

Molecular Cell, Volume 84

Supplemental information

**The HRI branch of the integrated stress response
selectively triggers mitophagy**

Yogaditya Chakrabarty, Zheng Yang, Hsiuchen Chen, and David C. Chan

Figure S1

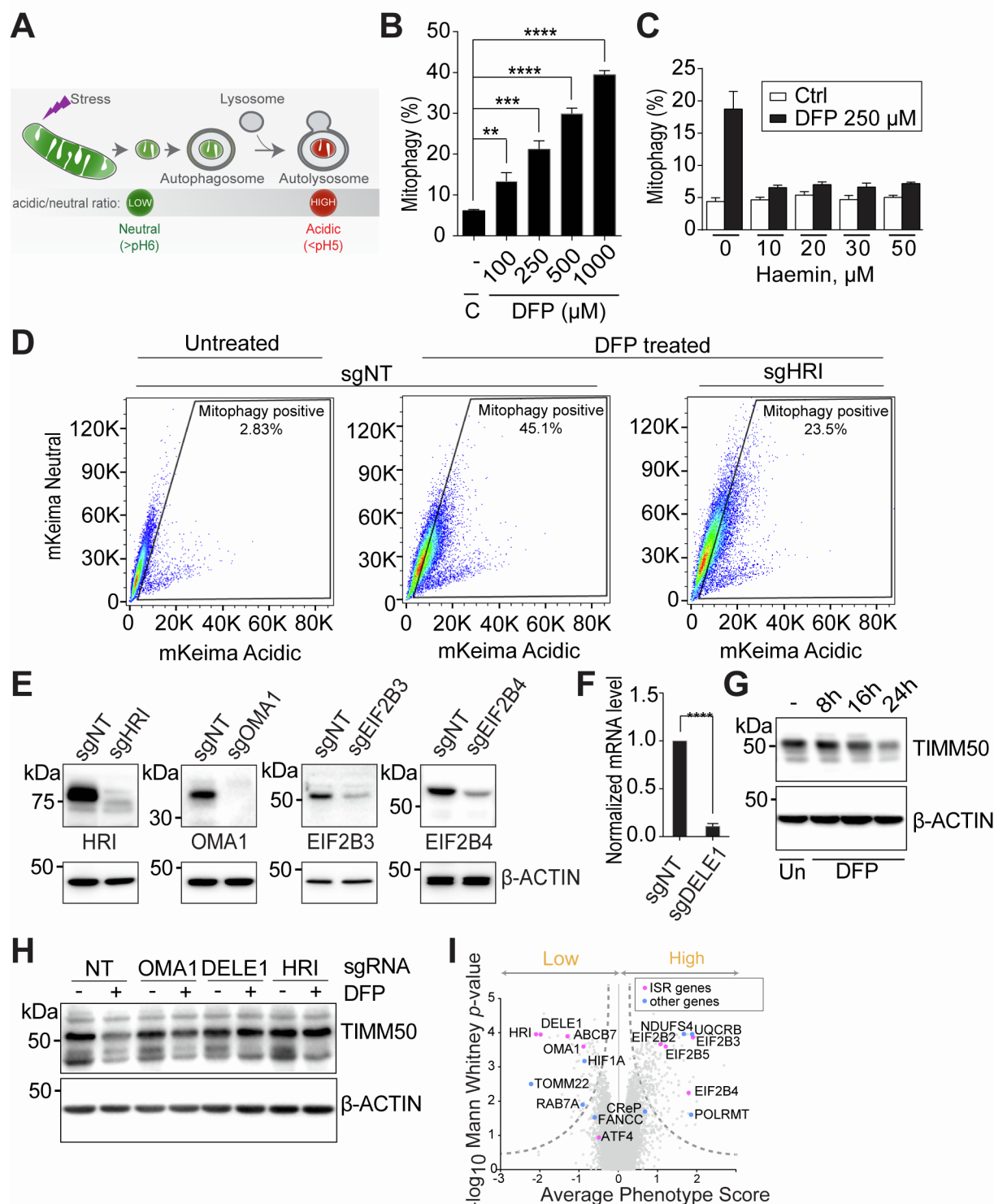


Figure S1. Quantification of DFP-induced mitophagy. Related to Figure 1.

(A) Schematic showing how mito-mKeima functions as a fluorescent mitophagy marker. Mito-mKeima has excitation properties that are pH sensitive. By tracking the acidic/neutral ratio of

mKeima, mitochondria within lysosomes can be distinguished from normal mitochondria. **(B)** Dose-dependence of DFP-induced mitophagy. K562 cells were treated with the indicated concentrations of DFP for 24 h, and mitophagy was quantified by flow cytometry as in Figure 1B (mean \pm s.d., $n \geq 3$). The following p -value designations are used in all figures: ****, $p \leq 0.0001$; ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$; ns, $p \geq 0.05$. **(C)** Inhibition of DFP-induced mitophagy by addition of haemin (mean \pm s.d., $n=2$). **(D)** Representative flow cytometry plots for K562 cells expressing mito-mKeima and either control sgRNA (sgNT) or sgRNA against HRI. The polygon indicates the gate used to score cells as positive for mitophagy. **(E)** Effectiveness of sgRNA knockdowns. K562 cells expressing the indicated sgRNA were treated with DFP and analyzed by immunoblotting. NT, non-targeting. **(F)** Effectiveness of sgRNA-DELE1 knockdown. Quantitative polymerase chain reaction (qPCR) was used to quantify DELE1 mRNA levels in K562 cells expressing non-targeting (NT) sgRNA or sgRNA against DELE1 (mean \pm s.d., $n=3$). **(G)** Time course of TIMM50 in DFP-treated K562 cells. Cell lysates were analyzed by immunoblotting against TIMM50. **(H)** Immunoblot analysis of TIMM50 in K562 cells expressing the indicated sgRNA, after 23 h DFP treatment. **(I)** Volcano plot of CRISPRi screen, showing select genes of interest.

A

kDa	NT		DELE1		HRI		:sgRNA		:DFP	ATF4	β-ACTIN
	-	+	-	+	-	+	-	+			
50											
50											

B

kDa	NT		ATF4		sgRNA	DFP	ATF4	β-ACTIN
	-	+	-	+				
50								
50								

C

kDa	shNT		shHRI		HRI	kDa	shNT		shOMA1		OMA1	β-ACTIN
	-	+	-	+			-	+	-	+		
75						30						
50						50						

D

Normalized mRNA level

shNT shDELE1

E

kDa	ATF4		NT		:shRNA	:DFP	ATF4	β-ACTIN
	-	+	-	+				
50								
50								

F

Mitophagy (%)

shNT shDELE1 shOMA1 shHRI

G

Mitophagy (%)

shRNA: ATF4 NT

H

Mitophagy (%)

CHX (μg/ml)

I

Mitophagy (%)

shRNA: ATF4 NT

(A) Inhibition of ATF4 upregulation by DELE1 and HRI knockdowns. K562 cells expressing non-targeting (NT) sgRNA or sgRNAs against DELE1 and HRI were treated with DFP and analyzed by immunoblotting for ATF4. **(B)** Effectiveness of ATF4 sgRNA knockdown by immunoblot analysis. DFP treatment was used to induce ATF4 expression, so that ATF4 knockdown efficiency could be evaluated. **(C)** Effectiveness of shRNA-HRI and shRNA-OMA1 knockdown by immunoblot analysis. **(D)** Effectiveness of shRNA-DELE1 knockdown by qPCR analysis (mean \pm s.d., $n=3$). The following p -value designations are used in all figures: ****, $p \leq 0.0001$; ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$; ns, $p \geq 0.05$. **(E)** Effectiveness of shRNA-ATF4 knockdowns by immunoblot

analysis. **(F)** Effect of knockdown of HRI components on DFP-induced mitophagy in HeLa cells. Mitophagy was quantified by flow cytometry (mean \pm s.d., $n \geq 3$). DFP was used at 1 mM. **(G)** Effect of ATF4 knockdown on DFP-induced mitophagy in HeLa cells, at both 250 μ M (top panel) and 1 M (bottom panel). **(H)** Effect of CHX on mitophagy. K562 cells expressing mito-mKeima were treated with different concentrations of CHX, and flow cytometry was used to measure mitophagy. DFP treatment was used as a positive control (mean \pm s.d., $n=2$). **(I)** Effect of translation inhibitors on protein puromycylation. After treatment of cells with the indicated compounds, puromycin was applied to release translating polypeptides, and cell lysates were analyzed by Western blotting with an anti-puromycin antibody.

Figure S3

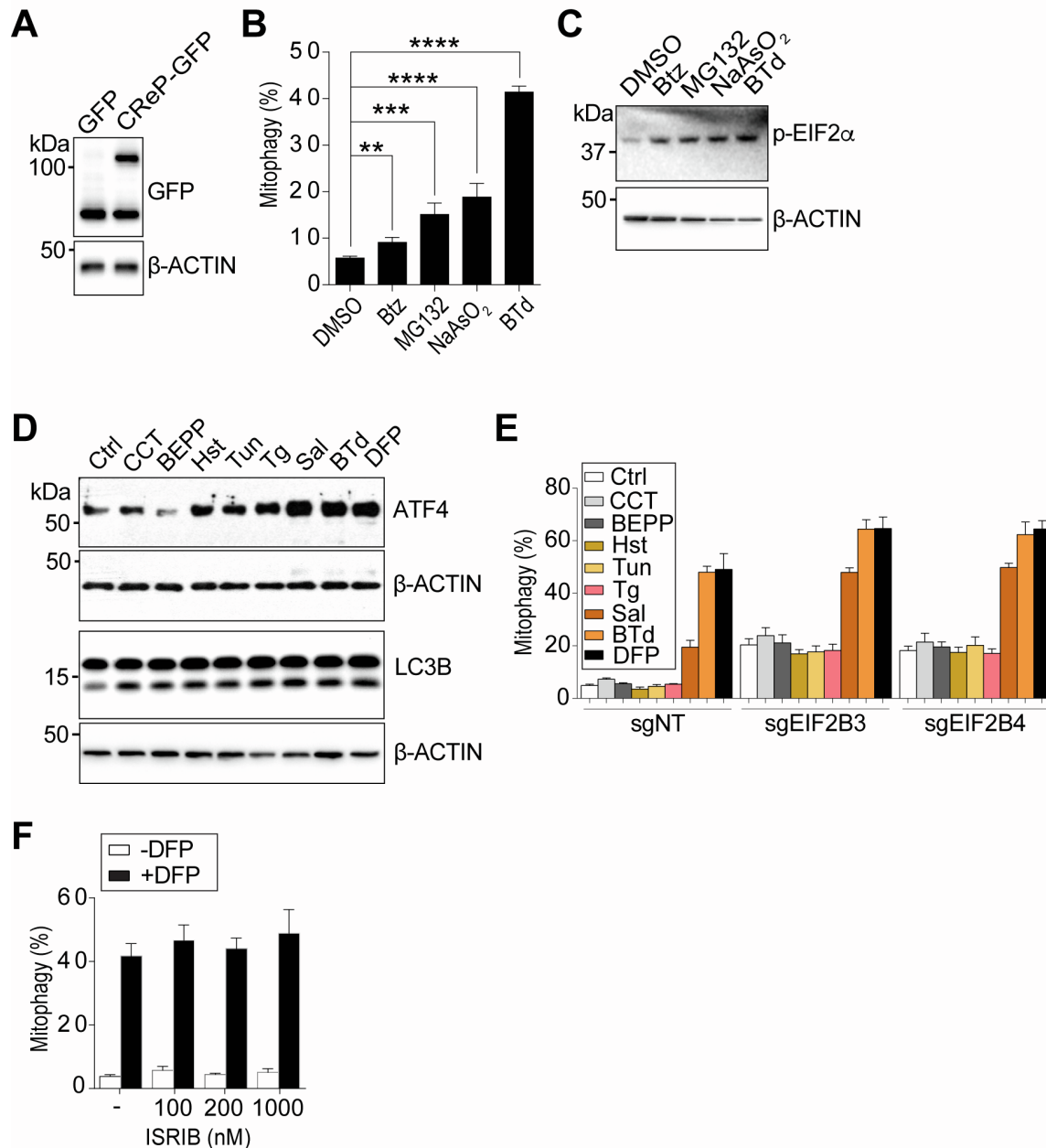


Figure S3. Effect of the ISR on mitophagy. Related to Figure 3.

(A) Immunoblotting of GFP (control) and CReP-GFP in K562 cells. (B) Mitophagy induction by drugs that activate the HRI pathway. Btz, bortezomib. (C) Induction of p-EIF2 α by the drugs in (B). (D) Accumulation of ATF4 and LC3B lipidation by ISR-inducing drugs. (E) Mitophagy levels in K562 cells expressing the indicated sgRNA, after treatment with ISR-inducing drugs. The control/untreated cells expressing sgNT were used to set the baseline level of mitophagy. (F) Effect of ISRIB treatment on mitophagy levels, with and without DFP. The following *p*-value designations are used in all figures: ****, *p* < 0.0001; ***, *p* < 0.001; **, *p* < 0.01; *, *p* < 0.05; ns, *p* > 0.05.

Figure S4

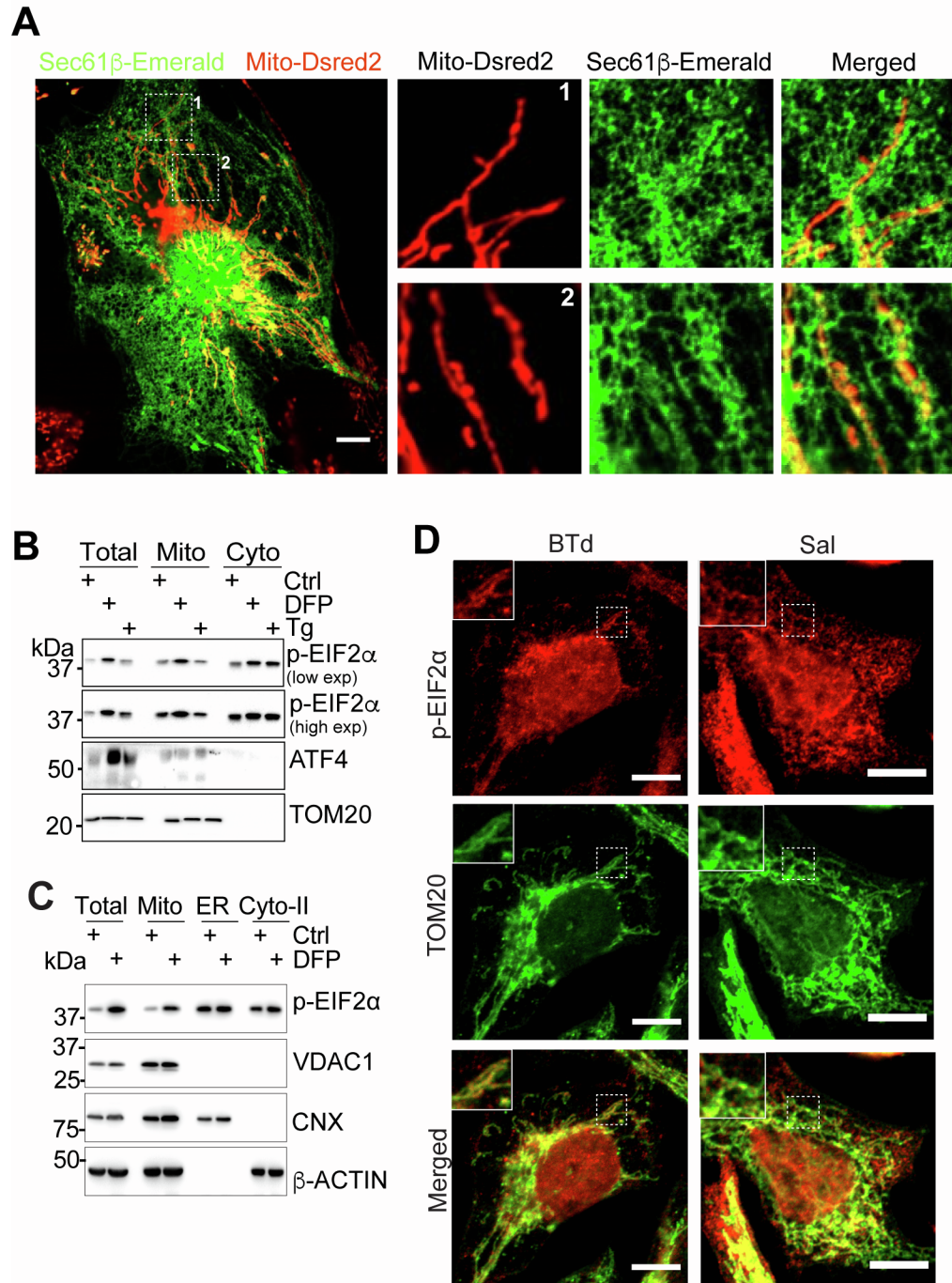


Figure S4. Subcellular localization of p-EIF2 α after BTd and Sal treatment. Related to Figure 4.

(A) Comparison of endoplasmic reticulum (Sec61 β -Emerald) and mitochondrial (mito-DsRed2) signals in HeLa cells. (B) Biochemical localization of p-EIF2 α to mitochondria after DFP treatment of HeLa cells. Samples were processed as in Figure 4A. TOM20, mitochondrial marker. (C) Comparison of p-EIF2 α levels in different cellular fractions of HeLa cells treated with DFP.

Endoplasmic reticulum (ER) enriched fractions were obtained by ultracentrifugation of post-mitochondrial supernatant at 100,000 *g*. Post-ER supernatant was used as the cytosolic (Cyto-II) fraction. CNX (Calnexin) and VDAC1 were used as ER and mitochondrial markers, respectively. **(D)** Immunolocalization of p-EIF2 α in HeLa cells after BTd and Sal treatment. Cells were treated with 10 mM BTd or 10 mM Sal for 24 h and analyzed by immunofluorescence. Insets show magnified image of boxed area.

Figure S5

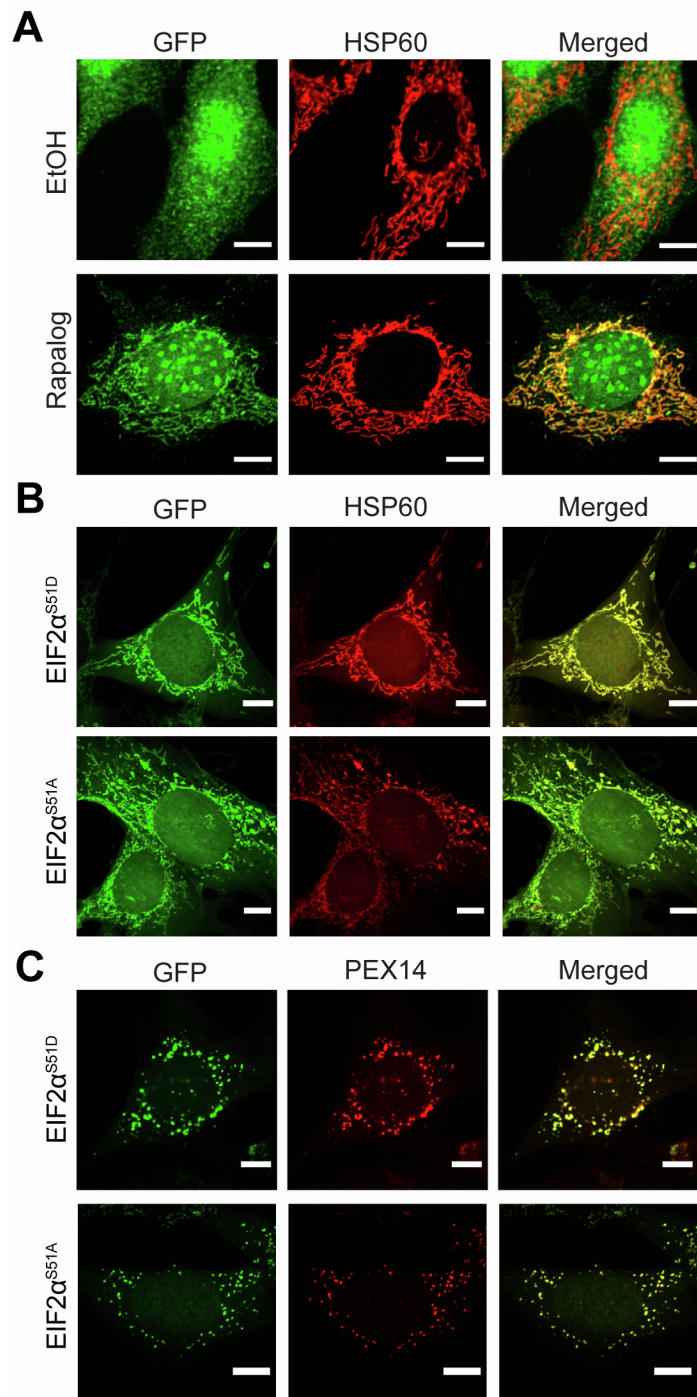
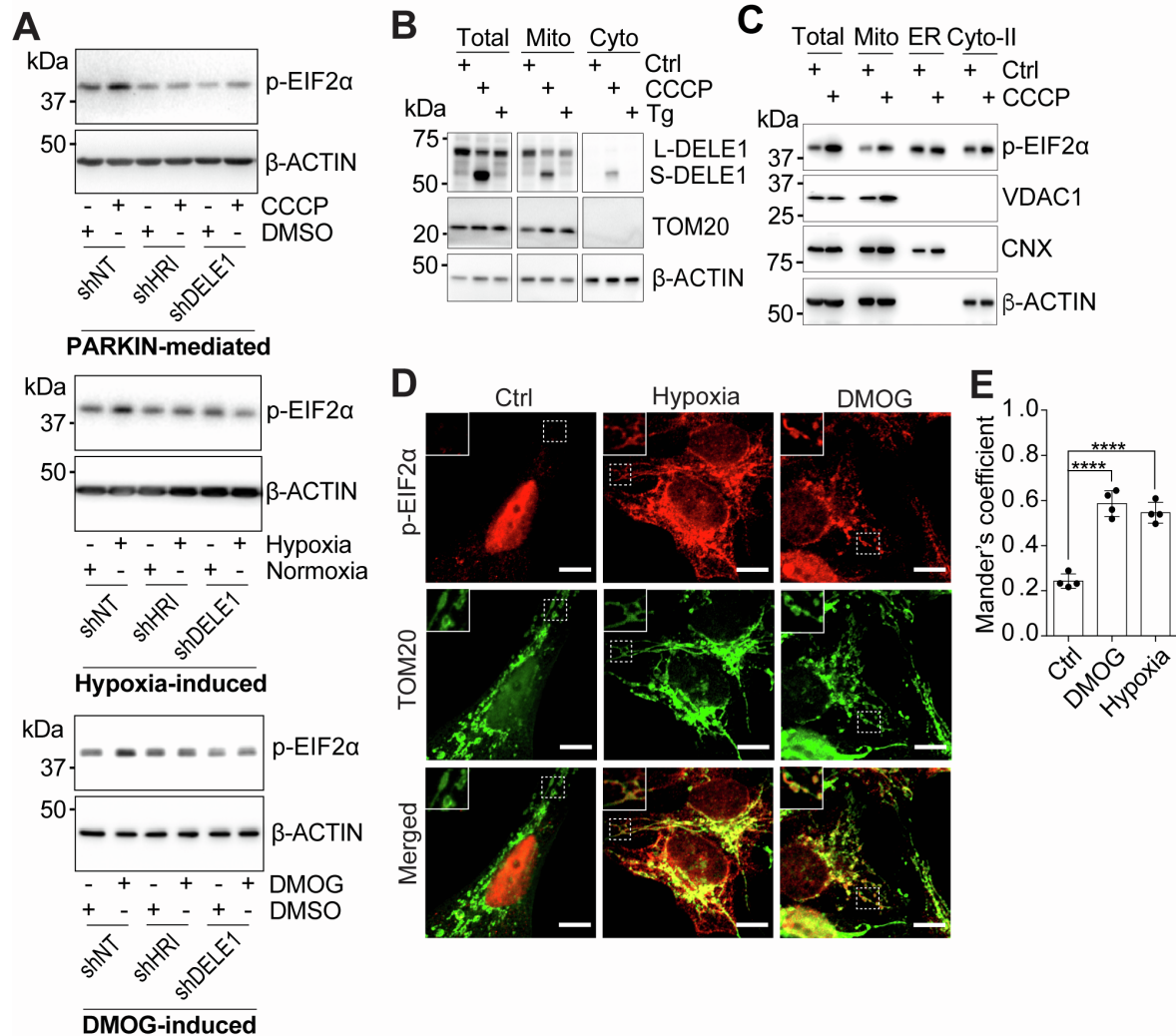


Figure S5. Induced mitochondrial and peroxisomal localization of EIFa fusion protein. Related to Figure 5.

(A) Localization of EIF2α in HeLa cells expressing the two constructs depicted in Figure 5A. Cells were treated with vehicle (EtOH) or rapalog, and then GFP fluorescence was imaged. HSP60 is a control for mitochondrial localization. (B) Localization of phosphomimetic or mutant versions of

EIF2 α , upon rapalog treatment (**C**) Similar to (**B**), except that cells contained an FRB construct fused to the peroxisomal targeting sequence from PEX26. PEX14 is a control for peroxisomal localization.

Figure S6

**Figure S6. Involvement of HRI in other forms of mitophagy. Related to Figure 6.**

(A) p-EIF2α accumulation during Parkin-, hypoxia-, and DMOG-induced mitophagy, and effect of HRI or DELE1 knockdown. p-EIF2α was monitored under the indicated conditions by immunoblotting. (B) Analysis of DELE1 cleavage during PARKIN-mediated mitophagy. DELE1-HA expressing cells were treated as indicated, and DELE1-HA was analyzed with an anti-HA antibody after subcellular fractionation. (C) Localization of p-EIF2α by subcellular fractionation. HeLa cells constitutively expressing PARKIN were treated with either DMSO or CCCP. p-EIF2α levels were analyzed by Western blotting, and CNX (Calnexin) and VDAC1 were used as ER and mitochondrial markers, respectively. (D) Immunofluorescent analysis of p-EIF2α during hypoxia- and DMOG-induced mitophagy. Cells were incubated at 1% O₂ (hypoxia) or treated with 1 mM DMOG for 24 h to induce mitophagy. Cells were then analyzed with antibodies against p-EIF2α and TOM20. (E) Quantification of colocalization of p-EIF2α with mitochondria using the Mander's coefficient, performed as in Figure 4D. ****, p ≤ 0.0001; ***, p ≤ 0.001; **, p ≤ 0.01; *, p ≤ 0.05; ns, p ≥ 0.05.

Figure S7

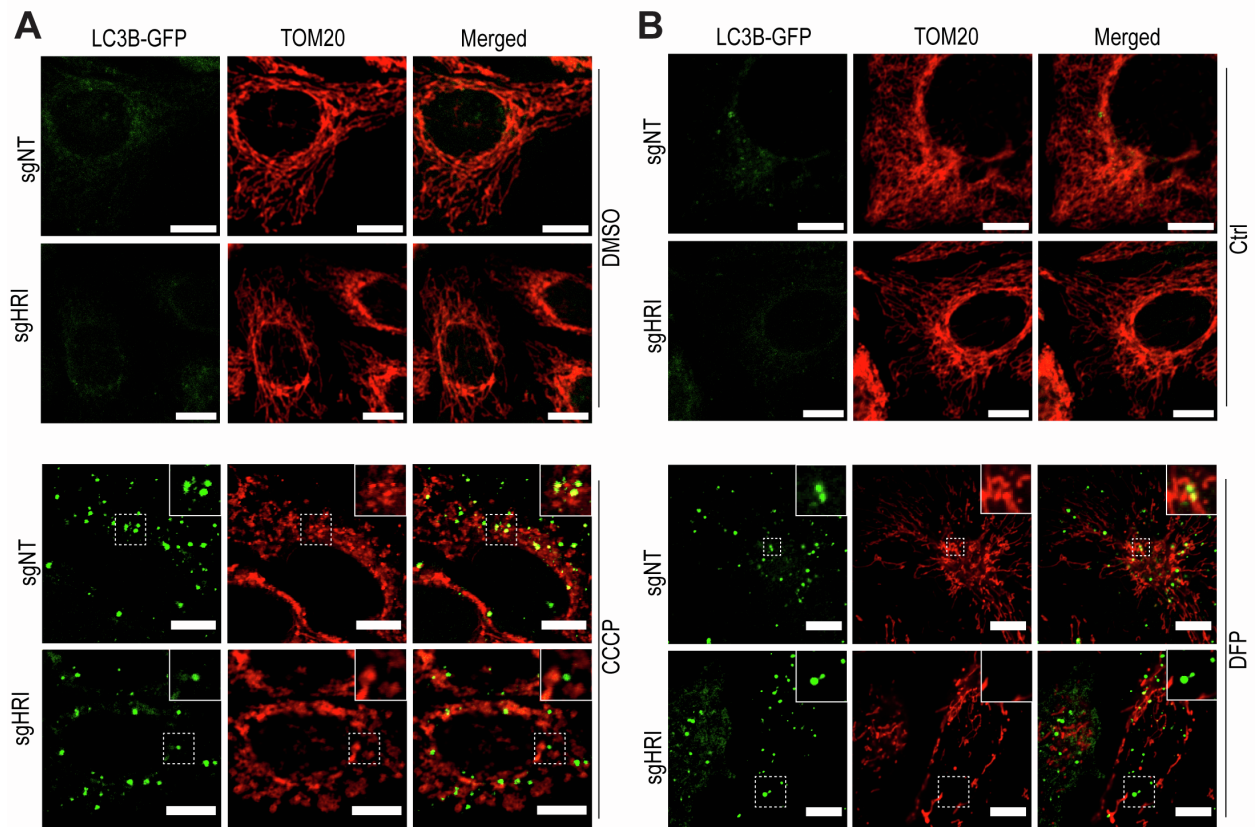


Figure S7. Colocalization of LC3B-GFP with mitochondria. Related to Figure 7.

(A) LC3B-GFP localization to mitochondria during PARKIN-mediated mitophagy. PARKIN- and LC3B-GFP-expressing HeLa cells transduced with control or HRI shRNA were treated with DMSO or CCCP as indicated. Immunofluorescence was performed to visualize LC3B-GFP and TOM20. (B) LC3B-GFP localization to mitochondria during DFP-induced mitophagy. HeLa cells transduced with control or HRI shRNA were treated as indicated, and immunofluorescence was performed to visualize LC3B-GFP and TOM20.