from endosomes to

75   mitochondria is mediated by physical interactions between the two organelles. Another classical

76   example of mitochondria-endolysosome interactions is autophagy. Autophagy is a clearance

77   mechanism whereby cells identify defective organelles following damage or stress, and eliminate them

78   via the formation of an autophagosome and subsequent fusion with lysosomes (Mizushima & Levine,

79   2010). The mechanism of degrading mitochondria has been termed macroautophagy or mitophagy.

80   Intriguingly, the expression of pro-apoptotic factors such as canonical BH3-only proteins drive Rab5-

81   and Rab7-positive endolysosomes into inner mitochondrial compartments via a pathway that appears

82   to differ from autophagy/mitophagy (Hamacher-Brady, Choe et al., 2014). Interestingly, our previous

83   conducted genome-wide RNAi screen of endocytosis (Collinet, Stoter et al., 2010) revealed that ~8%

84   of the hit genes had mitochondrial-related functions, pointing at hitherto unexplored molecular

85   connections between the endosomal system and mitochondria. This led us to hypothesize that other

86   mitochondrial functions may be regulated by endocytic components.

87   Here by exploring the interactions between EE and mitochondria, we unexpectedly found that

88   upon laser- or chemically-induced oxidative stress in mammalian cells, mitochondria outer membrane

89   permeabilization (MOMP) releases cytochrome c, and concomitantly, triggers the assembly of the

90   Rab5 machinery on mitochondria, in a process which is reversible and independent of mitophagy.

91   Remarkably, we found that the Rab5 GEF responsible for Rab5 activation is ALS2/Alsin, which is

92   necessary for efficient Rab5 recruitment to mitochondria. Our findings suggest that the Rab endocytic

93   machineries interact with mitochondria during oxidative stress as a cytoprotective mechanism, with

94   important implications for amyotrophic lateral sclerosis (ALS) and other neurodegenerative diseases.

95

96   **Results**

97   *Inter-organelle contacts between endosomes and mitochondria*

98   We first explored the potential physical link between the endosomal system and mitochondria

99   at steady state. HeLa cells stably expressing TagRFP-MTS (mitochondria targeting sequence)

100   (Takeuchi, Kim et al., 2013) were incubated with two types of endocytic cargo, Alexa-488-conjugated

101   transferrin (Tfn) or epidermal growth factor (EGF) for 10 min at 37°C, to visualize the

102   endocytic/recycling and degradative pathway, respectively. Cell were then fixed and imaged via

103   confocal microscopy. We observed a subset of endosomes that appeared to partially overlap with, or

104   were in close proximity to, mitochondria (Figure 1A,B). To avoid potential morphological changes

105   induced by the TagRFP-MTS overexpression, we also performed quantitative measurements in cells

106   stained for mitochondria with MitoTracker-Red CMXRos (Mito-Red) and labeled with Tfn-488 and

107   EGF-488 for 10 and 60 min at 37°C. All acquired images were subjected to chromatic shift correction,

108   deconvolution, and localization analysis (MotionTracking) based on subtraction of random

109   colocalization (Kalaidzidis, 2015). Tfn and EGF were efficiently internalized at 10 min, and further

110   sorted to distinct perinuclear compartments representing RE (Maxfield & McGraw, 2004) and

111   LE/lysosomes, respectively, at 60 min (Figure 1C). In both time points, Tfn-containing endosomes

112   consistently exhibited higher colocalization with Mito-Red (Figure 1D) compared to EGF-structures,

113   despite both having similar signal intensities (Figure 1E), suggesting that the interactions with

114   mitochondria may preferentially involve early/recycling endosomal structures.

115   Given that early endosomes are motile and omnipresent in the cytoplasm, we next asked

116   whether the observed physical proximity with mitochondria may reflect real MCS or be simply due to

117   random chance (Kalaidzidis, 2015). To further assess the interactions, we monitored the dynamics of

118   organelle contacts during early endocytic events by live-cell imaging. Cells were incubated with Tfn-

119 488 for 1 min to label EE and immediately imaged using a spinning disk confocal microscope. A

120 number of Tfn-containing endosomes and mitochondria labeled by TagRFP-MTS were observed in

121 close proximity, suggesting possible interactions (Video 1). Some endosomal vesicles remained in

122 close contact with mitochondria for 3-5 min and, interestingly, we could also observe interactions that

123 were followed by fission-like events (Video 2). Our data suggest that the occurrence of physical

124 contacts between EE and mitochondria observed in fixed and live cells may reflect *bona fide*, albeit

125 transient interactions, as suggested previously (Das, Nag et al., 2016, Sheftel, Zhang et al., 2007).

126
127 ***Acute mitochondrial stress recruits Rab5 and Rab5-positive endosomes to mitochondria***

128 Given the key role of mitochondria in sensing and responding to oxidative stress, we asked

129 whether acute perturbation on mitochondria may affect endosomes-mitochondria interactions. For this,

130 we used HeLa cells stably expressing GFP-Rab5 under its endogenous promoter with a bacterial

131 artificial chromosome (BAC) transgene (BAC GFP-Rab5) (Villasenor, Nonaka et al., 2015). These

132 cells were validated based on the lack of detectable alterations on endocytic trafficking. Live-cell

133 imaging of BAC GFP-Rab5 expressing RFP-MTS also confirmed the occurrence of Rab5-positive EE

134 (>200 nm) in close contact with mitochondria (Video 3).

135 To validate the results with the ectopically expressed mitochondrial marker, we tested

136 mitochondrial-selective dyes in our live-cell imaging. Unexpectedly, we found that photoirradiation in

137 cells labeled with Mito-Red not only robustly induced alterations in mitochondrial morphology but

138 also the appearance of GFP-Rab5 signals around mitochondria (Figure 2A). This is consistent with the

139 fact that certain rosamines and rhodamine-derived dyes used to assay mitochondrial functions possess

140 photosensitizing properties (Hsieh, Chu et al., 2015). Specifically, Mito-Red has been used to perturb

141 mitochondrial function (Minamikawa, Sriratana et al., 1999). Therefore, we hypothesized that the

142 change in Rab5 localization may be a consequence of alterations in mitochondrial function. Consistent

143 with previous reports, low exposure of Mito-Red labeled cells with a 561 nm laser (~5 J/cm^2) caused a

144 decrease in mitochondrial and an increase in cytoplasmic signal, indicative of MOMP, and

145 accompanied by a globular swelling of mitochondria within min (Figure 2A, Video 4). Remarkably,

146 laser treatment on Mito-Red labeled cells resulted in the translocation of GFP-Rab5 to mitochondria,

147 marked by increased colocalization with Mito-Red compared to the untreated (Figure 2B).

148 Furthermore, the frequency of GFP-Rab5 EE in close proximity to the stressed mitochondria increased

149 (Figure 2A, Post-laser arrowheads). This effect was specific to Mito-Red treatment, because cells

150 labeled with MitoTracker Green FM or transfected with RFP-MTS retained their tubular mitochondrial

151 structures under the same laser treatment and did not recruit GFP-Rab5 to the mitochondria (Figure 2–

152 figure supplement 1A,B, respectively). The presence GFP-Rab5 on mitochondria was independently

153 confirmed by staining with antibodies against Rab5 and the outer mitochondrial membrane (OMM)

154 protein TOM20 (Figure 2–figure supplement 1C).

155   Next, we examined the kinetics of GFP-Rab5 recruitment to Mito-Red labeled mitochondria

156 upon laser-induced stress. Time-lapse analysis revealed that the recruitment of Rab5 to rounded

157 mitochondria (Figure 2C, Pre- vs Post-laser) was visible within 5 min and reached its peak in signal

158 intensity at ~15 min post-laser treatment (Figure 2D,E; data correspond to the inset region of Figure

159 2C). The GFP-Rab5 ring-like signal persisted for >60 min (Figure 2–figure supplement 2, arrowheads).

160 In our time-lapse movies, we did not observe evidence of endosomal fusion with mitochondria (Figure

161 2E, Video 5), although we cannot exclude fusion with dim vesicles that may have escaped detection.

162 Our interpretation is that the bulk of Rab5 is recruited on mitochondria from the cytosolic pool. These

163 results suggest that upon acute oxidative stress, Rab5 translocates to mitochondria on the time-scale of

164 minutes.

165
166 ***Rab5 localizes to regions of mitochondria that are stressed***

167   We next asked if GFP-Rab5 translocation to mitochondria is a general response to the overall

168   cell stress or can be elicited locally on individual mitochondria. To test this, we photoirradiated a small

169   area of the cell labeled with Mito-Red (Figure 2F, Pre, inset) and monitored the GFP-Rab5 signal after

170   10 and 20 min. Despite the localized perturbation, the morphological alterations extended to most

171   mitochondria which changed from being tubular to rounded (Figure 2F, 10 min). This is consistent

172   with the fact that mitochondria form a dynamically interconnected network (Lackner, 2014, Wang, Du

173   et al., 2015) that appears to react to local damage as an ensemble. However, the recruitment of Rab5

174   was limited to only the laser-induced area (Figure 2F, 20 min, inset, arrowheads). Similar to Figure 2A,

175   distinct GFP-Rab5-positive endosomes contacting mitochondria became visible (Figure 2F, inset,

176   double arrowheads). These results suggest that Rab5 is recruited to mitochondria in response to

177   signal(s) originating from individually stressed mitochondria.

178
179   ***Membrane contacts between Rab5-positive mitochondria and endosomes***

180   The laser-induced stress to either an entire cell or a localized region led to the appearance of

181   Rab5-positive endosomes in close proximity to the swollen mitochondria (Figure 2A,F, arrowheads).

182   By live-cell imaging, these endosomes also appeared to dock stably onto mitochondria (Video 6, boxed

183   regions, Video 7). However, due to the diffraction limit of standard light microscopy, we could not

184   resolve distances that are closer than 200 nm and endosomes that are <200 nm in diameter. To confirm

185   that these are indeed organelle contacts, we complemented our study by correlative light and electron

186   microscopy (CLEM) in order to obtain ultrastructural details. We designed our experimental setup to 1)

187   perform live-imaging of an entire cell, 2) visualize the translocation of GFP-Rab5 and GFP-Rab5-

188   positive endosomes onto mitochondria upon laser-induced stress, and 3) re-image the same cell by

189   serial section transmission electron microscopy (TEM) (Figure 3A). GFP-Rab5 cells labeled with

190   Mito-Red were plated on gridded culture dishes, laser-treated and imaged live on the spinning disk

191   confocal microscope. Upon mitochondrial rounding, cells were immediately fixed and re-imaged to

192   assess organellar morphology following fixation. The fluorescent images showed many distinct GFP-

193   Rab5-positive puncta in close proximity to mitochondria (Figure 3A). Samples were then processed for

194   serial section TEM, and an inset area tracked by live-cell imaging (Figure 3B, red arrowhead) was re-

195   located in the thin sections by both nuclear membrane (Figure 3B, dotted line) and mitochondria

196   (Figure 3B, red stars) acting as fiducial markers. TEM images from three different serial sections of the

197   inset area revealed that the GFP-Rab5-positive structure corresponds to a tubular-cisternal structure

198   that is approximately 400 nm in diameter, with the typical morphology of an early endosome (Figure

199   3C, green). Serial section analysis showed that the endosomal membrane was in very close contact (<5

200   nm) with the adjacent mitochondrion of a diameter of ~1.5 µm (Figure 3C, red), which showed few

201   cristae. The tomogram showed the presence of membrane contact sites between the rounded

202   mitochondrion and a cisternal structure that could correspond to the ER, but did not reveal the presence

203   of double or multiple membranes indicative of mitophagy (Youle & Narendra, 2011). Our data suggest

204   that upon mitochondrial stress, two events involving early endosomes and mitochondria occur: 1) Rab5

205   translocates to mitochondria and 2) endosomes and mitochondria engage in membrane contacts.

206
207   ***The recruitment of Rab5 to mitochondria is not due to mitophagy***

208   Following mitochondrial stress, both the fast kinetics (min) of Rab5 recruitment on the rounded

209   mitochondria and the absence of wrapped double-membrane structures argue against mitophagy

210   (Narendra, Tanaka et al., 2008, Novak, Kirkin et al., 2010). We searched for additional evidence to

211   rule out this mechanism. Mitophagy requires the E3 ubiquitin ligase Parkin (Narendra et al., 2008),

212   which is normally located in the cytosol but recruited to damaged mitochondria, followed by the

213   formation of LC3-positive autophagosomes and fusion with Lamp1-positive lysosomes in a process

214   that occurs in hours (hr) to days (Dolman, Chambers et al., 2013). To test whether the swollen

215   mitochondria observed in our system undergo this process, we examined the localization of these

216   markers. We first labeled BAC GFP-Rab5 cells with Mito-Red in order to image mitochondria and

217   endosomes at steady state (Figure 4A,C,E,G, Untreated) prior to triggering laser-induced stress. Upon

218   fixation, cells were immunostained with specific antibodies to detect endogenous LC3, Lamp1, and

219   Parkin. The cells were seeded on a gridded dish in order to re-locate the same cells following the

220   immunostaining. This allowed us to assess changes to the localization of the markers as a result of

221   stress when compared to neighboring untreated cells (Figure 4–figure supplement 1). Cells were then

222   incubated for 60 min at 37°C in order to maximize the time window that these markers might be

223   recruited following GFP-Rab5 translocation to mitochondria. In all laser-induced cells, GFP-Rab5 was

224   specifically enriched around mitochondria when compared to the untreated (Figure 4B,D,F). Although

225   we observed a marginal increase in LC3 and Lamp1 colocalization with Mito-Red in stress-induced

226   cells, the signals were mostly concentrated near the perinuclear region (Figure 4B,D,I). On the other

227   hand, Parkin remained mostly cytoplasmic and did not show enrichment around mitochondria in stress-

228   induced cells (Figure 4F,I).

229   As an additional validation of the immunostaining result, we also tested all three markers in

230   HeLa BAC cell lines expressing GFP-tagged LC3, Lamp1, and Parkin by live-cell imaging (Figure 4–

231   figure supplement 2). Following laser irradiation, cells were monitored live for 60 min. Similar to the

232   endogenous LC3 and Lamp1 staining, a fraction of the puncta was enriched in the perinuclear region,

233   overlapping with small fragmented mitochondria, but not with the rest of the mitochondria (Figure 4–

234   figure supplement 2A,B), suggesting that a low level of autophagy is activated. The GFP-Parkin

235   signals were mostly cytoplasmic in untreated cells but showed a few GFP-Parkin-positive ring-like

236   structures around small fragmented mitochondria, whereas most were devoid of signal in the laser-

237   induced condition (Figure 4–figure supplement 2C, arrowheads). Even after 3 hr post treatment, we

238   observed no significant increase in the number of GFP-Parkin mitochondria (data not shown). The

239   GFP-Parkin ring-like recruitment to these small fragmented mitochondria could be a result of over-

240   expression, inducing some level of mitophagy (Rana, Rera et al., 2013). Nevertheless, similar to

241   endogenous staining, Parkin was not strongly recruited to the majority of mitochondria. Altogether, the

242   fast kinetics of Rab5 recruitment to stressed mitochondria (<10 min), the absence of a double

243   membranous structure (Figure 3C), and the lack of significant Parkin, LC3 and Lamp1 recruitment

244   argue for a mechanism distinct from autophagy and mitophagy.

245
246   ***Apoptotic signaling is partially activated in laser-induced oxidative stress***

247   The low level of autophagic and mitophagic response raise the question of whether apoptosis is

248   involved. Apoptosis is a programmed cell death pathway which occurs via one of two signaling

249   cascades termed intrinsic and extrinsic pathways (Tait & Green, 2010). The intrinsic pathway is

250   initiated through the activation of the Bax/Bak-mediated MOMP, which leads to the release of

251   cytochrome c to activate effector caspases (Tait & Green, 2010). Because photoirradiation with Mito-

252   Red engenders oxidative stress in mitochondria (Hsieh et al., 2015), we also examined the localization

253   of endogenous Bax via immunostaining. At steady state, Bax signals were visible as cytoplasmic

254   puncta (Figure 4G). Upon laser-induced stress, we observed increased Bax puncta around

255   mitochondria compared to the untreated (Figure 4H,I). As a positive control for the specificity of the

256   antibody, we treated cells with 10 µM protonophore carbonyl cyanide m-chlorophenyl hydrazone

257   (CCCP), which has been shown to cause Bax translocation to mitochondria (Mikhailov, Mikhailova et

258   al., 2001, Saikumar, Dong et al., 1998). Indeed, cells treated with CCCP exhibited higher Bax

259   fluorescence intensity and colocalization with Mito-Red compared to control cells (Figure 4–figure

260   supplement 3).

261   The observed enrichment of Bax on mitochondria upon laser-induced stress led us to ask

262   whether artificially activating apoptosis would also drive Rab5 translocation to mitochondria. One

263   method of triggering mitochondrial-associated apoptosis is the over-expression of the truncated BH3

264   interacting death domain agonist (tBid), which is a potent inducer of apoptosis by activating Bax

265   and/or self-oligomerization on mitochondria (Grinberg, Sarig et al., 2002). To this end, we infected

266   BAC GFP-Rab5 cells with an adenoviral vector expressing tBid (Ad-tBid) for 12 hr at 37°C followed

267   by TOM20 immunostaining. We found a strong enrichment of GFP-Rab5 on mitochondria only in

268   cells infected with Ad-tBid but not in Ad-control cells (Figure 4–figure supplement 4). Our results

269   suggest a mechanism of Rab5 translocation to mitochondria in response to apoptotic signals.

270
271   ***Hydrogen peroxide triggers Rab5 translocation to mitochondria and affects mitochondrial***

272   ***respiration without disrupting membrane potential***

273   What could be the signal that drives Rab5 recruitment? Several possible scenarios such as

274   morphological changes to mitochondria and/or release of mitochondrial-derived factor(s) may account

275   for this. Morphological changes such as matrix condensation or swelling of mitochondria are often

276   associated with MOMP by Bax activation, cytochrome c release and subsequent activation of caspases

277   (Gottlieb, Armour et al., 2003). However, this is not a prerequisite. For example, the mitochondrial

278   uncoupler CCCP causes mitochondrial swelling and rounding but without immediate cytochrome c

279   release or cell death (Gao, Pu et al., 2001, Lim, Minamikawa et al., 2001). On the other hand,

280   hydrogen peroxide (H_2O_2) induces mitochondrial rounding associated with increased Bax expression

281   (Gutierrez-Venegas, Guadarrama-Solis et al., 2015), cytochrome c release and caspase activation

282   (Gutierrez-Venegas et al., 2015, Takeyama, Miki et al., 2002). For these reasons, we first investigated

283   the effect of CCCP and H_2O_2 on Rab5 localization. Whereas mitochondria were mostly tubular in

284   control cells (Figure 5A,B, top panels), the exposure of cells to either CCCP or H_2O_2 for 2 hr resulted

285   in mitochondrial rounding and fragmentation (Figure 5A,B, bottom panels), as previously reported

286   (Narendra et al., 2008, Pletjushkina, Lyamzaev et al., 2006). Interestingly, CCCP did not cause Rab5

287   enrichment on mitochondria, which was only observed in H_2O_2-treated cells (Figure 5A,B,

288   arrowheads), quantified as colocalization with Mito-Red (Figure 5C,D). Consistent with previous

289   findings, we found that the release of cytochrome c into the cytosol was upregulated in H_2O_2-treated,

290   but not in CCCP-treated cells (Figure 5E). Since cytochrome c is a known factor for activating

291   caspase-dependent programmed cell death, we also assessed the activity of caspase 3/7 via a 4-amino

292   acid peptide (DEVD) conjugated to a DNA-binding dye. Cleavage of the DEVD peptide by caspase

293   3/7 releases the DNA-binding fragment, yielding a fluorescent signal. Using flow cytometry, we

294   detected ~62% of cells showing strong fluorescent signal in H_2O_2-treated cells, and merely ~0.6% and

295   ~6.2% in control and CCCP-treated cells, respectively. As additional controls of the treatment, we

296   showed that only CCCP, but not H_2O_2, resulted in the recruitment of GFP-Parkin as well as

297   endogenous Parkin on mitochondria (Figure 5–figure supplement 1A,B).

298   Given the primary role of mitochondria in cellular metabolism, we asked whether the observed

299   differences between CCCP and H_2O_2 could be related to altered mitochondrial respiration. To test this,

300   we measured the oxygen consumption rate (OCR) in live HeLa cells grown in the presence of

301   galactose in order to force cells to rely primarily on oxidative phosphorylation as opposed to glycolysis

302   (Aguer, Gambarotta et al., 2011). Upon the addition of CCCP, a sharp increase in OCR was recorded

303   compared to the media control (Figure 5G, purple vs orange curve). This is because CCCP causes the

304   collapse of the proton gradient and the disruption of the mitochondrial membrane potential. As a result,

305   electrons flow unhindered through the electron transport chain, boosting the OCR. In contrast,

306   injection of H_2O_2 resulted in an initially sharp decline in OCR, but quickly returned to its earlier steady

307   state within 20 min (Figure 5G, green curve), suggesting that the effect on OCR is reversible. The

308   decrease in respiration rate by H_2O_2 was similarly reported in intact cardiac mitochondria, in which α-

309   ketoglutarate was used as a respiratory substrate (Nulton-Persson & Szweda, 2001). Altogether, our

310   data show that, in addition to activating caspase-dependent apoptotic program, H_2O_2 also induces the

311   translocation of Rab5 to mitochondria without disrupting the membrane potential.

312
313   ***Hydrogen peroxide-induced stress triggers Rab5 translocation from EE to mitochondria, increases***

314   ***early endosomal-mitochondrial contacts, and interferes with transferrin uptake***

315   Given the key role of Rab5 in the biogenesis of the endosomal system (Zeigerer, Gilleron et al.,

316   2012, Zerial & McBride, 2001), the translocation of Rab5 to mitochondria upon oxidative stress by

317   H_2O_2 led us to investigate the connection between endocytosis and oxidative stress. Our live-cell

318   imaging data (Figure 2,3) argue that GFP-Rab5 is not delivered to mitochondria by fusion with EE.

319   Rather, it may be released from EE and recruited to mitochondria via a cytosolic intermediate. To test

320   this, we collected the total membrane (M) and cytosolic (C) fractions by subcellular fractionation from

321   HeLa cells treated with/out H_2O_2 and immunoblotted for Rab5, the endosomal tether Rab5 effector

322   EEA1, GAPDH (as cytosolic marker), and TOM20 (a mitochondrial marker) (Figure 6A). It was

323   previously shown that H_2O_2-induced stress causes increased Rab5-GDI complex in BHK cells (Cavalli,

324   Vilbois et al., 2001). Consistent with this, upon 1 hr H_2O_2 treatment, we detected a ~0.5-fold increase

325   in the amount of Rab5 in the cytosolic fraction compared to the control (Figure 6A, lane 2,4),

326   supporting the view that the cytosolic pool increases at the expense of the membrane-associated pool.

327   The cytosolic levels of Rab5 then decreased at 2 hr time point (Figure 6A, lane 4,6, Figure 6B). We

328   also detected an increase in the EEA1 pool to the cytosol in the long exposure upon H_2O_2 treatment

329   (Figure 6A, right blot). Since Rab5 in the M fraction includes both the EE and mitochondrial pool, we

330   specifically measured the EE-associated Rab5 by estimating the colocalization of Rab5 with EEA1 by

331   confocal immunofluorescence microscopy. We found a marked (~35%) decrease in the colocalization

332   in H_2O_2-treated compared to untreated cells (Figure 6C). Altogether, our results suggest that oxidative

333   stress induces the solubilization of a fraction of Rab5 from the endosomal membrane into the cytosol

334   and its translocation to the mitochondrial membrane.

335   The partial dissociation of Rab5 and EEA1 from the endosomal membrane suggests that

336   endocytic trafficking may be affected. To test this, we stimulated HeLa cells with Alexa-647 Tfn

337   continuously for 5 min at 37°C. In the absence of H_2O_2, transferrin was efficiently internalized into

338   endosomes (Figure 6D, Untreated). In contrast, cells pre-treated with H_2O_2 for 10 min showed a severe

339   block in Tfn uptake with an accumulation of Tfn signal on the cell surface (Figure 6D,E), confirming

340   the inhibition of endocytic uptake.

341   In the laser-induced stress conditions, we observed a high occurrence of EE contacting stressed

342   mitochondria (Figure 2A,F,Figure 3), and these interactions appeared to be specific to EE (Figure 1).

343   The effects by H_2O_2 prompted us to further probe whether differential interactions exist between

344   mitochondria and Tfn- vs. EGF-positive endosomes. To test this, HeLa cells stained with MitoRed

345   were continuously labeled with either Tfn-488 or EGF-488 for 10 min at 37°C and then incubated for

346   50 min with/out H_2O_2. There was a significant increase in the colocalization between Mito-Red and

347   both Tfn-488 and EGF-488 in H_2O_2-treated compared to non-treated cells (Figure 1C,D). Intriguingly,

348   we also found an increase in the total signal intensity in H_2O_2 conditions (Figure 1E), in which Tfn

349   signals accumulated more prominently in the perinuclear region (typical of RE) whereas EGF was

350   more evenly distributed compared to untreated (Figure 1C). Increased colocalization with H_2O_2 is

351   likely attributed to a block in recycling of Tfn (as uptake is inhibited) and sorting of EGF to

352   LE/lysosome, thereby resulting in cargo accumulation in EE.

353   Altogether, our data suggest that oxidative stress induced by laser irradiation or H_2O_2 leads to

354   Rab5 translocation from EE to mitochondria, increased EE-mitochondrial MCS, and a defect in

355   endosomal sorting.

356

357   ***Stress-induced Rab5 translocation to mitochondria blocks cytochrome c release***

358   By live-cell imaging, we found that mitochondria respond to H_2O_2 treatment with different

359   kinetics within a cell (Video 8). Distinct regions of the mitochondrial network were more prone to

360   rounding and membrane permeabilization than others, as revealed by the differential loss of Mito-Red

361   during H_2O_2 treatment (Figure 7A, inset image). Regions containing stressed rounded mitochondria

362   correlated exclusively with the Rab5 ring-like recruitment (Figure 7A, inset image, arrowheads),

363   suggesting that Rab5 may be involved in either facilitating or preventing the apoptotic process.

364   Therefore, we asked whether Rab5 plays a role in regulating cytochrome c release. For these

365   experiments, we transiently over-expressed GFP-Rab5 (or GFP as control) in HeLa cells and measured

366   the amount of cytosolic cytochrome c at different time points after incubation with H_2O_2. We found a

367   significant delay in the cytochrome c release from mitochondria in GFP-Rab5 over-expressing cells

368   compared to control cells (Figure 7B,C). Our results suggest that Rab5 plays a protective role in

369   mitochondrial-induced apoptosis by down-regulating the release of pro-apoptotic factor(s) to the

370   cytosol.

371

372   ***Rab5 enrichment on mitochondria is reversible***

373   We reasoned that if the translocation of Rab5 to mitochondria induced by oxidative stress is a

374   pro-survival response by lowering the apoptotic potential, then the process should be reversible when

375   stress is removed and mitochondria may recover their normal state. The fast recovery rate of

376   mitochondrial respiration following H_2O_2 injection (Figure 5E) also supports this prediction. We

377   initially tested a range of H_2O_2 concentrations (100 µM to 1 mM) and incubation times (2 to 24 hr), in

378   order to find an optimal balance between a measurable level of Rab5 translocation to mitochondria and

379   minimal cell death. We found that concentrations up to 500 µM did not cause a noticeable cell

380   rounding by 2 hr (data not shown). Therefore, we pre-incubated cells with 250 µM H_2O_2 over a period

381   of 24 hr and quantitatively measured TOM20 levels and Rab5-mitochondria colocalization as a means

382   to assess mitochondrial mass and interaction dynamics. Cells were incubated in the presence or

383   absence of H_2O_2 for 6, 12 and 24 hr, and either lysed for Western blot analysis or fixed for

384   immunostaining. Interestingly, we found that the levels of TOM20 started to increase after 6 hr,

385   suggesting that the mitochondrial mass increased (Figure 8A, left panels). In immunostained cells, we

386   found that the colocalization between endogenous TOM20 and Rab5 (Figure 8B) peaked at 6 hr and

387   started to taper off after 12 hr (Figure 8C). Decreased colocalization correlated with increased TOM20

388   protein levels (Figure 8A). Remarkably, the removal of H_2O_2 at 12 hr followed by an additional 12 hr

389   incubation in complete medium not only fully restored the morphology of mitochondria from rounded

390   to tubular (Figure 8B, bottom panel), but also returned TOM20:Rab5 colocalization to steady state

391   levels (Figure 8C). The response was dose-dependent as cells exposed to 500 µM H_2O_2 showed

392   arrested TOM20 levels and were unable to be rescued despite H_2O_2 removal at 12 hr (Figure 8A, right

393   panels). Our data show that Rab5 translocation to mitochondria induced by oxidative stress is a

394   reversible and protective process responding to apoptotic signals via the regulation of mitochondria.

395
396   ***Rab5 enrichment on mitochondria is accompanied by specific effector recruitment***

397   Because Rab5 translocates from EE to mitochondria with a consequent reduction in endocytic

398   uptake, we next asked whether the endosomal Rab5 effectors are also recruited to mitochondria. We

399   systematically assessed the localization of various endosomal effectors such as Rabenosyn-5, EEA1,

400   APPL1 and APPL2 in BAC GFP-Rab5 HeLa cells labeled with Mito-Red via immunostaining by pair-

401   wise combinations. We deliberately chose to detect the endogenous because the tagged proteins often

402   severely perturb the endosomal system, as assessed by quantitative endocytic trafficking (Kalaidzidis,

403   Miaczynska et al., 2015). Specific antibodies were first tested in untreated control cells, which showed

404   significant levels of colocalization with GFP-Rab5 (Figure 9–figure supplement 1). Upon laser-

405   induced stress, the appearance of GFP-Rab5 rings around mitochondria provided an immediate visual

406   cue and served as a positive control. Cells were fixed after 30 min incubation post-laser treatment. Of

407   the tested effectors, we detected a strong enrichment of Rabenosyn-5, but not EEA1, on mitochondria

408   in the same cell (Figure 9A,C). Neither APPL1 nor APPL2 showed enrichment around mitochondria,

409   despite a robust Rab5 recruitment (Figure 9B,C). Unlike Rabenosyn-5, EEA1, APPL1 and APPL2

410   remained well distributed in endosomal-like vesicles in both treated and untreated cells (Figure 9A,B,

411   Figure 9–figure supplement 1B,C,D). As Rabenosyn-5 and EEA1 are recruited to endosomes via both

412   Rab5 and PI(3)P-binding FYVE motifs (Nielsen, Christoforidis et al., 2000), we asked whether

413   phosphatidylinositol 3-phosphate (PI(3)P) was present on mitochondria in our stress conditions. To test

414   this, we over-expressed the PI(3)P probe GFP-2xFYVE^{Hrs} (Gillooly, Morrow et al., 2000) in HeLa

415   cells and monitored GFP signals in live cells upon laser-induced stress. Fluorescence signals were

416   present as vesicle-like puncta (Figure 9–figure supplement 2, 0 min), as previously reported (Gillooly,

417   Morrow et al., 2000). After 60 min, stressed and swollen mitochondria were observed, but these were

418   completely devoid of GFP signals, which remained on vesicle-like puncta (Figure 9–figure supplement

419   2, 60 min).

420   To corroborate the immunostaining data with an independent method, we also tested the effect

421   of H_2O_2 on Rab5 and Rab5 effectors in association with mitochondria by subcellular fractionation. We

422   isolated cytosolic (C) and mitochondrial fractions (Mito) via differential centrifugation and probed

423   them with different effector antibodies by Western blot (Figure 9D). Consistent with our

424   immunostaining results, Rab5 and Rabenosyn-5, but not EEA1 and APPL1/2, were found to be

425   specifically enriched in the mitochondrial fraction (marked by TOM20) treated with H_2O_2 compared to

426   non-treated (Figure 9D, lane 2,4).

427   Altogether, our findings reveal a selective mechanism of Rab5 translocation and activation on

428   mitochondria in the absence of PI(3)P.

429
430   ***The Rab5 GEF Alsin localizes to mitochondria upon stress induction***

431   Translocation and recruitment of effectors imply that Rab5 must be activated on the

432   mitochondrial membrane. Activation of Rab GTPases on organelle membranes depends on a family of

433   GEFs (Blumer et al., 2013, Pfeffer, 2013, Zerial & McBride, 2001, Zhen & Stenmark, 2015). We first

434   examined the localization of Rabex-5, a known GEF of Rab5 on the endosomal membrane, by

435   immunostaining in BAC GFP-Rab5 cells. For reasons described above, we visualized the endogenous

436   protein because tagged Rabex-5 constructs proved not to be functional as judged by their perturbations

437   on the endosomal system (Kalaidzidis and Zerial, unpublished data). The formation of GFP-Rab5 rings

438   upon laser treatment served as a positive control. Despite a modest enrichment on mitochondria upon

439   laser-induced stress, endogenous Rabex-5 remained mostly cytosolic and on cytoplasmic puncta

440   (Figure 10A), consistent with its endosomal localization (Figure 10–figure supplement 1A).

441   The localization pattern of Rabex-5 led us to hypothesize that another GEF might be principally

442   involved. We turned our attention to Alsin based on several lines of evidence. Alsin is the gene product

443   of ALS2, which is mutated in multiple neurodegenerative disorders such as juvenile amyotrophic

444   lateral sclerosis (ALS), juvenile primary lateral sclerosis (JPLS), and infantile-onset ascending

445   hereditary spastic paralysis (IAHSP). Alsin comprises three GEF domains: 1) a RCC1-like GEF

446   domain for Ran GTPase, 2) a DH-PH domain for Rho GTPase, and 3) a C-terminal VPS9 domain for

447   Rab5 (Topp, Carney et al., 2005) (Figure 10–figure supplement 1B). Functional studies in ALS mouse

448   models have associated Alsin with neuronal survival (Kanekura, Hashimoto et al., 2004, Panzeri, De

449   Palma et al., 2006) and endolysosomal trafficking (Hadano, Mitsui et al., 2016, Hadano, Otomo et al.,

450   2010). Moreover, corticospinal motor neuron (CSMN) in Alsin KO mice display selective defects in

451   mitochondrial morphology (Gautam, Jara et al., 2016). At steady state, Alsin localized to vesicular

452   structures, showing partial overlap with Rab5 (Figure 10–figure supplement 1C), consistent with the

453   reported localization of Alsin (Kanekura et al., 2004, Topp, Gray et al., 2004). However, after laser

454   treatment, Alsin exhibited a strong and uniform staining around mitochondria (Figure 10B), where it

455   showed significant colocalization with GFP-Rab5 and Mito-Red (Figure 10C).

456   The spatial and functional connection between Alsin and Rab5 suggest that Alsin may also be

457   implicated in stress-induced response on mitochondria. We tested this idea by over-expressing either

458   Alsin or WT Rab5 in HeLa cells and found that both prevented caspase 3/7 activation as revealed by

459   the weak fluorescence signals (due to the lack of cleavage on the DEVD-conjugated DNA-binding dye)

460   compared to control cells, when challenged with H_2O_2 (Figure 10–figure supplement 2A,B). Our

461   results point to Alsin as a candidate GEF for activating Rab5 on mitochondria upon stress induction.

462
463   ***Alsin regulates mitochondrial apoptotic signaling and is required for efficient Rab5 targeting to***

464   ***mitochondria***

465   Several mouse models have been generated for the studies on Alsin. However, these models

466   have failed to recapitulate the phenotypes observed in human patients (Cai, Shim et al., 2008). It has

467   recently been reported that the absence of Alsin appears to specifically affect the health of

468   corticospinal motor neurons (Gautam et al., 2016). Therefore, in order to directly probe the role of

469   Alsin in a more physiological background without compromising our ability for genetic and chemical

470   manipulations, we generated Alsin CRISPR knockout cells in human induced pluripotent stem cells

471   (iPSCs). We confirmed the deletion of the Alsin gene by sequencing (not shown) and RT-PCR, and the

472   encoded protein by Western blot (Figure 11–figure supplement 1A,B). We were then able to

473   differentiate both WT and mutant (Alsin$^{-/-}$) iPSCs into spinal motor neurons (iPSC-sMNs) using a

474   previously reported protocol (Reinhardt, Glatza et al., 2013). In short, we induced neural progenitor

475   cells (NPC) through embryonic bodies formation by growing iPSC in a medium supplemented with

476   transforming growth factor-ß (TGF- ß) and bone morphogen protein (BMP) small molecule inhibitors

477   (SB431542 and dorsomorphin, respectively), and WNT and Sonic Hedgehog signaling activators

478   (CHIR99021 and PMA, respectively). Differentiation and maturation stages were achieved by

479   culturing cells in retinoic acid (RA), cAMP, and neurotrophic factors (BDNF and GDNF) (Figure 11A).

480   As a quality control, high expression of pluripotency markers such as Oct4 and Lin28 were observed in

481   our iPSCs as well as Nestin, Sox2 and Pax6 expression in our neuro-progenitor cells (NPCs) (Figure

482   11–figure supplement 1C). Differentiation into mature spinal motor neurons was validated by the

483   expression of choline acetyltransferase (ChAT), HB9, and Islet-1 (ISL1) (Figure 11–figure supplement

484   1D,E). These cells showed extensive axonal network as revealed by the MAP2 staining (Figure 11–

485   figure supplement 1E). Finally, mature spinal motor neurons were re-stained for the expression of

486   Alsin in both WT and Alsin$^{-/-}$ cells (Figure 11–figure supplement 1F).

487   We first examined the steady state localization of Rab5 and morphology of mitochondria by

488   immunostaining for endogenous Rab5 and TOM20. Similar to hippocampal neurons (de Hoop, Huber

489   et al., 1994), Rab5 was ubiquitously present on endosomal-like vesicles in the soma, dendrites and

490   axon in iPSC-sMNs. The mitochondrial network in iPSC-sMNs was less tubular, and contained more

491   numerous and smaller rounded mitochondria than those in HeLa cells (Figure 11B, Ctrl). Next, we

492   tested whether iPSC-sMNs would exhibit the same mitochondrial response to oxidative stress as

493   observed in HeLa cells. Noticeably, we found iPSC-sMNs to be more susceptible to detachment and

494   cell rounding than HeLa cells when challenged with 250 µM H_2O_2 for 2 hr under the same conditions

495   (data not shown). We thus lowered the H_2O_2 concentration to 100 µM such that no immediate cell

496   detachment nor rounding were observed during the treatment. We then examined the morphology of

497   mitochondria, the association of Rab5 with mitochondria, and the release of cytochrome c into the

498   cytosol. At steady state, we did not observe significant alterations in mitochondria morphology in both

499   WT and Alsin$^{-/-}$ iPSC-sMNs. However, WT iPSC-sMNs challenged with H_2O_2 showed a robust

500   enrichment of Rab5 on mitochondria, but not in Alsin$^{-/-}$ iPSC-sMNs (Figure 11B, H_2O_2).

501   To corroborate these results, we also performed subcellular fractionation of iPSC-sMNs. In

502   control cells, endogenous Rab5 was detected primarily in the cytosolic fraction and minimally in the

503   mitochondrial fraction (Figure 11C, lane 1,2,5,6). On the other hand, cells challenged with H_2O_2

504   showed a strong enrichment of Rab5 co-fractionating with the mitochondrial fraction in WT iPSC-

505   sMNs, but only weakly in Alsin$^{-/-}$ iPSC-sMNs (Figure 11C, lane 3,7). The lack of Rab5 enrichment in

506   Alsin$^{-/-}$ cells also correlated with a greater susceptibility to H_2O_2-induced apoptotic signaling, as

507   assessed by the rapid release of cytochrome c into the cytosol within 1 hr and subsequent accumulation,

508   when compared to WT cells (Figure 11D). Collectively, our findings demonstrate that Alsin is a key

509   regulator for recruiting Rab5 to mitochondria, which altogether, impart a cytoprotective function for

510   cells against oxidative stress.

511

Discussion:

512

513   We discovered a novel cytoprotective mechanism during oxidative stress entailing the

514   translocation of Rab5 from EE to mitochondria. Interestingly, the activation of Rab5 requires Alsin,

515   which has been implicated in early onset ALS. Our results provide an unexpected mechanistic link

516   between the endosomal system and mitochondria that could be of primary importance for

517   understanding the mechanistic cause of ALS and other neurodegenerative diseases.

518   Different nutrient or environmental perturbations can affect mitochondria morphology and

519   metabolic activities such as oxidative phosphorylation and programmed cell death (Galloway & Yoon,

520   2013). Mitochondria can elicit adaptive responses to oxidative stress that may lead to hypoxia

521   adaptation, inflammation, or programmed cell death (Sena & Chandel, 2012). Our findings suggest

522   that the endocytic system is a primary responder to mitochondria under oxidative stress. Laser- or

523   exogenous ROS (e.g. H_2O_2)-induced damage causes MOMP, mitochondrial swelling, and release of

524   cytochrome c, leading to caspase activation and apoptosis. Under these conditions, the endosomal

525   system appears to rapidly respond to damaged mitochondria through a rescue pathway, which results

526   in the recruitment of Alsin and Rab5 to mitochondria, inhibition of cytochrome c release, decrease in

527   mitochondrial oxygen consumption and hence, increased overall cell viability (Figure 12). In the

528   course of this study, a mitochondrial clearance mechanism was reported where Rab5-positive EE

529   sequester mitochondria via the ESCRT machinery when cells are treated with the proton uncoupler

530   FCCP (Hammerling, Najor et al., 2017), an analog of CCCP. Our mechanism appears to be distinct

531   from this as well as the canonical autophagic/mitophagic mechanisms. First, we did not observe the

532   engulfment of mitochondria into Rab5-positive EE but rather, the recruitment of Alsin, Rab5, and

533   Rabenosyn-5 on mitochondria, as well as an increase in early endosomal-mitochondrial MCS in

534   response to stress. This is also distinct from the intra-mitochondrial recruitment of Rab5 and endo-

535 lysosomes upon over-expression of the apoptotic factors (Hamacher-Brady et al., 2014). Second, we

536 did not observe engulfing membraneous structures around stressed mitochondria nor upon CCCP

537 treatment. One explanation could be the use of different cell types and the lower concentration of

538 CCCP employed in our experiments. Third, the recruitment of Rab5 to damaged mitochondria occurs

539 rapidly, i.e. within min, well preceding any autophagic components that we analyzed in this study. We

540 found that autophagy is restricted to only a subset of small mitochondrial fragments that are LC3$^+$

541 whereas the majority are devoid of known autophagic markers such as Parkin, LC3 and Lamp1. We

542 could not rule out that mitochondrial clearance mechanism may still be activated at a later time.

543 However, our data with H_2O_2 show that the mechanism described here is reversible (Figure 8) and

544 argue for a mitochondrial-protective role rather than a degradative process. In fact, the reversal and

545 recovery of cells from late-stage apoptosis (i.e. following cytochrome c release and caspase activation)

546 have recently been reported in multiple cells lines including HeLa cells and brain cells in a process

547 called "anastasis" (Sun, Guzman et al., 2017, Tang, Tang et al., 2012), suggesting that this may be a

548 general mechanism to cope with cellular stress. We attempted to track the fate of individually damaged

549 mitochondria in a localized region after laser treatment (data not shown), but the continuous

550 photoirradation required to achieve a high spatio-temporal resolution also led to a quick decrease in

551 MitoTracker Red signal and undesirable additional stress to the cell over time, preventing us from

552 determining its precise outcome. The loss of Mito-Red signal is likely due to MOMP, as evident by the

553 release of cytochrome c, and not a result of mitochondrial clearance because the outer mitochondrial

554 membrane can be stained by TOM20 and visualized by the presence of Rab5 ring-like formation.

555   Which molecular mechanism is responsible for the dissociation of Rab5 from EE and its

556 recruitment to mitochondria? On EE, the levels of Rab5 depend on the equilibrium between the

557 cytosolic pool of Rab5 complexed to Rab GDI and the membrane-associated pool sustained by the

558   Rabex-5/Rabaptin-5 complex and a plethora of Rab5 effectors (Del Conte-Zerial, Brusch et al., 2008,

559   Lippe, Miaczynska et al., 2001, Zerial & McBride, 2001, Zhang, Zhang et al., 2014). Prior studies have

560   shown that such equilibrium, and consequently endocytic trafficking, is adaptive to stress and

561   apoptotic signal. For example, Rabaptin-5 is selectively cleaved by caspase-3 during apoptosis, thus

562   affecting its interaction with Rab5 and reducing the overall endocytic capacity (Cosulich, Horiuchi et

563   al., 1997, Swanton, Bishop et al., 1999). The activation of p38 MAPK by H_2O_2 also stimulates the

564   formation of the GDI:Rab5 complex, thus favoring the extraction of Rab5 from the early endosomal

565   membrane (Cavalli et al., 2001). In addition, p38 MAPK modulates the endosomal function via

566   phosphorylation and membrane association of Rab5 effectors (Mace, Miaczynska et al., 2005). Such a

567   mechanism may account for the mobilization of Rab5 from the endosomal membrane, which is

568   suggested by increased in cytosolic Rab5 and decreased colocalization of Rab5 with the endosomal

569   EEA1 (Figure 6A-C). These phenotypes correlated to a defect in endosomal sorting (Figure 1E) and a

570   block in transferrin uptake (Figure 6D,E), which may be yet another protective mechanism in order to

571   avoid iron overload and toxicity associated with neurodegeneration (Nunez, Urrutia et al., 2012).

572   Interestingly, hippocampal HT-22 neurons exposed to excess iron exhibit mitochondrial fragmentation

573   and a decrease in cell viability (Park, Lee et al., 2015). The metabolic effect of H_2O_2 on OCR is

574   intriguing. Mitochondria require oxygen to produce ATP to drive energy-consuming reactions (Bratic

575   & Trifunovic, 2010). The observed decrease in OCR (Figure 5E) may serve to lower cellular

576   respiration and prevent further ROS production.

577   If the stress response triggers solubilization of Rab5 from EE, then it must also catalyze the

578   activation of Rab5 on mitochondria. We discovered that this step depends on Alsin. The C-terminal

579   VPS9 domain of Alsin has GEF activity towards Rab5, and plays a role in Rab5 endosomal

580   localization and dynamics (Otomo, Hadano et al., 2003, Topp, Gray et al., 2004). However, the

581   physiological role of Alsin with respect to Rab5 and endosomal activity has remained somewhat

582   mysterious. At steady state, Alsin is mainly cytosolic with a fraction localizing to vesicular-like

583   structures (Figure 10–figure supplement 1C) (Millecamps, Gentil et al., 2005). In stress-induced

584   conditions, however, Alsin re-localizes to mitochondria. The N-terminal RLD of Alsin has been shown

585   to exhibit an autoinhibitory effect on its VPS9 domain (Otomo et al., 2003). We posit that

586   mitochondrial-induced stress triggers structural changes in the protein, releasing the autoinhibitory

587   effect of RLD, thereby exposing the VSP9 domain for Rab5 activation and recruitment. Alsin was

588   required for Rab5 translocation to mitochondria as this was severely diminished in Alsin$^{-/-}$ iPS-sMN

589   cells. The presence of (low levels) Rabex-5 on mitochondria (Figure 10A,C) detected in HeLa cells

590   suggests that other GEFs may contribute to some Rab5 activation, depending on the cell types, but

591   cannot fully compensate for the loss of Alsin function. A homologous gene, ALS2CL, containing only

592   the carboxyl-terminal half of ALS2, may also play a role by specifically binding to Rab5 and forming a

593   homodimer with the full-length Alsin to membranous compartments (Hadano, Otomo et al., 2004,

594   Suzuki-Utsunomiya, Hadano et al., 2007).

595   What is the function of the assembly of Rab5 and Rab5 machinery (Alsin and Rabenosyn-5) on

596   mitochondria besides its protective role? The stress response triggers remodeling of mitochondria to

597   confer molecular features characteristic of the endocytic system. The Rab5 machinery may be used to

598   bring mitochondria in close proximity to EE and form MCS (Figure 1). These MCS may mediate the

599   transfer of lipids and metabolites (Helle, Kanfer et al., 2013), or involve in "patching" up

600   mitochondrial wounds by recruiting the ESCRT machinery for closure, or other endomembranes for

601   fusion, both of which are observed in the PM-repair response (Jimenez, Maiuri et al., 2014, Reddy,

602   Caler et al., 2001). Worth noting from our EM study, we observed ER-like membranous structure in

603   contact with stressed mitochondria (Figure 3C) and considering the role of ER-mitochondria contacts

604   in Ca^{2+}-regulated apoptosis (Pinton, Giorgi et al., 2008), one may postulate that an orchestrated three-

605   way organelle crosstalk exists.

606   Considering that Rab5 is necessary for the biogenesis of the endolysosomal system (Zeigerer et

607   al., 2012), the Rab5 translocation may be a priming step of a stress response pathway that subjects

608   mitochondria to interact with the entire endolysosomal system, in order to modulate the mitochondrial

609   apoptotic potential. One quality control mechanism is the formation of mitochondrial-derived vesicles

610   (MDVs), which are involved in the transport of oxidized or damaged cargo to LE and lysosomes for

611   degradation (Soubannier, McLelland et al., 2012). This process depends on PINK1/Parkin (McLelland,

612   Soubannier et al., 2014), but can also occur independently (Matheoud, Sugiura et al., 2016). Rab5 may

613   play a role in MDV formation, although we could not detect vesicle budding events in our

614   experimental conditions. Once recruited onto mitochondria, Rab5 activity may not be limited to the

615   recruitment of its effectors, but initiate a more extensive endosomal Rab cascade via the Rab

616   coupling/conversion mechanism. On EE, Rab5 interacts with divalent effectors, coupling its activity to

617   other Rab proteins (e.g. Rab4, Rab11) that are required for receptor recycling (de Renzis, Sonnichsen

618   et al., 2002, Vitale, Rybin et al., 1998). Rab5 also initiates the activation of Rab7, resulting in the

619   conversion of EE into LE (Rink, Ghigo et al., 2005). The Rab coupling/conversion may also be

620   initiated on the mitochondria. Therefore, it is possible that the mitochondria-endosome MCS may

621   evolve over time leading to a Rab7-dependent mitophagic pathway (Jimenez-Orgaz, Kvainickas et al.,

622   2017), the engulfment of mitochondria by the EE (Hammerling, Najor et al., 2017), or conventional

623   autophagic processes (Ao, Zou et al., 2014, Stolz, Ernst et al., 2014). Future work exploring the

624   dynamics of other endosomal Rab GTPases in relation to Rab5 will be necessary to elucidate the

625   precise role of the endosomal system on mitochondria.

626   The physiological role of Alsin, although elusive, has been linked to both endosomes and

627   mitochondria. Cultured hippocampal neurons from Alsin knockout mice display an accumulation of

628   enlarged Rab5 endosomes and a reduced endosomal motility (Lai, Xie et al., 2009). Mutational and

629   linkage analysis of Alsin from human patients show that the VPS9 domain is critical for Alsin function

630   (Daud, Kakar et al., 2016, Verschuuren-Bemelmans, Winter et al., 2008). A recent EM study on the

631   corticospinal motor neurons (CSMN) from Alsin KO mice reveals a selective morphological defect in

632   mitochondria with enlarged core and broken cristae (Gautam, Jara et al., 2016). Interestingly, WT vs

633   Alsin KO CSMN show no change in Parkin expression, suggesting that mitophagy does not play a

634   major role (Gautam et al., 2016). We postulate that the pathological condition of mitochondrial defects

635   in Alsin KO cells is related to a deficiency in Rab5 recruitment to mitochondria, thereby leading to a

636   decline in protection from ROS and oxidative stress associated with aging. In ALS patients, motor

637   neurons likely accumulate more damaged mitochondria as age progresses, which eventually become an

638   overburden for cells.

639   The primary cause for ALS is still unclear, but oxidative stress is considered to be a major

640   contributor. Mutations in the anti-oxidant enzyme, superoxide dismutase 1 (SOD1), are associated with

641   motor neuron degeneration. In mouse models, an accumulation of the SOD1 mutant proteins results in

642   mitochondrial swelling and increased oxidative damage (Jaarsma, Rognoni et al., 2001). Interestingly,

643   loss of Alsin in the mutant SOD1 transgenic mice exacerbates and accelerates disease progression

644   (Hadano, Otomo et al., 2010). These studies, along with our findings, corroborate the protective role of

645   Alsin during oxidative stress. The mechanistic link between Rab5 and Alsin may present a general or

646   related mechanism in other neurodegenerative diseases. In Parkinson disease, the most common

647   mutation in the multidomain Leucine-rich repeat kinase 2 (LRRK2) protein leads to a hyper-activation

648   of the kinase domain, resulting in hyper-phosphorylation of a number of Rab GTPase substrates

649   including Rab5 (Steger, Diez et al., 2017, Steger, Tonelli et al., 2016). This may present yet another

650   mechanism of regulating Rab5 localization and function on mitochondria. Future work using different

651   neurodegenerative disease models in differentiated human neurons will provide deeper insights into the

652   disease etiology.

653

Materials and Methods:

Cell lines, cell culture, and growth conditions

The following cell lines have been validated and tested negative for mycoplasma contamination: HeLa (Kyoto) cell line, BAC HeLa GFP expressing cell lines, and human KOLF_C1 iPSC (kindly provided by Bill Skarnes, Sanger Institute). HeLa cells were cultured in high glucose DMEM (Gibco) with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine (all reagents from Sigma-Aldrich) with 5% CO_2 at 37°C. All plasmids were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. All bacterial artificial chromosome (BAC) transgene HeLa cell lines expressing different markers were obtained from the BAC recombineering facility at MPI-CBG (Dresden, Germany) and generated using the method previously described (Poser, Sarov et al., 2008).

Plasmids and chemical reagents

Construction of the pEGFP-C3-2xFYVE was made using mouse Hrs FYVE domain containing a linker (QGQGS) (Raiborg, Bremnes et al., 2001). Human Rab5c cDNA was subcloned into the pEGFP-C3 plasmid (Addgene). Human Alsin cDNA subloned into the pEF1/Myc-His (Invitrogen) plasmid was a kind gift from Dr. Ikuo Nishimoto (Kanekura, Hashimoto et al., 2004). Alexa-conjugated transferrin (Invitrogen; T13342) and EGF (Invitrogen; E13345) were used at 25 µg/ml and 2 µg/ml, respectively. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was purchased from Sigma Aldrich (C2759). Stock solution was prepared to a final concentration of 10 mM in DMSO. 100 mM Hydrogen peroxide (H_2O_2) (Merck Millipore; 7722-84-1) stock solution was prepared in PBS.

677 **Live-cell imaging**

678 Cells were seeded either in a 35-mm glass bottom dish or ibidi µ-Dish 35-mm, high Grid-500. Before

679 imaging, medium was replaced with HEPES-buffered DMEM without phenol red (Gibco). Time-lapse

680 imaging was performed using the Nikon TiE inverted stand microscope equipped with spinning disc

681 scan head (CSU-X1; Yokogawa), fast piezo objective z-positioner [Physik Instrumente], and back-

682 illuminated EMCCD camera (iXon EM+ DU-897 BV; Andor). Imaging was done with an Olympus

683 UPlanSApo 100x 1.4 Oil and Nikon Apo 100x 1.49 Oil DIC 0.13–0.20 objectives (illumination by

684 lasers: DPSS-488nm, DPSS-561nm, DPSS-640nm). Individual planes were recorded at ~11 frames per

685 sec with Z-stacks of three planes (step 0.3 µm).

686

687 **Photosensitization of mitochondria**

688 Cells were incubated with MitoTracker Red CMXRos (ThermoFisher; M7512) at a final concentration

689 of 100 nM at 37°C for 30 min, 5% CO_2 incubator, and followed by 2X PBS wash before irradiating

690 with 561 nm laser on the spinning disc Andor-Olympus-IX71 at a low power dosage of ~5 J/cm^2 for 60

691 sec.

692

693 **Correlative light electron microscopy**

694 Cells were grown on a gridded dish (ibidi µ-Dish 35-mm, high Grid-500). Cells in different locations

695 were laser-treated with 561 nm laser for 30 sec. Cells were fixed in 2.5% glutaraldehyde/PBS for 30

696 min at room temperature. Post-fixation and embedding were performed using 1% osmium

697 tetroxide/1.5% potassium ferrocyanide and Epon Lx112, respectively. Sectioning of 150 nm thick UA

698 sections was performed on a Leica Ultracut UCT (Leica Microsystem, Wetzlar, Germany) with a

699   diamond knife. Samples were post-stained with 2% uranyl acetate and lead citrate. 2D images were

700   acquired on a Tecnai T12 (FEI, Hillsboro, Oregon, USA).

701

702   **Immunofluorescence and antibodies**

703   Cells were seeded on a ibidi Grid-500 glass bottom. After laser or H_2O_2 treatment, cells were fixed in

704   4% paraformaldehyde/PBS for 15 min at room temperature. Cells were washed twice with PBS and

705   permeabilized in PBS containing 0.1% saponin, and 1% BSA for 30 min at room temperature. Cells

706   were immunostained with the corresponding primary antibodies: anti-rabbit Rabenosyn-5/ZFYVE20

707   (Sigma Aldrich: HPA044878), anti-mouse EEA1 (BD Biosciences: 610457), anti-rabbit TOM20

708   (Santa Cruz Biotechnology: sc-11415), anti-rabbit APPL1 (Abcam: ab59592), anti-mouse APPL2

709   (home-made), anti-mouse Rab5 (BD Biosciences: 610724), anti-mouse cytochrome c (Abcam:

710   ab6311), and anti-rabbit Alsin (Novus Biological: NBP2-14284) antibodies. Alexa fluor-conjugated

711   (ThermoFisher) were used as secondary antibodies. Samples were mounted with Mowiol (Sigma-

712   Aldrich) on glass slides and examined using the Zeiss LSM 880 inverted single photon point scanning

713   confocal system with Quasar detector (32 spectral detection channels in the GaAsP detector plus

714   2PMTs) and transmitted light detector. Acquired images were processed and saved using the Zeiss

715   ZEN software. For immunofluorescence on iPSCs, smNPCs, and sMNs, cells were fixed with 4%

716   formaldehyde for 15 min, washed three times with wash buffer (0.3% Triton-X in PBS) for 5-10 min,

717   and blocked with blocking buffer (5% goat serum, 2% BSA, and 0.3% Triton-X in PBS) at room

718   temperature for 1 hr. Cells were incubated with primary antibodies in blocking buffer overnight at 4 °C.

719   After three washes with PBS for 10 min, cells were incubated with secondary antibodies in wash

720   buffer for 2-3 hr at room temperature followed by three washes in PBS for 10 min. Primary antibodies

721   used include: goat anti-ChAT (1:100) (Millipore, #AB144P), mouse anti-HB9 (1:50) (DSHB,

722 #81.5C10, conc.), rabbit anti-ISL1 (1:100) (Abcam, #ab20670), mouse anti-LIN28 (1:1000) (Cell

723 signaling, #5930S), chicken anti-MAP2 (1:1000) (Novus Biologicals, #NB300-213), mouse anti-

724 Nestin (1:150) (R&D Systems, #MAB1259), rabbit anti-OCT4 (1:500) (Abcam, #ab19857), rabbit

725 anti-PAX6 (1:300) (Covance, #PRB-278P), and rabbit anti-SOX2 (1:500) (Abcam, #ab97959). Ad-Ctrl

726 and Ad-tBid (a kind gift from Dr. Heidi McBride) were used at 1:200 PFU/cell.

727

728 **Transferrin uptake**

729 Cells were seeded in a 384-well plate and incubated with either complete medium or in the presence of

730 250 μM H_2O_2 at 37ºC for 2 hr. Cells were then pulsed with Alexa-647 Tfn (10 μg/ml) for 5 min,

731 followed by 3x PBS wash, fixed with 3.7% PFA for 15 min, and then stained with DAPI (1:1000) and

732 CellMask Blue (1:2000) (ThermoFisher). Image acquisition was performed via the automated confocal

733 imaging system, CV7000S Yogokawa. Images analysis were performed using MotionTracking

734 software.

735

736 **Subcellular fractionation**

737 Cytosolic and mitochondrial fractions were performed using the mitochondria isolation kit, according

738 to the manufacturer's protocol with minor modification (ThermoFisher: cat89874). Cells (~1 x 10^7)

739 were resuspended in 400 μl Mitochondrial Isolation Reagent A. Cells were chemically lysed by adding

740 5 μl of Reagent B. After 5 min incubation on ice, 400 μl of Reagent C was added to each sample and

741 centrifuged at 720 x g for 10 min. The post nuclear supernatant (PNS) was transferred to a new

742 eppendorf tube and centrifuged at 3000 x g for at 4ºC for 15 min. For the total membrane and purified

743 cytosolic fractions, the PNS was clarified at 200,000g at 4ºC for 1 hr. The resulting supernatant was

744 collected and trichloroacetic acid (TCA)/acetone precipitation was performed to obtain the final

745   cytosolic fraction. The remaining pellet was washed by adding 500 µl of Reagent C and centrifuged at

746   15,000 x g for 5 min. Final samples were resuspended in SDS loading buffer.

747

748   **Cytochrome c release assay and Western blot**

749   Cells were seeded on a 12-well plate. For hydrogen peroxide treatment, reagent was added directly into

750   the well to achieve the appropriate concentration. Separation of mitochondrial and cytosolic fractions

751   were performed using the mitochondrial isolation kit from ThermoFisher (cat:89874) with an

752   additional step of trichloroactic acid precipitation of the cytosolic fraction. The final pellet was dried in

753   a 95ºC heat block for 2-3 min before resuspending it in the SDS loading buffer. Cell lysates were

754   separated by SDS-PAGE, transferred onto the nitrocellulose membrane and blocked in 5% milk in

755   PBS containing 0.1% Tween. Primary and secondary antibodies were diluted in theblocking buffer and

756   incubated at room temperature for 2 hr. The bands were detected using the electrochemiluminescence

757   reagent and exposure onto x-ray films. The following antibodies were used in Western blot: anti-

758   mouse cytochrome c (Abcam: ab13575), anti-rabbit gamma tubulin (Sigma-Aldrich: T6557), anti-

759   rabbit Rabenosyn-5/ZFYVE20, anti-rabbit Alsin (Sigma Aldrich: SAB4200137), anti-mouse EEA1

760   (BD Biosciences: 610457), anti-rabbit APPL1 (Abcam: ab59592), anti-mouse APPL2 (home-made),

761   anti-mouse GAPDH (Sigma Aldrich: G8795), anti-mouse gamma tubulin (Sigma Aldrich: T6557), and

762   anti-rabbit TOM20 (Santa Cruz Biotechnology: sc-11415).

763

764   **The caspase-3/7 activation assay**

765   The caspase-3/7 activation in CCCP- vs H_2O_2- treated cells were measured using the caspase-3/7 green

766   flow cytometry assay kit (ThermoFisher: C10427). After 2 hr treatment, cells were scraped off per well

767   from a 6-well dish, gently resuspended in 1 ml of PBS containing 1 µl of green detection reagent, and

768   incubated at 37ºC for 20 min. Samples were analyzed using the 488-nm excitation with standard

769   fluorescence compensation and emission filter (530/30 BP) in the FACS Calibur (Beckton Dickinson).

770   The gating was set based on the background signal in the DMSO control. The total cell count was set

771   to 5000. The caspase-3/7 activation in Alsin and Rab5 over-expressed cells was measured using the

772   caspase-3/7 green detection reagent (ThermoFisher: C10423). Cells were incubated in a complete

773   medium containing 5 µM of green detection reagent at 37ºC for 30 min prior to fixation. Cells were

774   imaged using the Zeiss LSM 880 microscope.

775

776   **Measurements of mitochondrial oxygen consumption rates**

777   20,000 HeLa cells per well were seeded in a XFe96-well plate (Seahorse Bioscience), and grown to

778   ~80% confluency after overnight incubation. Cells were then equilibrated with a bicarbonate-free

779   DMEM medium containing 4 mM glutamine and 10 mM galactose in a 37ºC ambient CO_2 incubator

780   for 1 hr, before starting the experiment. CCCP and H_2O_2 compounds were prepared fresh and diluted

781   in the assay medium, and were injected from the reagent ports at the indicated time. Oxygen

782   consumption rates (OCR) were measured using a Seahorse Extracellular Flux Analyzer.

783

784   **Generation of CRISPR/Cas9 knockout in human induced pluripotent cells**

785   Human KOLF_C1 iPSC were cultured in feeder-free conditions on Matrigel with TeSR-E8 media

786   (StemCell, Germany). For ALS2/Alsin knockout using CRISPR/Cas9 genome editing, 350,000 cells

787   were detached using Accutase, washed once with PBS and electroporated using the Neon Transfection

788   System (Invitrogen, Germany, 10ul kit, 1000V, 20ms, 3 pulses). The genomic sequence of human

789   Alsin was analyzed for CRISPR/Cas9 target sites by Geneious 8.1.6 software (Biomatters), and two

790   pairs of guides flanking a critical exon (exon3) were selected (5'-GCTAAAGTACTGAATTTTGG-3'

791   and 5'-AATAAAATCAGCAGGTGTGG-3'; 5'-GAATTTCTACAAAGTGCAGG-3' and 5'-

792   TAGCCTGGATGATGGCCGTT-3') and were used together to cause a frame shifting exon deletion.

793   The in vitro efficiency of these gRNAs was assessed by generating a genomic PCR cleavage template

794   of 3.4 bp (primers used: for-CCTCCCTTCCCAGGATCTGA and rev-

795   TGCTCAACTCGAGTGCCTTT; for-CAGGGTGAGCATCCCACATT and rev-

796   AGGAGTTCCAGTCAACCAGT) and incubating with recombinant Cas9. All gRNAs used in vitro

797   were identical in sequence to the DNA sense strand and not complementary to the mRNA sequence.

798   The RNAs employed in this method were chemically-modified and length optimized variants of the

799   native guide RNAs (Alt-R^{TM} CRISPR crRNAs and tracrRNA, Integrated DNA Technologies,

800   Coralville, IA, USA). The recombinant Cas9 (provided by Protein Expression Facility at MPI-CBG)

801   protein from *Streptococcus pyogenes* was used. The crRNAs were mixed with trRNA and NLS-Cas9

802   (1 µg/µl). The guide RNA complex was formed by mixing the crRNAs and tracrRNAs in equal

803   amounts in Buffer R (Invitrogen, Germany) at 100 µM. Five days after electroporation, cells were

804   pooled and seeded for clonal dilution. Single clones were mechanically picked and amplified. Next,

805   genomic DNA was isolated using QuickExtract DNA Extraction Solution (EpiCentre, USA).

806   Homozygous deletions were verified by PCR and sequencing.

807

808   **Generation of iPSC-derived smNPC, and differentiation of smNPCs to sMNs**

809   All procedures were performed as previously described (Reinhardt, Glatza et al., 2013). Briefly, for

810   smNPC generation, iPSC colonies detached from Matrigel-coated wells (by 1 mg/ml dispase) were

811   resuspended in hESC medium (DMEM/F12, 20% KnockOUT Serum Replacement, 1%

812   Penicillin/Streptomycin/Glutamine, 0.1 mM Non-Essential Amino Acids Solution, 0.05 mM beta-

813   mercaptoethanol, without bFGF supplemented with 10 µM SB431542 (Tocris, #1614), 1 µM

814   dorsomorphin (Tocris, #3093), 3 μM CHIR99021 (Axon Medchem, #Axon-1386) and 0.5 μM

815   purmorphamine (STEMCELL Technologies, #72202), and cultured in non-coated petri dishes. After

816   two days, hESC medium was replaced by N2B27 medium (1:1 mixture of DMEM/F12 and Neurobasal

817   medium, 1% Penicillin/Streptomycin/Glutamine, 1:100 B-27 supplement minus vitamin A, 1:200 N-2

818   supplement) and supplemented with the same small molecules as listed above. After another two days,

819   culture medium was replaced by smNPC expansion medium (N2B27 medium supplemented with 150

820   μM ascorbic acid (Sigma, #A4403), 3 μM CHIR99021 and 0.5 μM purmorphamine). On day 6 of

821   neural induction, embryonic bodies were broken into smaller clumps by titration and plated in 6 wells

822   of a Matrigel-coated 12-well plate. On day 9, cells were passaged for the first time using Accutase at a

823   1:3 split ratio and seeded in 4 wells of a Matrigel-coated 6-well plate. Afterwards, cells were passaged

824   once a week and seeded at a density of 1 x 10^6 cells per well. To obtain a highly pure smNPC culture,

825   smNPCs were propagated for at least 10 passages in smNPC expansion medium. For differentiation of

826   smNPC to MNs, smNPCs were seeded at a density of 1.5 x 10^6 cells per one well of a Matrigel-coated

827   6-well plate and cultured in N2B27 medium supplemented with 1 μM purmorphamine for the first two

828   days of differentiation. The cells were then cultured in N2B27 medium supplemented with 1 μM

829   purmorphamine and 1 μM retinoic acid (Sigma, #R2625) until day 9 of differentiation. On day 9, cells

830   were dissociated using Accutase and plated on polyornithine/laminin-coated ibidi μ-slides (at a density

831   of 150000 cells per well) or Nunc 4-well plates (at a density of 300.000 cells per well) in maturation

832   medium (N2B27 medium supplemented with 0.5 mM cAMP (Sigma, #D0627), 10 ng/ml BDNF

833   (Peprotech, #450-02-10), and 10 ng/ml GDNF (Peprotech, #450-10-10)). Cells were maintained in

834   maturation medium until analysis on day 28.

835

836   **Image and statistical analysis**

837   Image resizing, cropping and brightness were uniformly adjusted in Fiji (http://fiji.sc/). Colocalization

838   analysis was performed using MotionTracking software (Rink et al., 2005) (http://motiontracking.mpi-

839   [cbg.de/get/](http://motiontracking.mpi-cbg.de/get/)) and described previously (Gilleron, Querbes et al., 2013). The y-axis is expressed as the

840   ratio of co-localized objects (eg. A to B) to total objects found in A. Final images were assembled

841   using Adobe Photoshop and Illustrator. Densitometry quantification were performed in Fiji following

842   the previously described protocol (http://www.yorku.ca/yisheng/Internal/Protocols/ImageJ.pdf). P-

843   values were calculated by two-tailed t-test using GraphPad Prism7.

844

855

856   **Competing interests**

857   No competing interests exist.

858

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Figure Legends:

Figure 1. Endosomal contacts with mitochondria. (**A**) and (**B**) HeLa cells were transfected with

TagRFP-MTS (mitochondria targeting sequence) and labeled with either Alexa-488 transferrin (Tfn)

or epidermal growth factor (EGF) at 37°C for 10 min, respectively. (**C**) HeLa cells stained with Mito-

Red were labeled with Tfn-488 or EGF-488 at 37°C for 10, 60 min, or treated with 250 µM H_2O_2 after

10 min labeling followed by additional 50 min incubation. Inset regions are shown. Scale bars, 10 µm.

(**D**) Colocalization analysis based on subtraction of random colocalization was performed between

Mito-Red and Tfn-488 or EGF-488 from images obtained in Figure 1C, $n = 50$. (**E**) Quantification of

total intensity per cell, $n = 50$. Error bars represent SEMs. P-values based on two-tailed t-tests.

Figure 2. Recruitment of Rab5 to mitochondria upon laser-induced stress. (**A**) Live BAC GFP-

Rab5 HeLa cells were labeled with 100 nM Mito-Red at 37°C for 30 min. Cells were photoirradiated

with a low dosage of 561 nm laser (~5 J/cm^2) for 60 sec. A snapshot of a cell before (Pre) and 20 min

post-laser were taken. Arrowheads indicate GFP-Rab5-positive endosomes that are in close proximity

to mitochondria. (**B**) Colocalization analysis between Mito-Red and GFP-Rab5 after photoirradiation

as performed in (A), $n = 5$. Error bars represent SEMs. P-values based on two-tailed t-tests. (**C**) Live-

cell imaging of a BAC GFP-Rab5 HeLa cell treated the same way as in (**A**). (**D**) Quantification of the

GFP fluorescent signal over a period of 30 min from the inset region in (**C**). (**E**) Time-lapsed images of

Mito-Red, GFP-Rab5, and merged from the inset region in (**C**). (**F**) Live-cell imaging of a BAC GFP-

Rab5 HeLa cell photoirradiated only to the marked region (white box). The cell before laser irradiation

is shown (Pre). Snapshots of the cell were taken at 10 min and 20 min post-laser. Inset images show

the effect of laser treatment on mitochondrial morphology and network. Arrowheads indicate the

1030 recruitment of GFP-Rab5 (filled) and GFP-Rab5-positive endosomes near mitochondria (double-head).

1031 Scale bars, 10 μm.

1032

1033 **Figure 2–figure supplement 1. Rab5 recruitment to mitochondria is specific to Mito-Red.** (**A**)

1034 HeLa cells were labeled with 100 nM MitoTracker-Green FM (Mito-Green) at 37°C for 30 min and

1035 photoirradiated with 561 nm laser (~10 J/cm^2) for 60 sec. Image was taken at 20 min post-laser

1036 treatment. Mitochondrial network remained tubular. (**B**) HeLa BAC GFP-Rab5 cells were transfected

1037 with TagRFP-MTS and photoirradiated with 561 nm laser (~10 J/cm^2) for 60 sec. Image was taken at

1038 20 min post-laser treatment. (**C**) BAC GFP-Rab5 cells were seeded on gridded glass dishes, labeled

1039 with Mito-Red for 30 min, photoirradiated as before, and fixed after 30 min post-laser. Cells were

1040 immunostained with specific antibodies against Rab5 or TOM20. Inset regions are shown. Scale bars,

1041 10 μm.

1042

1043 **Figure 2–figure supplement 2. GFP-Rab5 enrichment on mitochondria persist for <60 min.**

1044 Time-lapse images of a cell following laser treatment as in Figure 2F. Inset images are shown at

1045 different time points (min). Arrowheads indicate the recruitment of GFP-Rab5 to mitochondria within

1046 15 min, which sustain for at least 60 min. Scale bars, 10 μm.

1047

1048 **Figure 3. Membrane contacts between Rab5-positive mitochondria and endosomes.**

1049 Ultrastructural analysis of cells upon laser-induced stress. (**A**) Flow chart of the experimental set up.

1050 BAC GFP-Rab5 cells were seeded on gridded dishes, labeled with 100 nM Mito-Red at 37ºC for 30

1051 min. A cell of interest was then photoirradiated with a low dosage of 561 nm laser (~5 J/cm^2) for 60

1052 sec. Live-cell imaging between GFP-Rab5-positive endosomes and mitochondria was assessed for

1053   organelle dynamics. The same cell was re-imaged for post-fixation analysis before processing for EM.

1054   (**B**) Fluorescent images of GFP-Rab5 and (Mito-Red) after fixation with glutaraldehyde. Box region

1055   shows a GFP-Rab5 endosome (red arrowhead) next to a swollen Rab5-positive mitochondrion. The

1056   transmission electron micrograph (TEM) of the same cell is shown (right panel). The nuclear

1057   membrane (dotted line) and mitochondria (red star) were used as fiducial markers. (**C**) Zoom-in

1058   images of the box region in (**B**). Colored images indicate the GFP-Rab5-positive structure in green and

1059   mitochondrion in red. Raw images are shown below. Mitochondrial cristae are visible. The

1060   membranous structure adjacent to the mitochondrion likely corresponds to ER. Scale bars, 500 nm.

1061

1062   **Figure 4. Localization of endogenous LC3, Lamp1, Parkin, and Bax upon laser-induced stress.**

1063   BAC GFP-Rab5 cells were labeled with 100 nM Mito-Red at 37ºC for 30 min. Cells were acquired

1064   prior to laser irradiation as controls (Untreated) (**A**), (**C**), (**E**), and (**G**). Same cells were photoirradiated

1065   as before, fixed after 60 min post-laser treatment, and immunostained with specific antibodies against

1066   LC3 (**B**), Lamp1 (**D**), Parkin (**F**), and Bax (**H**). Inset images show the area before and after laser

1067   treatment. (**I**) Colocalization analysis of Mito-Red to Rab5, LC3, Lamp1, Parkin, and Bax, in untreated

1068   vs laser-induced conditions ($n = 3$). Untreated cells correspond to cells outside of the laser treated area.

1069   Error bars represent SEMs. P-values based on two-tailed t-tests. Scale bars, 10 μm.

1070

1071   **Figure 4–figure supplement 1. Localization of endogenous LC3, Lamp1, and Parkin in untreated**

1072   **cells.** BAC GFP-Rab5 cells were seeded on gridded glass dishes, and labeled with 100 nM Mito-Red at

1073   37ºC for 30 min. Cells were fixed and immunostained with antibodies against LC3, Lamp1, and Parkin.

1074   Images were taken from the area outside of laser-treated cells. Scale bars, 10 μm.

1075

1076 **Figure 4–figure supplement 2. BAC GFP -LC3, -Lamp1, and -Parkin localization upon laser-**

1077 **induced stress.** Live-cell imaging of BAC GFP-LC3 (**A**), GFP-LAMP1 (**B**), and GFP-Parkin (**C**) cells.

1078 Cells were labeled with 100 nM Mito-Red at 37ºC for 30 min and photoirradiated as before. Images of

1079 the same cell before (untreated) and 60 min post-laser treatment are shown. Inset images indicate the

1080 approximate area before and after laser treatment.

1081

1082 **Figure 4–figure supplement 3. CCCP induces Bax protein expression and localization on**

1083 **mitochondria.** (**A**) HeLa cells labeled with 100 nM Mito-Red were treated with either DMSO (Ctrl) or

1084 10 µM CCCP at 37ºC for 2 hr. Cells were fixed and immunostained with antibody against Bax. Scale

1085 bars, 10 µm. (**B**) Fluorescence fold change of Bax signals between Ctrl and CCCP-treated cells; $n = 50$.

1086 Values in controls cells are normalized to one. (**C**) Colocalization analysis between Mito-Red and Bax

1087 in Ctrl vs CCCP. Error bars represent SEMs. P-values based on two-tailed t-tests.

1088

1089 **Figure 4–figure supplement 4. tBid over-expression leads to Rab5 enrichment on mitochondria.**

1090 BAC GFP-Rab5 cells were infected with control adenovirus (Ad-Ctrl) or Ad-tBid at 37ºC for 12 hr.

1091 Cells were fixed and immunostained with TOM20 antibody. Inset regions are shown. Scale bars,

1092 10 µm.

1093

1094 **Figure 5. Effects of CCCP vs H_2O_2 on Rab5 recruitment to mitochondria.** BAC GFP-Rab5 HeLa

1095 cells labeled with 100 nM of Mito-Red were treated with either DMSO (Ctrl), 10 µM CCCP (**A**), or

1096 250 µM H_2O_2 at 37ºC for 2 hr (**B**). Cells were fixed and imaged by confocal microscopy. Inset regions

1097 reveal the effect on mitochondrial morphology and GFP-Rab5 localization upon treatment.

1098 Arrowheads indicate rounded and stressed mitochondria in both CCCP- and H_2O_2- treated conditions.

1099   Scale bars, 10 μm. (**C**) and (**D**) Colocalization analysis between Mito-Red and GFP-Rab5 in (**A**) and

1100   (**B**), respectively; n = 50. Error bars represent SEMs. P-values based on two-tailed t-tests. (**E**)

1101   Subcellular fractionation was performed in HeLa cells treated with DMSO (Ctrl), 10 μM CCCP, or 250

1102   μM H_2O_2 at 37°C for 2 hr. Protein samples from purified mitochondria (Mito) and cytosolic (C)

1103   fractions were loaded onto SDS-PAGE and imunoblotted with cytochrome c antibody. (**F**) Cells were

1104   treated the same way as in (**E**). Cells were then resuspended in live-cell imaging solution containing

1105   500 nM caspase-3/7 green flow cytometric reagent and incubated at 37°C for 30 min before subjecting

1106   to flow cytometric analysis. FITC signal (x-axis) is plotted against the total cell count (y-axis). The

1107   gating was set based on the background signal in DMSO control. (**G**) HeLa cells were seeded in the

1108   Seahorse 96-well plate format and incubated overnight. Growth medium was exchanged to

1109   bicarbonated-free low-buffered assay medium (provided by the manufacturer) supplemented with 10

1110   mM galactose immediately before the start of the experiment. Oxygen consumption rate measurements

1111   were measured in the Seahorse XFe96 Analyzer from cells injected with media only (purple), 250 μM

1112 H_2O_2 (green), or 10 μM CCCP (orange). Error bars represent SEMs.

1113

1114   **Figure 5–figure supplement 2. CCCP, but not H_2O_2, leads to Parkin recruitment.** (**A**) BAC GFP-

1115   Parkin or WT HeLa cells (**B**) were treated with either DMSO (Ctrl), 10 μM CCCP, or 250 μM H_2O_2 at

1116   37°C for 2 hr. Cells were fixed and imaged by confocal microscopy. HeLa cells were immunostained

1117   with Parkin antibody. Inset regions are shown. Scale bars, 10 μm.

1118

1119   **Figure 6. Hydrogen peroxide (H_2O_2) treatment decreases Rab5 membrane association on EE,**

1120   **increases early endosomal-mitochondrial contacts, and reduces transferrin uptake.** (**A**)

1121   Subcellular fractionation was performed in HeLa cells treated with H_2O_2 for 1 and 2 hr. The total

1122   membrane (M) fraction was obtained by centrifugation of the post nuclear supernatant at 200,000 g at

1123   4°C for 1 hr, and supernatant was taken as cytosolic (C) fraction. Protein samples were loaded onto

1124   SDS-PAGE and imunoblotted with antibodies against Rab5, EEA1, GAPDH, and TOM20. The long

1125   exposure blot for EEA1 is shown (right panel). (**B**) Densitometric quantification of Rab5 in (**A**). Band

1126   intensities were calculated as normalized ratio between Rab5 to TOM20 in the M fraction, and Rab5 to

1127   GAPDH in the C fraction. Fold change is plotted on the y-axis. Error bars represent SEMs from 3

1128   independent experiments. (**C**) BAC GFP-Rab5 HeLa cells were incubated with/out 250 µM H_2O_2 at

1129   37°C for 2 hr. Cells were fixed and immunostained with EEA1 antibody. Colocalization analysis was

1130   performed between GFP-Rab5 and EEA1. (**D**) HeLa cells were seeded in a 384-well plate and pre-

1131   treated with either PBS (control) or 250 μM H_2O_2 at 37°C for 10 min. Cells were then pulsed with

1132   Alexa-647 Tfn (10 μg/ml) for 5 min, washed with PBS, fixed, and stained with DAPI (nuclear) and

1133   CellMask Blue (cytoplasmic) dyes. Images were acquired by the Yogokawa confocal microscope.

1134   Scale bars, 10 μm. (**E**) Quantification of the cytoplasmic fluorescence intensity per cell, $n = 50$. P-

1135   values based on two-tailed t-tests.

1136

1137   **Figure 7. Rab5 regulates cytochrome c release during H_2O_2-induced stress.** (**A**) Live-cell imaging

1138   of a BAC GFP-Rab5 cell stained with 100 nM Mito-Red before (0 min) and after treatment (60 min)

1139   with 250 μM H_2O_2 at 37°C. Inset images are shown and arrowheads indicate GFP-Rab5 recruitment to

1140   MOMP events (right panel). Scale bars, 10 μm. (**B**) HeLa cells over-expressing either GFP or GFP-

1141   Rab5 were treated with 250 μM H_2O_2 at 37°C for 1 to 4 hr. Protein samples from cytosolic fractions

1142   were obtained and immunoblotted for cytochrome c and tubulin (as a loading control). (**C**)

1143   Densitometric quantification of cytochrome c release in (**B**). Data were collected from three

1144 independent experiments. Y-axis corresponds to the normalized ratio intensity of cytochrome c to the

1145 tubulin loading control. P-values based on two-tailed t-tests.

1146

1147 **Figure 8. H$_2$O$_2$-induced translocation of Rab5 to mitochondria is reversible.** HeLa cells were

1148 seeded in 24-well plates (**A**) or on glass coverslips (**B**), and after overnight incubation, were treated

1149 with either 250 µM or 500 µM H$_2$O$_2$ at 37ºC for 0, 6, 12, 24 hr, or replaced with a fresh medium at 12

1150 hr followed by additional 12 hr incubation. (**A**) Cells were then harvested in SDS loading buffer and

1151 protein lysates were loaded onto SDS-PAGE and immunoblotted with TOM20 and GAPDH antibodies.

1152 (**B**) Cells were fixed and immunostained with Rab5 and TOM20 antibodies. Inset regions are shown.

1153 Scale bars, 10 µm. (**C**) Colocalization analysis between TOM20 and Rab5 in (**B**), $n = 50$. P-values

1154 based on two-tailed t-tests.

1155

1156 **Figure 9. Localization of Rab5 effectors upon mitochondrial stress.** BAC GFP-Rab5 cells seeded

1157 on gridded dishes were labeled with Mito-Red and photoirradiated as before. Cells were fixed after 30

1158 min post-laser treatment and immunostained with antibodies against ZFYVE20 and EEA1 (**A**), or

1159 APPL1 and APPL2 (**B**). Inset regions are shown. Scale bars, 10 µm. (**C**) Colocalization analysis from

1160 untreated and laser-treated cells in (**A**) and (**B**), $n = 3$. P-values based on two-tailed t-tests. (**D**)

1161 Subcellular fractionation was performed in HeLa cells treated with either PBS (control) or 250 µM

1162 H$_2$O$_2$ at 37°C for 2 hr. Protein samples from purified mitochondria (Mito) and cytosolic (C) fractions

1163 were loaded onto SDS-PAGE and imunoblotted with antibodies against Rab5, EEA1, Rabenosyn-5,

1164 APPL1, APPL2, and TOM20.

1165

1166 **Figure 9–figure supplement 1. Localization of endogenous Rabenosyn-5, EEA1, APPL1 and**

1167 **APPL2.** BAC GFP-Rab5 cells were seeded on glass coverslips, fixed, and immunostained with

1168 antibodies against Rabenosyn-5 (**A**), EEA1 (**B**), APPL1 (**C**), and APPL2 (**D**). Inset regions are shown.

1169 Scale bars, 10 μm.

1170

1171 **Figure 9–figure supplement 2. Localization of GFP-2xFYVE^{Hrs} upon laser-induced stress.** HeLa

1172 cells were transfected with GFP-2xFYVE^{Hrs} for 24 hr, labeled with 100 nM Mito-Red, photoirradiated

1173 as before, and then imaged live. Images were acquired immediately post-laser (0 min) and after 60 min.

1174

1175 **Figure 10. Localization of Rabex-5 and Alsin upon mitochondrial stress.**

1176 BAC GFP-Rab5 cells seeded on gridded dishes were labeled with 100 nM Mito-Red and

1177 photoirradiated as before. Cells were fixed after 30 min post-laser treatment and immunostained with

1178 antibody against Rabex-5 (**A**) or Alsin (**B**). Inset regions are shown. Scale bars, 10 μm. (**C**)

1179 Colocalization analysis from untreated and laser-treated cells in (**A**) and (**B**), $n = 3$. P-values based on

1180 two-tailed t-tests.

1181

1182 **Figure 10-figure supplement 1. Endogenous localization of Rabex-5 and Alsin.** BAC GFP-Rab5

1183 cells were seeded on glass coverslips, fixed, and immunostained with specific antibodies against

1184 Rabex-5 (**A**) or Alsin (**C**). Inset regions are shown. Scale bars, 10 μm. (**B**) A schematic representation

1185 of the domain organization of human Alsin. The regulator of chromosome condensation 1 (RCC1), B-

1186 cell lymphoma (Dbl) homology (DH) and pleckstrin homology (PH), membrane occupation and

1187 recognition nexus (MORN) motif, and vacuolar protein sorting 9 (VPS9) domains are labeled. Three

1188 putative GEF domains are annotated.

1189

1190 **Figure 10-figure supplement 2. Over-expression of Alsin or Rab5 blocks caspase-3/7 activation.**

1191 (**A**) HeLa cells transiently expressing empty vector, Alsin, or Rab5 were incubated in the presence of

1192 500 μM H_2O_2 and 5 μM CellEvent caspase-3/7 green detection reagent at 37ºC for 2 hr. Nucleus was

1193 stained with DAPI (blue). Images were acquired by confocal microscopy. Caspase-activated cells

1194 displayed green signals, while non-activated cells (PBS control) showed no signal. Percent of cells

1195 with positive signals were quantified in (**B**), $n = 50$. Error bars represent SEMs. Scale bars, 10 μm.

1196

1197 **Figure 11. Alsin is required for Rab5 recruitment and regulates cytochrome c release.** (**A**) Flow

1198 chart depicting the different stages and time (in days) from induced-pluripotent stem cells (iPSC), to

1199 neuroprogenitor cells (NPC), and to mature spinal motor neurons (sMN). The small molecules and

1200 compounds used at different stages are shown and color-coded. (**B**) WT and Alsin^{-/-} cells were

1201 challenged with either PBS (Ctrl) or 100 μM H_2O_2 at 37°C for 1 hr. Cells were fixed and

1202 immunostained with Rab5 and TOM20 antibodies. Inset images show are shown. Scale bars, 10 μm.

1203 (**C**) Subcellular fractionation of mitochondrial (Mito) and cytosolic (C) fractions from WT and Alsin^{-/-}

1204 iPSC-sMN challenged with either PBS or 100 μM H_2O_2 at 37°C for 1 hr. Protein samples were loaded

1205 onto SDS-PAGE and imunoblotted with antibodies against Rab5, tubulin and TOM20. (**D**) Cytosolic

1206 fractions were prepared from WT and Alsin^{-/-} iPSC-sMN challenged with 100 μM H_2O_2 at 37°C for 1

1207 hr. Densitometric quantification of cytosolic cytochrome c were collected from three independent

1208 experiments. Y-axis corresponds to normalized ratio intensity of cytochrome c to tubulin. Error bars

1209 represent SEMs. P-values based on two-tailed t-tests.

1210

1211 **Figure 11–figure supplement 1. Validation of the CRISPR/Cas9 Alsin$^{-/-}$ cells in iPSC, NPC and**

1212 **iPSC-sMN.** (**A**) Electrophoresis result of the PCR reaction using primers flanking exon 3 and within

1213 exon 3. Homozygous deletion was confirmed by the absence of a 2.8 kb. (**B**) Protein lysates from WT

1214 KOLF and Alsin$^{-/-}$ iPSC were loaded onto SDS-PAGE and immunoblotted with Alsin antibody. A

1215 band detected at ~184 kDa, but absent in Alsin$^{-/-}$, corresponds to full-length Alsin. (**C**) WT KOLF,

1216 Alsin$^{-/-}$ iPSC, and neuroprogenitor cells (NPC) were immunostained with pluripotency markers Oct4

1217 and Lin28, and neuroprogenitor markers Sox2 and Pax6, respectively. (**D-E**) WT KOLF and Alsin$^{-/-}$

1218 iPSC-sMN were immunostained with motor neuron markers such as ChAT, HB9, and ISL1, nuclear

1219 dye DAPI, and cytoskeletal marker MAP2. (**F**) WT KOLF and Alsin$^{-/-}$ iPSC-sMN were immunostained

1220 with antibodies against Rab5 and Alsin, along with DAPI (nuclear) and phalloidin (actin) stains. Scale

1221 bars, 10 μm.

1222

1223 **Figure 12. Schematic model depicting the role of Rab5-Alsin-mitochondria during oxidative**

1224 **stress.** In the normal condition, mitochondria (Mito, red) are elongated and tubular (left). Rab5 (green)

1225 are localized on early endosomes (EE) to assemble the Rab5 machinery for endosomal maturation and

1226 membrane trafficking. At steady state, some EE make transient contacts with mitochondria. During

1227 oxidative stress (e.g.. laser- or chemically-induced), mitochondria undergo MOMP and a dramatic

1228 morphological transformation into rounded and swollen structures (right). The release of the apoptotic

1229 factor, cytochrome c, from mitochondria into the cytosol is associated with a re-localization event of

1230 Rab5 from EE to mitochondria via a cytosolic intermediate, accompanied by an increase in EE-

1231 mitochondria MCS. The recruitment and activation of Rab5 on mitochondria depend on the Rab5 GEF

1232 Alsin (blue), which leads to a selective recruitment of Rabenosyn-5 (light green). This signaling

1233   cascade on mitochondria is a reversible process that regulates the apoptotic program (e.g. cytochrome c

1234   release and caspase activation) and thus, promotes overall cell survival.

1235

1236   **Video 1. Dynamics of transferrin and mitochondria.** HeLa cells were labeled with Alexa-488

1237   transferrin (Tf; green) and Mito-Red (Mito; red). Images were acquired using a spinning disk confocal

1238   microscope at 11 frames/sec for ~6 min. Time stamp corresponds to min:sec:msec.

1239

1240   **Video 2. Dynamics of transferrin and mitochondria.** Zoom-in of a HeLa cell labeled with Alexa-

1241   488 transferrin (Tf; green) and Mito-Red (Mito; red). The movement of endosomes during a

1242   mitochondria fission event is shown. Images were acquired using a spinning disk confocal microscope

1243   at 11 frames/sec for ~5 min. Time stamp corresponds to min:sec:msec.

1244

1245   **Video 3. Dynamics of Rab5-positive endosomes and mitochondria.** Zoom-in of a BAC GFP-Rab5

1246   (green) cell labeled with Mito-Red (Mito; red). Images were acquired using a spinning disk confocal

1247   microscope at 11 frames/sec for ~4 min. Time stamp corresponds to min:sec:msec.

1248

1249   **Video 4. Mitochondria dynamics during laser-induced stress.** HeLa cells were labeled with Mito-

1250   Red and photoirradiated via 561 nm laser for 1 min, and immediately imaged. Time-lapse was

1251   acquired using a spinning disk confocal microscope at 11 frames/sec for ~5 min.

1252

1253   **Video 5. GFP-Rab5 recruitment to mitochondria during laser-induced stress.** Zoom-in of a BAC

1254   GFP-Rab5 (green) cell labeled with Mito-Red (Mito; red). Images were acquired using a spinning disk

1255   confocal microscope at 11 frames/sec with 1 min increment for ~30 min.

Video 6. Endosomes and mitochondria interactions upon laser-induced stress. BAC GFP-Rab5 (green) cells were labeled with Mito-Red (red) and photoirradiated via 561 nm laser for 1 min, and then imaged after 5 min. Time-lapse was acquired using a spinning disk confocal microscope for 20 frames with 5 sec increment for ~3 min. Boxed regions indicate GFP-Rab5 endosomes docking onto mitochondria.

Video 7. Endosomes and mitochondria interactions upon laser-induced stress. Zoom-in of a BAC GFP-Rab5 (green) cell labeled with Mito-Red (Mito; red) and photoirradiated via 561 nm laser for 1 min, and then imaged continuously for ~5 min. Images were acquired using a spinning disk confocal microscope at 11 frames/sec.

Video 8. Mitochondria dynamics during H_2O_2-induced stress. HeLa cells were labeled with Mito-Red and then imaged in the presence of 250 µM H_2O_2 for ~60 min. Time-lapse was acquired using a spinning disk confocal microscope for 50 frames with 2 min increment.

Figure 1D,E–source data. Numerical data corresponding to the graphs presented in Figure 1D,E.

Figure 2B–source data. Numerical data corresponding to the bar graphs presented in Figure B.

Figure 2D–source data. Numerical data corresponding to the line trace presented in Figure 2D.

Figure 4I–source data. Numerical data corresponding to the bar graphs presented in Figure 4I.

1279

1280 **Figure 4–figure supplement 3B,C-source data.** Numerical data corresponding to the bar graphs

1281 presented in Figure 4–figure supplement 3B,C.

1282

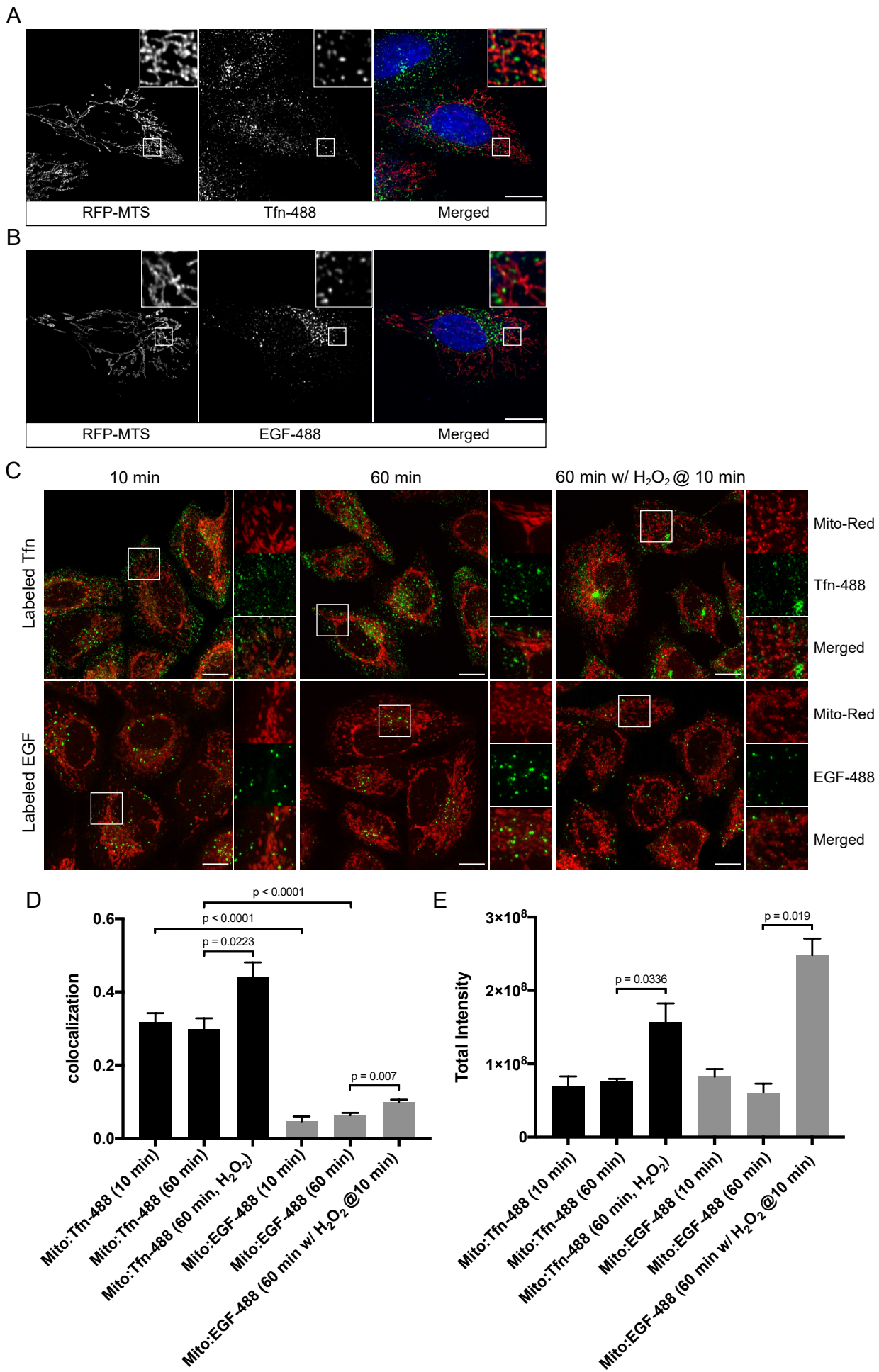1283 **Figure 5C,D–source data.** Numerical data corresponding to the bar graphs presented in Figure 5C,D.

1284

1285 **Figure 5G–source data.** Numerical data corresponding to the line traces presented in Figure 5G.

1286

1287 **Figure 6B–source data.** Numerical data corresponding to the bar graph presented in Figure 6B.

1288

1289 **Figure 6C–source data.** Numerical data corresponding to the chart presented in Figure 6C.

1290

1291 **Figure 6E–source data.** Numerical data corresponding to the bar graphs presented in Figure 6E.

1292

1293 **Figure 7C–source data.** Numerical data corresponding to the line traces presented in Figure 7C.

1294

1295 **Figure 8C–source data.** Numerical data corresponding to the bar graphs presented in Figure 8C.

1296

1297 **Figure 9C–source data.** Numerical data corresponding to the bar graphs presented in Figure 9C.

1298

1299 **Figure 10C–source data.** Numerical data corresponding to the bar graphs presented in Figure 10C.

1300

1301   **Figure 10–figure supplement 2B-source data.** Numerical data corresponding to the bar graphs

1302   presented in Figure 10–figure supplement 2B.

1303

1304   **Figure 11D–source data.** Numerical data corresponding to the line traces presented in Figure 11D.

1305

Figure 1

Figure 2

A

Gridded dish

I. Locate a cell of interest

II. Apply stress by photoirradiation & track endosomes-mitochondria interactions

III. Re-image cell after fixation

IV. Relocate the same cell & organelle of interest by serial section EM

B

5 µm

5 µm

C

Colored

500 nm

Raw

500 nm
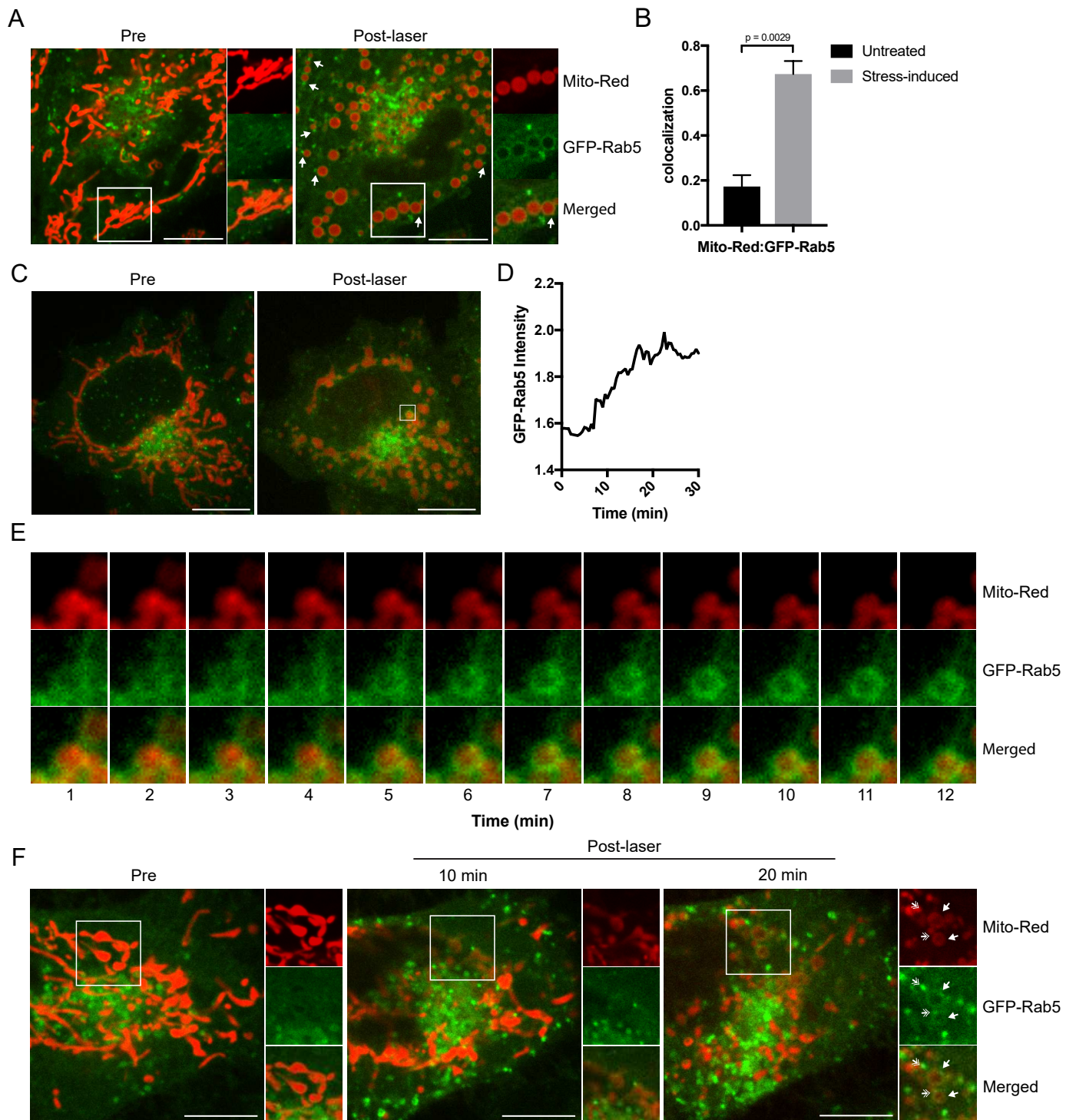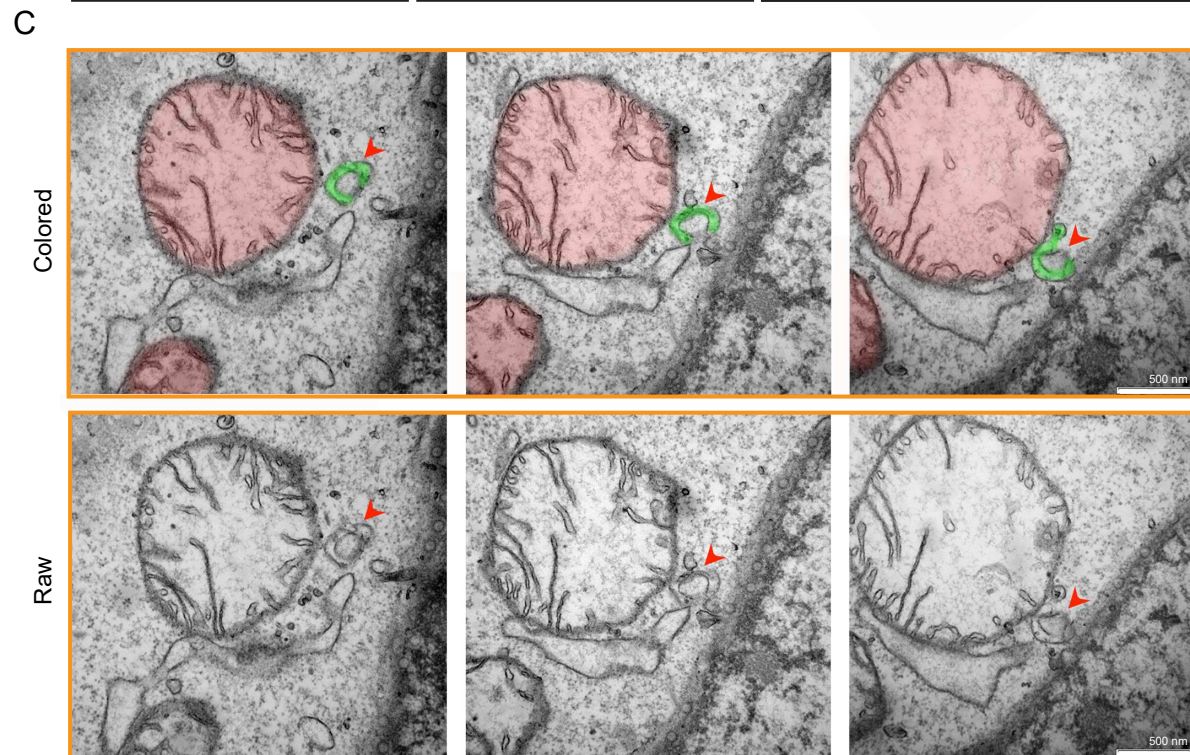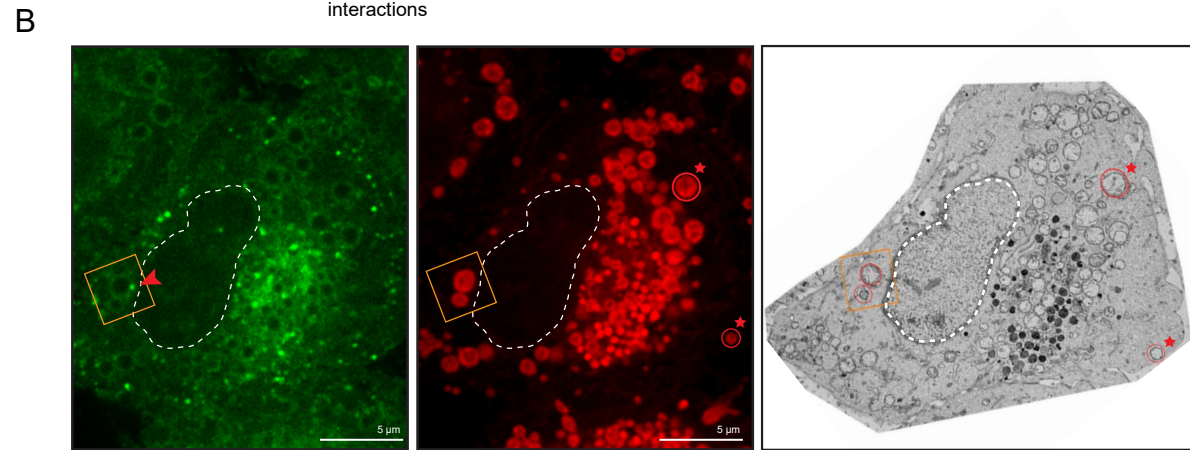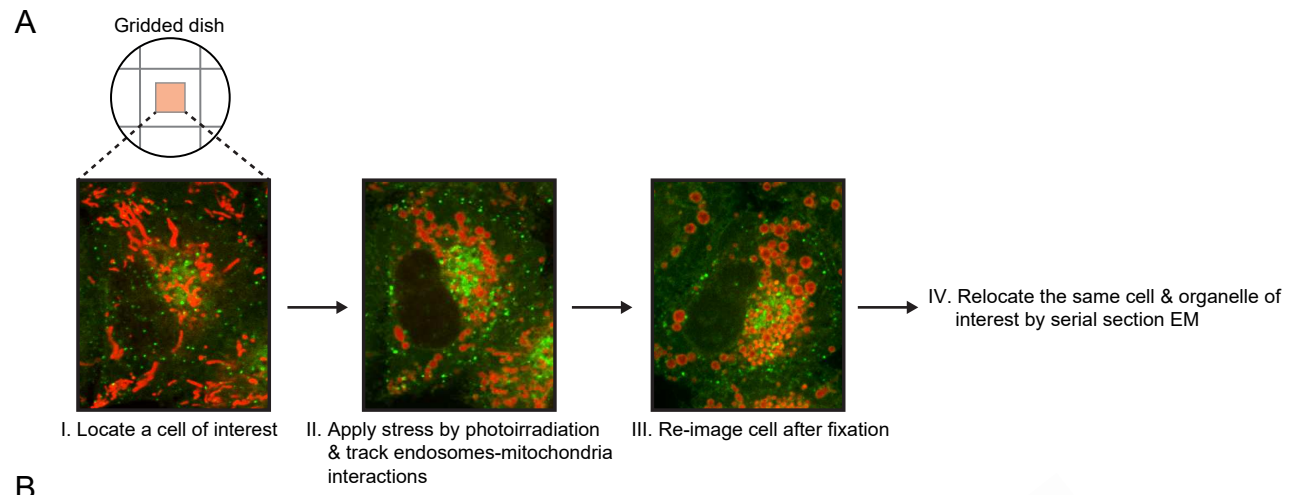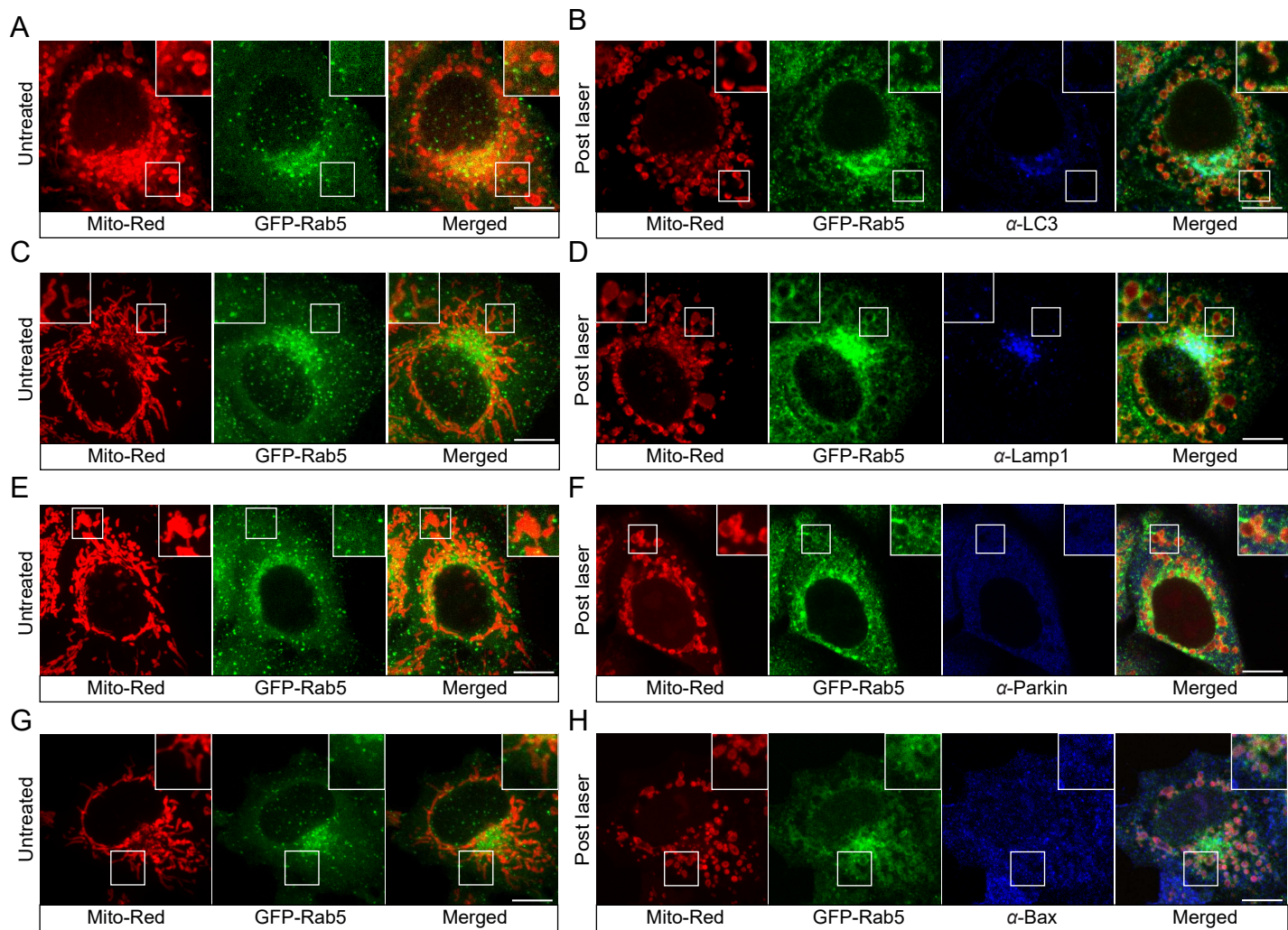
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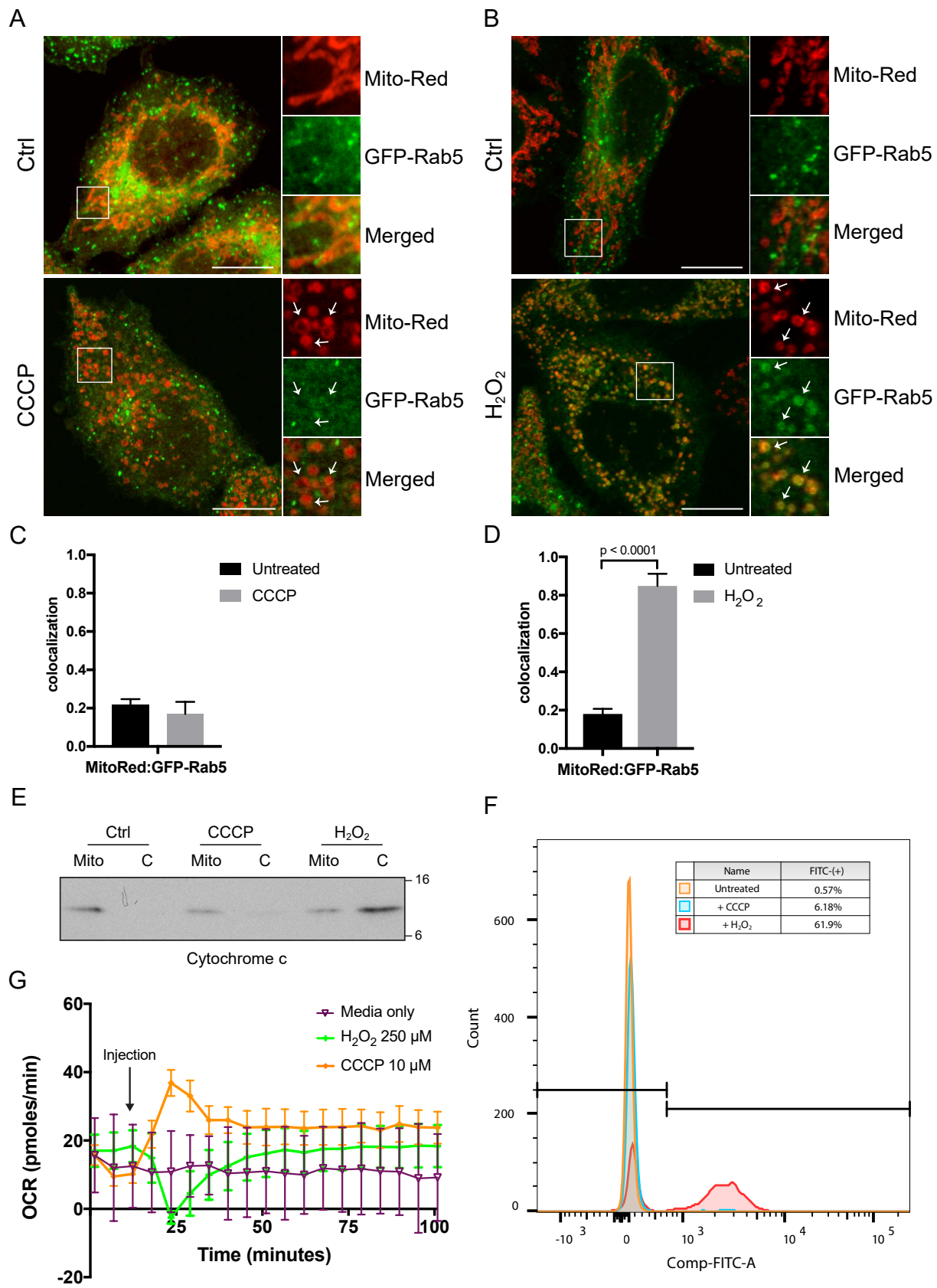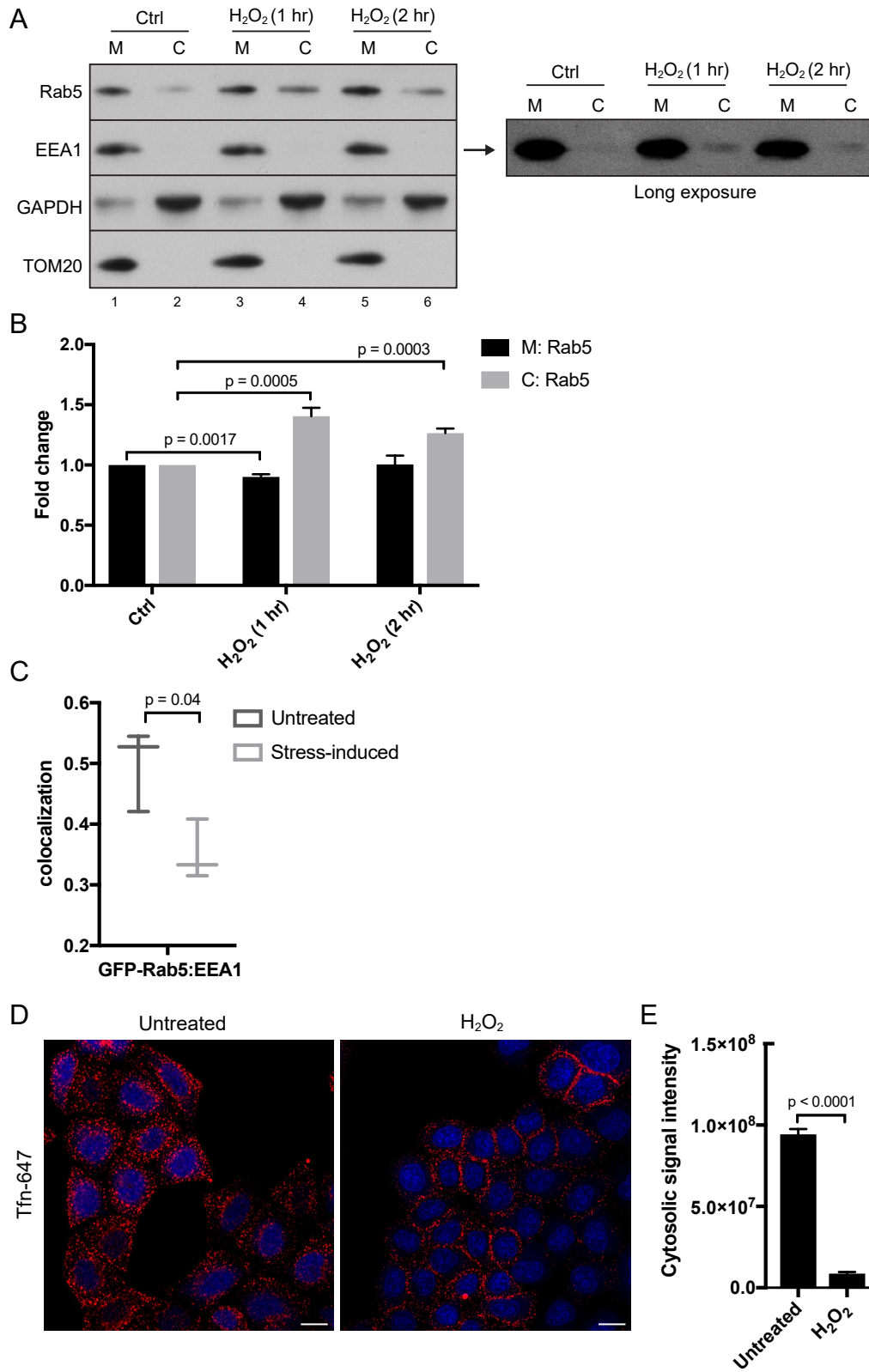
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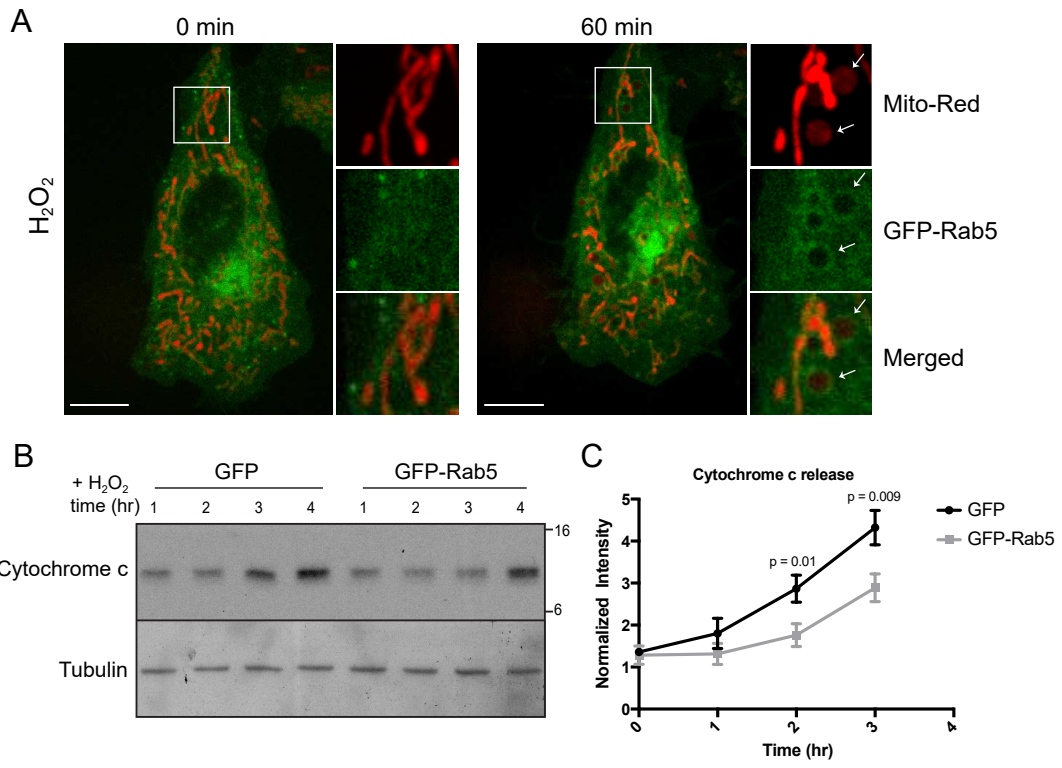
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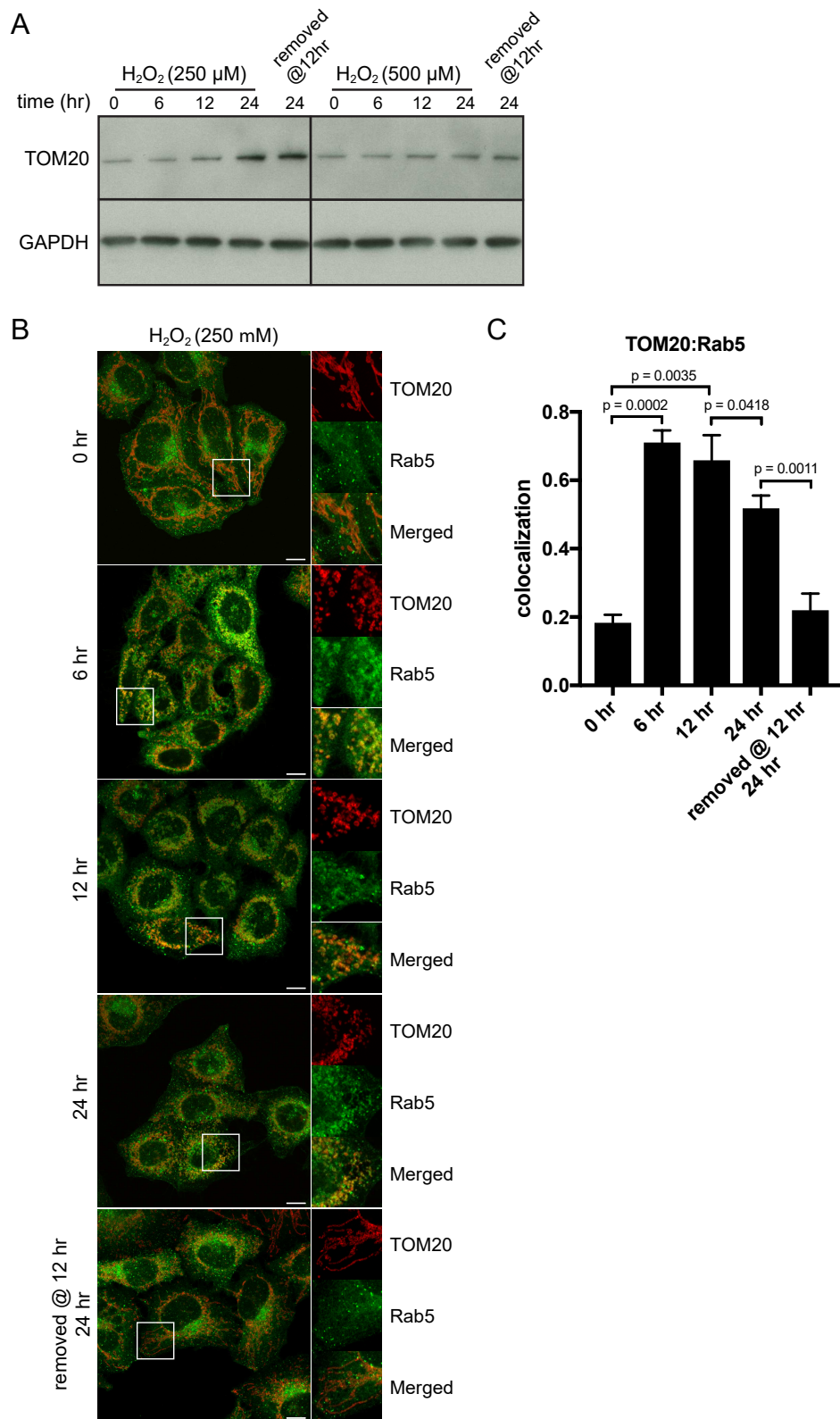
Figure 6

Figure 7

Figure 8
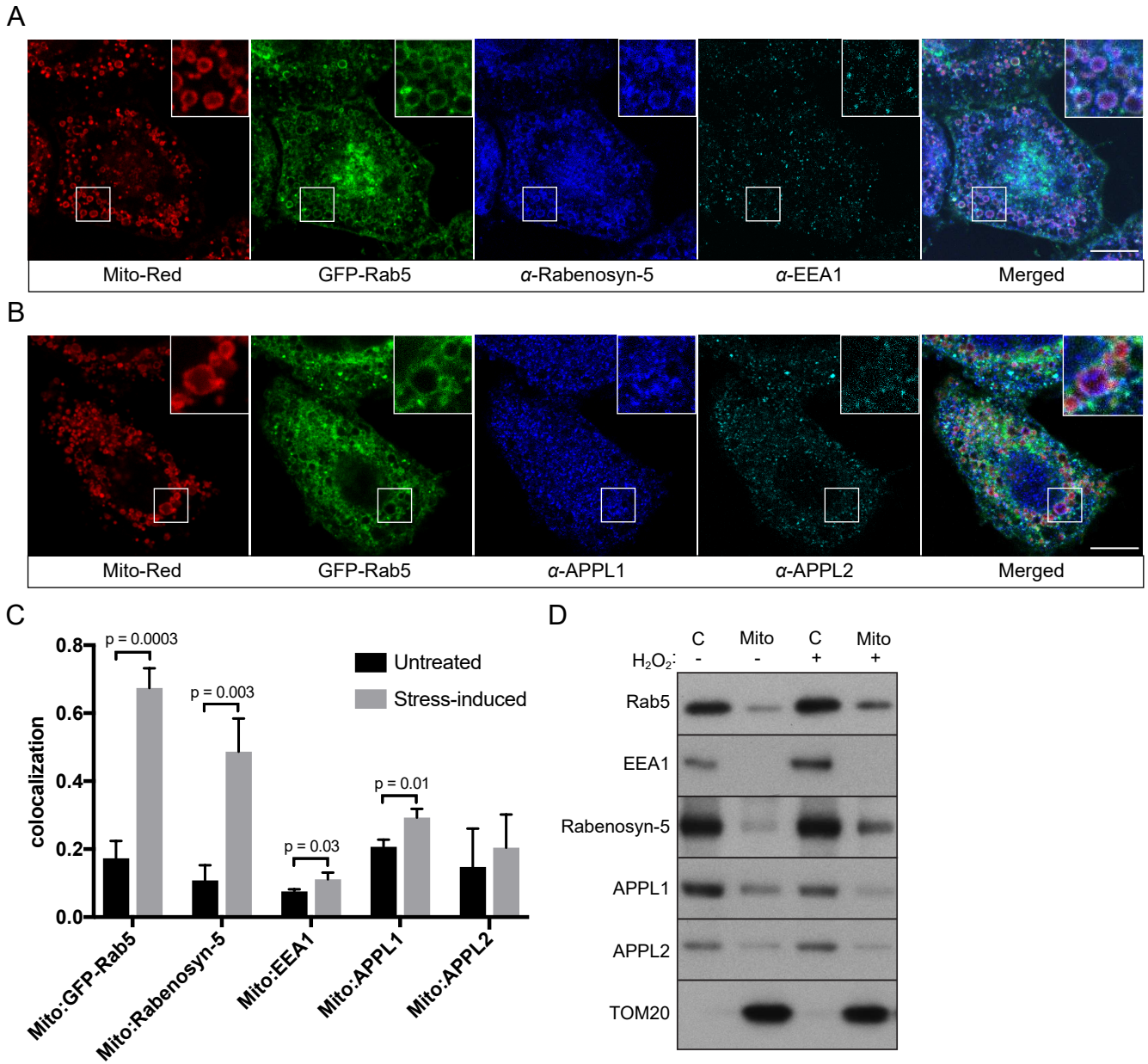
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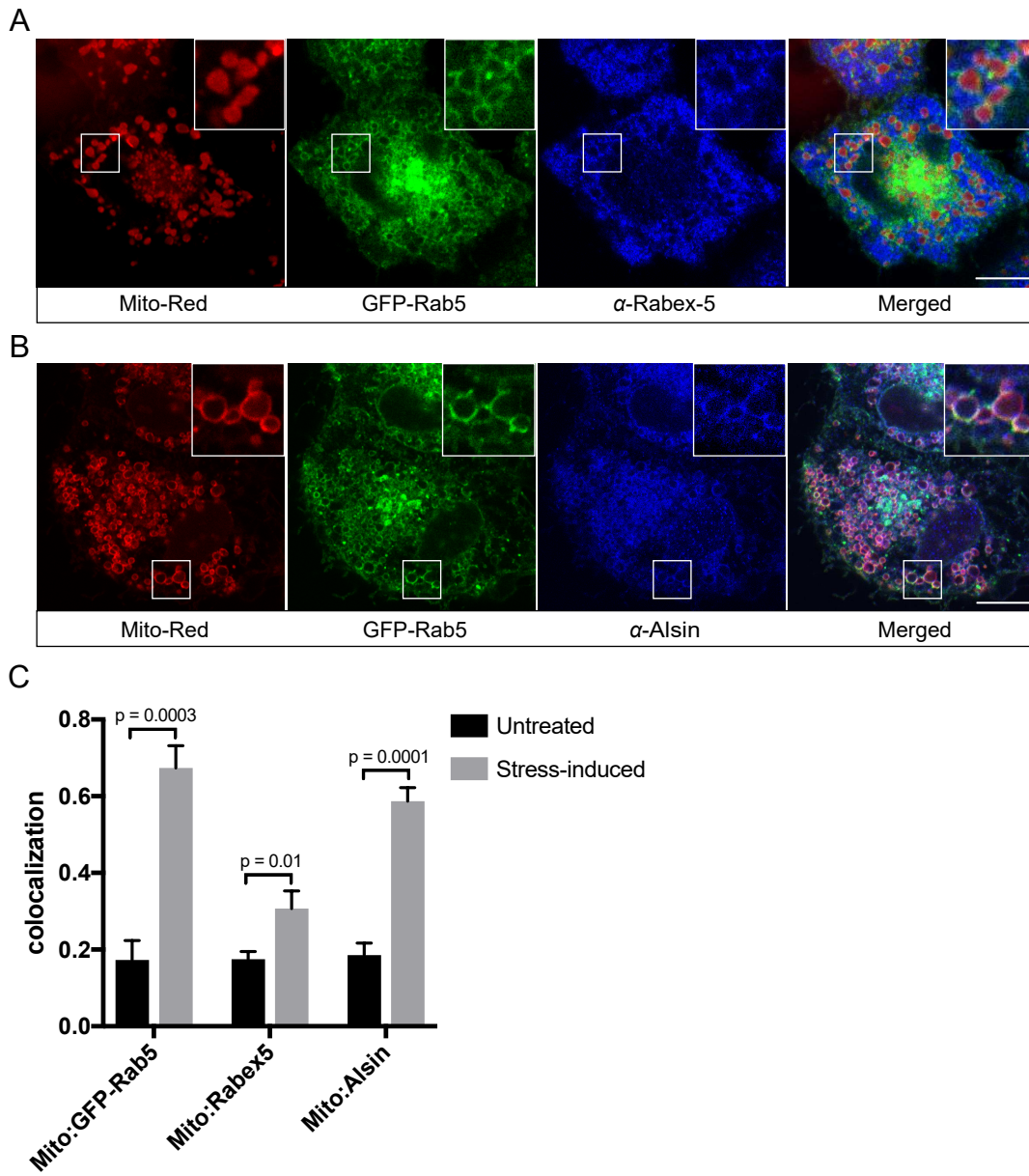
Figure 10

Figure 11

Figure 12