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ARTICLE

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Phosphorylation of eukaryotic initiation factor-2 α (elF2 α) in autophagy

Juliette Humeau^{1,2,3}, Marion Leduc^{1,2}, Giulia Cerrato ^{1,2,3}, Friedemann Loos^{1,2}, Oliver Kepp ^{1,2} and Guido Kroemer^{1,2,4,5,6}

Abstract

The integrated stress response is characterized by the phosphorylation of eukaryotic initiation factor-2 α (elF2 α) on serine 51 by one out of four specific kinases (ElF2AK1 to 4). Here we provide three series of evidence suggesting that macroautophagy (to which we refer to as autophagy) induced by a variety of distinct pharmacological agents generally requires this phosphorylation event. First, the induction of autophagic puncta by various distinct compounds was accompanied by elF2 α phosphorylation on serine 51. Second, the modulation of autophagy by >30 chemically unrelated agents was partially inhibited in cells expressing a non-phosphorylable (S51A) mutant of elF2 α or lacking all four elF2 α kinases, although distinct kinases were involved in the response to different autophagy inducers. Third, inhibition of elF2 α phosphatases was sufficient to stimulate autophagy. In synthesis, it appears that elF2 α phosphorylation is a central event for the stimulation of autophagy.

Introduction

The so-called integrated stress response^{1–3} is characterized by the phosphorylation of eukaryotic initiation factor- 2α (eIF2 α). The phosphorylation of eIF2 α occurs on serine 51 in response to the activation of one out of four eIF2 α kinases^{4–6}. EIF2AK1, commonly known as heme-regulated inhibitor (HRI), is activated by oxidative, osmotic, and heat stress, as well as by arsenic and redaporfin-mediated photodynamic therapy^{7–10}. EIF2AK2, commonly known as protein kinase R (PKR), is activated by viruses and alcohol^{11,12}. EIF2AK3, commonly known as protein kinase Rlike endoplasmic reticulum (ER) kinase (PERK), is activated by ER stress, because unfolded proteins in the ER lumen occupy the chaperone GRP78, which then releases PERK from inhibition^{13,14}. EIF2AK4, commonly known as general control nonderepressible 2 (GCN2), is activated by nutrient deprivation^{15,16}. Thus a variety of rather distinct stressors converge on eIF2 α phosphorylation (peIF2 α).

peIF2a causes a block in cap-dependent translation, thus reducing general protein synthesis while favoring that of cap-independent, often internal ribosome entry site-dependent proteins¹⁷. This results in a global shift of translational programs, favoring, for example, the (capindependent) synthesis of activating transcription factor 4 (ATF4), which then translocates to the nucleus to transactivate a transcriptional program¹⁸, allowing cells to adapt to stress, for example, by enhancing the expression of chaperones (which help refolding misfolded proteins), by stimulating the removal of unfolded proteins by endoplasmic reticulum associated protein degradation (ERAD), by activating autophagy^{19,20}, which is the most efficient pathway for the removal of damaged organelles, or by igniting apoptotic pathways for whole-cell removal when stress is chronic and cannot be overcome. Thus peIF2a indeed stimulates a coordinated ensemble of stress responses that provide cytoprotection when the intensity and duration of the stress is limited, yet allows for the controlled elimination of cells that are irreversibly or chronically damaged²¹.

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The central role of peIF2 α for autophagy induction was discovered by Beth Levine's group in the context of starvation of yeast or mouse cells²⁰. Since autophagy is (one of) the most important stress-adaptive mechanisms and even determines organismal longevity^{22,23}, we wondered whether autophagy would generally rely on peIF2 α to be induced. Here we performed a systematic analysis of mammalian cells responding to a vast panel of autophagy modulators to determine their dependence on peIF2 α and eIF2 α kinases.

Results and discussion

Correlation of pelF2a and autophagy

As a first approach to investigate the interdependence between autophagy and peIF2 α , we measured the phosphorylation of eIF2α in human osteosarcoma U2OS cells by means of an immunofluorescence staining protocol with a phosphoneoepitope-specific antibody recognizing eIF2 α phosphorylated on serine 51 (Figs. S1 and 1a). We also quantified autophagic puncta in cells stably transfected with red fluorescent protein (RFP) fused to microtubule-associated proteins 1A/1B light chain 3B (hereafter referred to as LC3) by fluorescence microscopy (Fig. 1b). We took advantage of the Enzo library of autophagy modulators (all used at $10 \,\mu$ M) that was complemented by a series of caloric restriction mimetics including several polyamines and chalcones²⁴⁻²⁶, several microtubule inhibitors²⁷ as well as crizotinib^{26,28}, all of which were added at doses that are expected to stimulate autophagy. As shown in Fig. 1c, many of the strong inducers of autophagic puncta also stimulated peIF2a. Similar results were obtained in mouse embryonic fibroblasts (MEFs) exposed to the same collection of compounds (Fig. 2a, b). Of note, in both U2OS and MEF cells, torin 1, which is a direct inhibitor of mechanistic target of rapamycin (mTOR), induced autophagy without enhancing peIF2 α , in line with a prior report²⁹. If the intensity of peIF2a and the surface of RFP-LC3 dots were measured on a cell-per-cell basis in U2OS, the two parameters were found to correlate among each other, both in baseline condition and after pharmacological autophagy induction (Fig. S2). Thus induction of LC3 puncta is often, but not always, coupled to peIF2 α .

Dependency of autophagy on pelF2a

In order to understand to which extent peIF2 α is required for autophagy induction, U2OS cells stably transduced with RFP-LC3 were subjected to CRISPR-Cas9-driven mutagenesis to render eIF2 α nonphosphorylable (due to the exchange of serine 51 by an alanine residue yielding EIF2 α ^{S51A}). We validated three *EIF2\alpha*^{S51A} homozygous clones (C25, C59, and C70) by genomic sequencing and immunofluorescence analysis (Fig. S3). Of note, all the three clones exhibited reduced



Fig. 1 EIF2a phosphorylation and autophagy in U2OS cells. Human osteosarcoma U2OS cells stably expressing GFP-LC3 were treated with the custom arrayed library of autophagy-modulating agents and controls for 6 h. After fixation, the cells were stained with a phosphoneoepitope-specific elF2a antibody followed by an AlexaFluor-568 secondary antibody. Nuclei were counterstained with Hoechst 33342, and phosphorylation was assessed by fluorescence microscopy (a). U2OS RFP-LC3 cells were treated as described above. After fixation and staining with Hoechst 33342, images were acquired (b). Representative images are shown for control (Ctrl), torin 1, brefeldin A (BFA), niclosamide (Niclo), and PI-103 (a, b). For the assessment of autophagy, the average surface of RFP-LC3 dots per cell was quantified. The phosphorylation of eIF2a was evaluated by measuring the cytoplasmic fluorescence intensity of the immunostaining. Data were subjected to a z-score transformation centered on control. For LC3 dot surface, the mean of technical quadruplicates from one experiment is shown. For cytoplasmic pelF2a fluorescence intensity, the mean of three independent experiments is depicted. The correlation (R) between LC3 dot surface and pelF2a fluorescence intensity was calculated employing Spearman's rank test. A threshold to select autophagy- and pelF2a-inducing agents was established based on the 60% quartile and distribution, respectively (c).



RFP-LC3 puncta in response to many of the pharmacological autophagy modulators with the notable exception of torin 1 (Fig. 3). As a complementary approach, we used MEFs that had been subjected to the knockout of all eIF2 α kinases (EIF2AK1 commonly known as HRI, EIF2AK2 commonly known as PKR, EIF2AK3 commonly known as PERK, EIF2AK4 commonly known as GCN2)⁵, finding again that most pharmacological autophagy enhancers display a lower pro-autophagic potential in the absence of peIF2 α (Fig. 4). When all results were combined and subjected to hierarchical clustering, three major clusters (1-3) emerged (Fig. 5a). Of note, the strongest inducers of autophagic puncta that depended in their activity on both phosphorylable eIF2 α and the eIF2 α kinases (cluster 3) exhibited a significantly higher phosphorylation level of eIF2 α than the weak autophagy inducers (cluster 1) (Fig. 5b-d).

We also measured the contribution of each individual eIF2 α kinase to autophagy by knocking them out individually in U2OS cells stably expressing green fluorescent protein (GFP)-LC3 (Fig. S4). For this, we concentrated on those pharmacological agents that were the strongest inducers of RFP-LC3 puncta and tended to depend in their activity on peIF2 α , contrasting with torin 1 (Fig. S5). For these compounds, the knockout of EIF2AK1 and EIF2AK4 had the highest autophagy-inhibitory impact, although EIF2AK3 appeared to be relevant for some autophagy inducers as well (Fig. 6). Altogether, these results suggest that distinct pharmacological autophagy enhancers rely on different eIF2 α kinases to be efficient and that some functional redundancy among such kinases exists.

Autophagy induction by eIF2a phosphatase inhibitors

We next addressed the question whether the stimulation of peIF2 α might be sufficient for the induction of autophagy. For this, we took advantage of nelfinavir, an inhibitor of the phosphatase that constitutively dephosphorylates eIF2 $\alpha^{30,31}$. Nelfinavir did not only cause the hyperphosphorylation of $eIF2\alpha$ but also stimulated the generation of GFP-LC3 puncta (Fig. 7a-c). Of note, nelfinavir combined with bafilomycin A1, an inhibitor of vacuolar-type H⁺-ATPase and hence an inhibitor of the final stage of autophagy³², induced more GFP-LC3 puncta than in cells treated with bafilomycin A1 alone, a finding indicating that nelfinavir stimulates autophagic flux. The induction of GFP-LC3 puncta depended on the phosphorylability of $eIF2\alpha$ (because the effect was lost in $EIF2\alpha^{S51A}$ cells) and the activity of $eIF2\alpha$ kinases (because the effect was suppressed in cells lacking the four eIF2 α kinases) (Fig. 7d, e). Importantly, a variety of additional

on the 60% quartile and distribution, respectively (b).

select autophagy- and pelF2a-inducing agents was established based



inhibitors of eIF2 α dephosphorylation (such as guanabenz, salubrinal, and sephin 1) shared the capacity of nelfinavir to simultaneously stimulate peIF2 α and autophagy (Fig. 7f).

Concluding remarks

As mentioned in the "Introduction" section, peIF2 α is part of the integrated stress response, which is connected to autophagy. However, a systematic study of the requirement of peIF2 α and the involvement of distinct eIF2 α kinases has been elusive. Here we used a collection of approximately 200 compounds to show that most inducers of LC3 puncta require peIF2 α and eIF2 α kinases to be efficient. There are three major arguments that favor the hypothesis that peIF2 α is important for the activation of autophagy: (i) the stimulation of autophagic puncta and peIF2 α correlates for many autophagy modulators; (ii) cells bearing a non-phosphorylable eIF2 α mutant or lacking all known eIF2 α kinases are refractory to autophagy induction by most stimuli; and (iii) activation of peIF2 α by inhibition of a specific set of phosphatases suffices to trigger autophagy.

In accord with previous studies, the proximal mode of action of distinct autophagy enhancers may depend on distinct eIF2a kinases. Thus EIF2AK4 (GCN2) is important for autophagy induction by nutrient deprivation^{15,16}, contrasting with the fact that primary ER stress mediated by, for example, polyglutamine repeats, tends to require EIF2AK3 (PERK)³³ and saturated fatty acids (such as palmitate) rely on EIF2AK2 (PKR) to stimulate autophagic puncta. This latter pathway is complex, requiring the phosphorylation of heat shock protein 27, resulting in its binding to STAT3, which then releases EIF2AK2 from its inhibition to facilitate peIF2a and autophagy



induction^{34,35}. Thus rather different proximal stimuli can converge on distinct $eIF2\alpha$ kinases to trigger a similar integrated stress response leading to autophagy.

It should be noted that peIF2 α is not only important for autophagy induction but also contributes to a variety of stress pathways including the induction of ER stress³⁶, the formation of stress granules³⁷, and the translocation of calreticulin to the surface of stressed cells where it acts as "eat me" signal for the removal of the stressed cells by phagocytosis³⁸. This latter phenomenon is highly important for the dendritic cell-dependent immune recognition of cancer cells in the context of "immunogenic cell death"³⁹ meaning that peIF2 α is actually a central hallmark of this cell death modality²⁷. Thus peIF2 α can be viewed as a central hub that signals in favor of immune responses, that minimizes the replication of intracellular pathogens (through inhibition of protein synthesis in the ER), sequesters and eliminates them in the cytoplasm (through autophagy/xenophagy), facilitates the destruction of infected cells (through phagocytosis/phagoptosis), or connects them to T lymphocyte-mediated immune recognition (subsequent to antigen cross-presentation by dendritic cells). Importantly, viruses may encode proteins that either inhibit eIF2α kinases or dephosphorylate eIF2α to inhibit peIF2 α and to escape from cell-autonomous and immune $recognition^{40-42}$. From this point of view, it appears intriguing that prominent antiretroviral agents such as nelfinavir, which is usually considered as a HIV-1 inhibitor³⁰, may have off-target effects that favor peIF2 α and hence may stimulate a broad immune response. Future work must determine to what extent this effect may contribute to the clinical efficacy of nelfinavir and similar compounds.

Materials and methods Cell lines

Human osteosarcoma U2OS were purchased from the ATCC. U2OS cells stably expressing GFP-LC3 and RFP-LC3 were generated by our group in the past^{27,35}. MEF wild type (WT) and knockout for the four $eIF2\alpha$ kinases (MEF 4KO) were provided by Professor Seiichi Oyadomari from Tokushima University. MEF WT RFP-LC3 and 4KO RFP-LC3 were constructed from the aforementioned cell lines, which were transduced with LentiBright lentiviral particles coding for RFP-LC3 (17-10143, Millipore, Burlington, MO, USA). The day following transduction, cells were supplemented with fresh medium and, 1 day later, single cell sorted by flow cytometry based on RFP fluorescence. U2OS cells stably expressing RFP-LC3 bearing a mutant non-phosphorylable version of $eIF2\alpha$ (EIF2 α^{S51A}) were constructed using the CRISPR-Cas9 technology as previously detailed⁴³. Briefly, we designed a guide RNA (gRNA) targeting eIF2a and inserted them into the pX458 vector (containing a tracrRNA and Cas9 fused with 2A-GFP)⁴⁴ following the manufacturer's protocol (New England Biolabs, Ipswich, MA, USA). This plasmid was used together with a homology repair template that targets serine in position 51 of eIF2 α for an exchange to alanine to transfect RFP-LC3 expressing U2OS cells by means of Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Two days later, single cells were sorted by flow cytometry. DNA of clones that grew was extracted, amplified by PCR, and analyzed for homozygous knockin by sequencing (Eurofins Scientific, Luxembourg). They were further validated by immunofluorescence (Fig. S3). U2OS GFP-LC3 having



(see figure on previous page)

Fig. 5 elF2a phosphorylation participates in the induction of autophagy in certain contexts. The heatmap summarizes the effects of the agents of the custom arrayed library of autophagy modulators in MEF and U2OS on LC3 dot surface and pelF2a in addition to the dependency of LC3 dot surface on elF2a phosphorylation. Agents that caused toxicity in both cell lines were excluded from the analysis and marked with gray color when toxic in only one cell line. The geometric distances of each point in LC3 dot surface between U2OS WT and *ElF2a^{551A}* (from Fig. 3b–d) as well as between MEF WT and knockout for *elf2ak1–4 (4KO)* (Fig. 4b) were calculated. It reflects the dependency of autophagy induction on elF2a phosphorylation, with a positive distance allocated to points located under the regression curve (corresponding to agents that requires pelF2a for complete autophagy induction). In U2OS, the mean of the distances of the three tested clones was calculated. Distances were subjected to a *z*-score transformation centered on control. Then the *z*-score of LC3 dot surface and pelF2a cytoplasmic fluorescence intensity (from Fig. 1c for U2OS and Fig. 2b for MEF) as well as geometric distances were independently scaled between 0 and 1 with a sigmoidal transformation and represented. Agents that are among the 40% most potent autophagy and in purple positive controls for pelF2a in U2OS (**b**) and in MEFs (**c**) as well as of LC3 dot surface in U2OS (**d**) and in MEFs (**e**) are shown as boxplots with median \pm quartiles \pm 95% confidence intervals. Statistical significance was analyzed using a Kolmogorov–Smirnov test. Differences to cluster 1 are depicted as **p < 0.01 and ***p < 0.001 and differences between clusters 2 and 3 as **p < 0.05 and ***p < 0.05 and **p < 0.05 and **p < 0.01 (**b–e**).

one eIF2 α kinase knocked out (*EIF2AK1^{-/-}*, *EIF2AK2^{-/-}*, *EIF2AK3^{-/-}*, and *EIF2AK4^{-/-}*) were constructed using an U6gRNA-Cas9-2A-RFP plasmid containing gRNAs (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocol as previously described⁴³. In short, U2OS GFP-LC3 cells were transfected, and 2 days later, single cells were sorted by flow cytometry. Clones were validated by immunoblot with specific antibodies against human EIF2AK1 (HRI), EIF2AK2 (PKR), EIF2AK3 (PERK), and EIF2AK4 (GCN2) (Fig. S4A) and by immunofluorescence (Fig. S4B).

Cell culture

Human osteosarcoma U2OS cells and MEF cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco by Thermo Fisher Scientific), 1% nonessential amino acids (Thermo Fisher Scientific), 1% nonessential amino acids (Thermo Fisher Scientific), and 1% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Thermo Fisher Scientific) in a humidified incubator with 5% CO₂ at 37 °C. Cell culture plastics and consumables were purchased from Greiner Bio-One (Kremsmünster, Austria) or Corning (NY, USA).

Antibodies

Rabbit monoclonal phosphoneoepitope-specific antibody against phospho-eIF2 α (Ser51) (ab32157) (used with U2OS cells) and mouse monoclonal antibody against β-actin (ab49900) were purchased from Abcam (Cambridge, UK). Rabbit polyclonal antibody against HRI (sc-30143) and mouse monoclonal antibody against PKR (sc-6282) were purchased from Santa Cruz biotechnology Rabbit (Dallas, TX. USA). mouse monoclonal phosphoneoepitope-specific antibody against phosphoeIF2 α (Ser51) used with MEF cells (#3597), rabbit monoclonal antibody against PERK (#3192), and rabbit polyclonal antibody against GCN2 (#3302) came from Cell Signaling Technology (Danvers, MA, USA). Anti-rabbit and anti-mouse AlexaFluor-488, -568, and -647 secondary antibodies came from Thermo Fisher Scientific.

Compounds

The Enzo autophagy library came from Enzo Life Science (Farmingdale, NY, USA). In addition, crizotinib (PZ0191), docetaxel (01885), doxorubicin (D1515), paclitaxel (T7191), rapamycin (R0395), thapsigargin (T9033), tunicamycin (T7765), vinblastine sulfate (V1377), vincristine sulfate (V0400000), 3,4-dimethoxychalcone abbreviated as 3.4DC (S798126), 4,4'-dimethoxychalcone abbreviated as 4.4 DMC (S617237), sodium arsenate dibasic hepta-hydrate (A6756), spermidine (740780), anacardic acid (A7236), C646 (SML0002), quercetin hydrate (337951), sodium salicylate (S3007), potassium hydroxycitrate tribasic monohydrate (59847), phenformin hydrochloride (SC219590), 1-cyano-4dimethylaminopyridinium tetrafluoroborate abbreviated as CPT613 (C2776), UK5099 (PZ0160), epigallocatechingallate (E4143), curcumin (C1386), resveratrol (R5010), 1,3,5-benzenetricarboxylic acid abbreviated as BTC (8012990025), nelfinavir mesylate hydrate (PZ0013), sephin1 (SML1356), guanabenz acetate (G110), and salubrinal (SML0951) have been bought from Sigma-Aldrich. Garcinol (BML-GR343) have been purchased from Enzo Life Science. SB204990 (4962) and torin 1 (4247) have been purchased from Tocris (Bristol, UK).

A custom arrayed library used for various experiments in the study (Figs. 1–6 and S5) was made from the compounds of the Enzo autophagy library at 10 μ M (except for bafilomycin A1, which was used at 1 μ M) supplemented with 3,4-dimethoxychalcone at 30 μ M, 4,4'dimethoxychalcone at 50 μ M, hydroxycitrate at 10 mM, phenformin at 3 mM, salicylate at 5 mM, spermidine at 100 μ M, anacardic acid at 50 μ M, C646 at 10 μ M, epigallocatechingallate at 50 μ M, CPT613 at 100 μ M, curcumin at 50 μ M, resveratrol at 50 μ M, SB204990 at 100 μ M, benzenethicarxylic acid at 5 mM, UK5099 at 1 μ M, garcinol at 10 μ M, quercetin at 50 μ M, taxotere at



Fig. 6 Role of the elF2a kinases 1–4 in autophagy. Human osteosarcoma U2OS cells stably expressing GFP-LC3 either wild type (WT) or knockout for *ElF2AK1, 2, 3, 4* were treated with the custom arrayed library of autophagy-modulating agents and controls for 6 h. After fixation, nuclei were counterstained with Hoechst 33342 (**a**). The samples were acquired by fluorescence microscopy and LC3 dot surface was quantified. The data were normalized as percentage of induction with the untreated condition as negative control and torin 1 as positive control. The mean of LC3 dot surface from three independent experiments was plotted for *ElF2AK1^{-/-}* (**a**), *ElF2AK2^{-/-}* (**b**), *ElF2AK3^{-/-}* (**c**), and *ElF2AK4^{-/-}* (**d**) versus the WT cell line. A linear regression was performed for control and torin 1 in each *ElF2AK^{-/-}* cell line to the WT. For the agents that were identified as requiring elF2a phosphorylation for complete autophagy induction in Fig. 5, the geometric distances to the linear regression were calculated, transformed to *z*-scores, independently scaled between 0 and 1 with a sigmoidal transformation, and represented (**e**).



Hoechst 33342, and autophagy and phosphorylation of elF2a were assessed by fluorescence microscopy (a-c). The different conditions were normalized as percent of control (Ctrl). Representative images and mean ± SD of triplicates (among four replicates, the one that had the highest deviation from the mean was excluded) from one representative experiment among three is shown. Statistical significance was analyzed using a Student's t test. Differences to respective controls (with or without BAF) are depicted as **p < 0.01 and ***p < 0.001 (**b**, c). U2OS RFP-LC3 WT and three clones of U2OS knockin for EIF2a^{S51A} were treated with torin 1 (TOR) at 300 nM as a positive control and nelfinavir (NLF) at 40 mM for 6 h. LC3 dot surface was measured and normalized as percent of Ctrl. Depicted are mean ± SD of quadruplicates and statistical significance was analyzed using Student's t test. Differences to controls are depicted as ***p < 0.001 in the same cell line and as ***p < 0.001 between WT and EIF2a^{SS1A} clones (d). Mouse embryonic fibroblasts (MEFs) stably expressing RFP-LC3 either WT or knockout for eif2ak1-4 (4KO) were treated with torin 1 (TOR) at 300 nM as a positive control and nelfinavir (NLF) at 40 mM for 6 h. After fixation, the cells were stained with a phosphoneoepitope-specific eIF2a antibody followed by an AlexaFluor-488 secondary antibody. Nuclei were counterstained with Hoechst 33342 and autophagy and phosphorylation were assessed by fluorescence microscopy. LC3 dots surfaces for each condition were normalized as percent of their respective Ctrl in the same cell line. Depicted are mean ± SD of quadruplicates and statistical significance was analyzed using the Student's-test. Differences to controls are depicted as ***p < 0.001 in the same cell line and as ###p < 0.001 between WT and 4KO (e). U2OS GFP-LC3 were treated with nelfinavir at 40 mM, sephin 1 at 50 mM, salubrinal at 80 mM, guanabenz at 50 mM and torin 1 at 300 nM as a positive control for 6 h. After fixation, the cells were stained with a phosphoneoepitope-specific eIF2a antibody followed by an AlexaFluor-568 secondary antibody. Nuclei were counterstained with Hoechst 33342 and autophagy and phosphorylation were assessed by fluorescence microscopy. LC3 dot surface and pelF2a fluorescence intensity in the cytoplasm were measured, submitted to a z-score transformation and depicted (f).

3 μ M, paclitaxel at 3 μ M, vincristine at 3 μ M, crizotinib at 15 μ M, torin 1 at 0.3 μ M, and rapamycin at 20 μ M. Staurosporine and SU1152 were excluded from the analysis because of their toxicity that induced a phenotype preventing a relevant analysis of the LC3 aggregation.

Tunicamycin and thapsigargin at 3 or 10 μ M were used all along the study as positive controls for peIF2 α and are

written in purple in the figures. Rapamycin at $10 \,\mu\text{M}$ and torin 1 at $0.3 \,\mu\text{M}$ were used as positive controls for autophagy and are written in orange, with torin 1 as peIF2 α -independent autophagy inducer. The hits that are shown in Figs. 5 and S5 as peIF2 α -dependent autophagy inducers are depicted in dark blue in all the figures. Untreated control is shown in green.

Fluorescence microscopy, image acquisition, and analysis

One day prior to treatment, 2500 U2OS cells (RFP-LC3 WT or $EIF2\alpha^{S51A}$, GFP-LC3 WT, $EIF2AK1^{-/-}$ *EIF2AK2^{-/-}, EIF2AK3^{-/-}*, or *EIF2AK4^{-/-}*) or 2000 MEF cells (RFP-LC3 WT or 4KO) were seeded in 384-well µClear imaging plates (Greiner BioOne) and let to adhere. The next day, cells were treated for 6 h to assess peIF2 α and autophagy levels. Following this, cells were fixed with 3.7% formaldehyde (F8775, Sigma Aldrich) supplemented with $1 \mu g/ml$ Hoechst 33342 for 1 h at room temperature. When measuring LC3 aggregation for assessing the level of autophagy, the fixative was exchanged to phosphatebuffered saline (PBS), and the plates were analyzed by automated microscopy. peIF2 α was assessed by further immunostaining after fixation: unspecific antibody interaction was blocked by 2% bovine serum albumin (BSA) for 1 h at room temperature and followed by incubation with primary antibody overnight at 4 °C. After several washing steps with PBS, cells were stained with AlexaFluor-568 (or 488)-coupled secondary antibody (Thermo Fisher Scientific) for 2 h at room temperature and washed with PBS before acquisition. For automated fluorescence microscopy, a robot-assisted IXM XL BioImager and a IXM-C confocal BioImager (Molecular Devices, Sunnyvale, CA, USA) equipped with either a SpectraX or an Aura II light source (Lumencor, Beaverton, OR, USA), adequate excitation and emission filters (Semrock, Rochester, NY, USA), and a 16-bit monochromes sCMOS PCO.edge 5.5 camera (PCO Kelheim, Germany) or an Andor Zyla camera (Belfast, Northern Ireland) and a 20X PlanAPO objective (Nikon, Tokyo, Japan) were used to acquire a minimum of four view fields per well, followed by automated image processing with the custom module editor within the MetaXpress software (Molecular Devices). The primary region of interest (ROI) was defined by a polygon mask around the nucleus allowing for the enumeration of cells and the detection of morphological alterations of the nucleus and nuclear fluorescence intensity. Secondary cytoplasmic ROIs were used for the quantification of peIF2 α intensity. To quantify LC3 aggregation, a mask of high-intensity dots was drawn in the cytoplasm of cells. After exclusion of cellular debris and dead cells from the data set, parameters of interest were normalized, statistically evaluated, and graphically depicted using the R software. Of note, when <30 cells per condition were in good shape, the corresponding condition was excluded from the data set. Using R, images were extracted, and pixel intensities were scaled to be visible (to the same extent for all images of a given experiment). Scale bars represent 20 µm. When needed to compare autophagy among different cell lines, the surface of LC3 dots was normalized as the percentage of induction $((x - c^+)/(c^- - c^+))$, with x test, c^+ positive control torin 1, and c^- negative control)⁴⁵.

Method to determine the effect of a genetic modification on the induction of autophagy

In order to compare cell lines, a linear regression of LC3 dot surface (subjected to *z*-score transformation centered on control) between control and torin 1 was performed. Then the geometric distance to this regression was calculated and considered positive when lower in the genetically modified cell line. For plotting heatmaps, data were independently scaled between 0 and 1 with a sigmoidal transformation with control at 0.5. Then hierarchical clustering was performed.

Protein immunoblot

Protein was extracted with RIPA buffer (#89900; Thermo Fisher Scientific) supplemented with phosphatase and protease inhibitors (#88669; Thermo Fisher Scientific) followed by sonication. Then protein concentration was measured by means of the Bio-rad laboratory DC Protein Assay (#500-0113, #500-0114 and #500-0115, Hercules, CA, USA) following the manufacturer's protocol. Twenty µg of proteins were resuspended in Laemmli buffer (Thermo Fisher Scientific), denaturated at 100 °C, and separated by means of polyacrylamide gel electrophoresis using 4-12% Bis-Tris precasted gels (Thermo Fisher Scientific) in MOPS buffer (Thermo Fisher Scientific). Then proteins were electrotransferred to EtOH-activated polyvinylidene difluoride membranes (Merck Millipore IPVH00010) in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol in H₂O) at 200 mA and 120 V for 1.5 h. Membranes were washed in Tris-buffered saline with Tween20 buffer (TBST; 20 mM Tris, pH 7.5 150 mM NaCl 0.1% Tween 20 in H_2O), and then non-specific sites were blocked with 5% BSA in TBST for 1 h. Membranes were incubated with primary antibody diluted in 5% BSA in TBST overnight at 4 °C. Following this, membranes were washed with TBST and then incubated with appropriate horseradish peroxidase-coupled secondary antibody (Southern Biotech, Birmingham, AL, USA) for 1 h at room temperature. Using ECL (GE Healthcare, Chicago, IL, USA), proteins on the membranes were revealed. Beta-actin was quantified to ensure equal loading.

Statistical analyses

Statistical analyses were performed using the freely available software R (https://www.r-project.org). To compare the effect of treatments on one biological parameter, data were depicted in barcharts with mean \pm SD. The statistical significance was evaluated using one-sided unpaired Student's *t* test with the *t.test* function from the *stats* R package. Kolmogorov–Smirnov test was used to compare distributions of drugs belonging to different clusters, using the *ks.test* function from the *stats* R package. Such data are depicted as boxplot with median \pm quartiles \pm 95%

confidence intervals. Correlations between two parameters were performed by Spearman's rank test, using the *ggscatter* function from the *ggpubr* R package.

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Conflict of interest

G.K. and O.K. are cofounders of Samsara Therapeutics.

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References

- Harding, H. P. et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* 11, 619–633 (2003).
- Ron, D. Translational control in the endoplasmic reticulum stress response. J. Clin. Invest. 110, 1383–1388 (2002).
- Kroemer, G., Marino, G. & Levine, B. Autophagy and the integrated stress response. *Mol. Cell* 40, 280–293 (2010).
- Nakamura, T. et al. Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis. *Cell* 140, 338–348 (2010).
- Taniuchi, S., Miyake, M., Tsugawa, K., Oyadomari, M. & Oyadomari, S. Integrated stress response of vertebrates is regulated by four elF2alpha kinases. *Sci. Rep.* 6, 32886 (2016).
- Pathak, V. K., Schindler, D. & Hershey, J. W. Generation of a mutant form of protein synthesis initiation factor eIF-2 lacking the site of phosphorylation by eIF-2 kinases. *Mol. Cell. Biol.* 8, 993–995 (1988).
- Lu, L., Han, A. P. & Chen, J. J. Translation initiation control by heme-regulated eukaryotic initiation factor 2alpha kinase in erythroid cells under cytoplasmic stresses. *Mol. Cell. Biol.* 21, 7971–7980 (2001).

- Chen, J. J. et al. Cloning of the cDNA of the heme-regulated eukaryotic initiation factor 2 alpha (eIF-2 alpha) kinase of rabbit reticulocytes: homology to yeast GCN2 protein kinase and human double-stranded-RNA-dependent eIF-2 alpha kinase. *Proc. Natl Acad. Sci. USA* 88, 7729–7733 (1991).
- Chen, J. J. & London, I. M. Regulation of protein synthesis by heme-regulated eIF-2 alpha kinase. *Trends Biochem. Sci.* 20, 105–108 (1995).
- Gomes-da-Silva, L. C. et al. Photodynamic therapy with redaporfin targets the endoplasmic reticulum and Golgi apparatus. *EMBO J.* https://doi.org/10.15252/ embj.201798354 (2018).
- Meurs, E. et al. Molecular cloning and characterization of the human doublestranded RNA-activated protein kinase induced by interferon. *Cell* 62, 379–390 (1990).
- Levin, D. H., Petryshyn, R. & London, I. M. Characterization of double-stranded-RNA-activated kinase that phosphorylates alpha subunit of eukaryotic initiation factor 2 (eIF-2 alpha) in reticulocyte lysates. *Proc. Natl Acad. Sci. USA* 77, 832–836 (1980).
- Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P. & Ron, D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat. Cell Biol.* 2, 326–332 (2000).
- Harding, H. P., Zhang, Y. & Ron, D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* **397**, 271–274 (1999).
- Zhang, P. et al. The GCN2 elF2alpha kinase is required for adaptation to amino acid deprivation in mice. *Mol. Cell. Biol.* 22, 6681–6688 (2002).
- Dever, T. E. et al. Phosphorylation of initiation factor 2 alpha by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. *Cell* 68, 585–596 (1992).
- Thakor, N. & Holcik, M. IRES-mediated translation of cellular messenger RNA operates in elF2alpha- independent manner during stress. *Nucleic Acids Res.* 40, 541–552 (2012).
- Baird, T. D. & Wek, R. C. Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. *Adv. Nutr.* 3, 307–321 (2012).
- B'Chir, W. et al. The elF2alpha/ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res.* 41, 7683–7699 (2013).
- Talloczy, Z. et al. Regulation of starvation- and virus-induced autophagy by the elF2alpha kinase signaling pathway. *Proc. Natl Acad. Sci. USA* 99, 190–195 (2002).
- Kadowaki, H. & Nishitoh, H. Signaling pathways from the endoplasmic reticulum and their roles in disease. *Genes* 4, 306–333 (2013).
- Madeo, F., Zimmermann, A., Maiuri, M. C. & Kroemer, G. Essential role for autophagy in life span extension. J. Clin. Invest. 125, 85–93 (2015).
- Mizushima, N., Levine, B., Cuervo, A. M. & Klionsky, D. J. Autophagy fights disease through cellular self-digestion. *Nature* 451, 1069–1075 (2008).
- Pietrocola, F. et al. Caloric restriction mimetics enhance anticancer immunosurveillance. *Cancer Cell* 30, 147–160 (2016).
- Pietrocola, F. et al. Spermidine induces autophagy by inhibiting the acetyltransferase EP300. *Cell Death Differ.* 22, 509–516 (2015).
- Chen, G. et al. 3,4-Dimethoxychalcone induces autophagy through activation of the transcription factors TFE3 and TFEB. *EMBO Mol. Med.* 11, e10469 (2019).
- 27. Bezu, L. et al. elF2alpha phosphorylation is pathognomonic for immunogenic cell death. *Cell Death Differ.* **25**, 1375–1393 (2018).
- You, L. et al. Crizotinib induces autophagy through inhibition of the STAT3 pathway in multiple lung cancer cell lines. *Oncotarget* 6, 40268–40282 (2015).
- Yu, X. & Long, Y. C. Autophagy modulates amino acid signaling network in myotubes: differential effects on mTORC1 pathway and the integrated stress response. *FASEB J.* 29, 394–407 (2015).
- De Gassart, A. et al. An inhibitor of HIV-1 protease modulates constitutive elF2alpha dephosphorylation to trigger a specific integrated stress response. *Proc. Natl Acad. Sci. USA* **113**, E117–E126 (2016).
- Bruning, A., Rahmeh, M. & Friese, K. Nelfinavir and bortezomib inhibit mTOR activity via ATF4-mediated sestrin-2 regulation. *Mol. Oncol.* 7, 1012–1018 (2013).
- Mauvezin, C. & Neufeld, T. P. Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCAdependent autophagosome-lysosome fusion. *Autophagy* **11**, 1437–1438 (2015).
- Kouroku, Y. et al. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. *Cell Death Differ.* 14, 230–239 (2007).
- Shen, L. et al. Phosphorylated heat shock protein 27 promotes lipid clearance in hepatic cells through interacting with STAT3 and activating autophagy. *Cell. Signal.* 28, 1086–1098 (2016).

- Shen, S. et al. Cytoplasmic STAT3 represses autophagy by inhibiting PKR activity. *Mol. Cell* 48, 667–680 (2012).
- Wek, R. C., Jiang, H. Y. & Anthony, T. G. Coping with stress: eIF2 kinases and translational control. *Biochemical Soc. Trans.* 34, 7–11 (2006).
- Anderson, P. & Kedersha, N. Stress granules. *Curr. Biol.* **19**, R397–R398 (2009).
 Obeid, M. et al. Calreticulin exposure dictates the immunogenicity of cancer
- cell death. Nat. Med. **13**, 54–61 (2007).
- Galluzzi, L., Buque, A., Kepp, O., Zitvogel, L. & Kroemer, G. Immunogenic cell death in cancer and infectious disease. *Nat. Rev. Immunol.* 17, 97–111 (2017).
- Mulvey, M., Poppers, J., Sternberg, D. & Mohr, I. Regulation of elF2alpha phosphorylation by different functions that act during discrete phases in the herpes simplex virus type 1 life cycle. J. Virol. 77, 10917–10928 (2003).
- Verpooten, D. et al. Dephosphorylation of elF2alpha mediated by the gamma134.5 protein of herpes simplex virus 1 facilitates viral neuroinvasion. J. Virol. 83, 12626–12630 (2009).
- Schneider, R. J. & Mohr, I. Translation initiation and viral tricks. *Trends Biochem. Sci.* 28, 130–136 (2003).
- Humeau, J. et al. Inhibition of transcription by dactinomycin reveals a new characteristic of immunogenic cell stress. *EMBO Mol. Med.* 12, e11622 (2020).
- 44. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).
- Malo, N., Hanley, J. A., Cerquozzi, S., Pelletier, J. & Nadon, R. Statistical practice in high-throughput screening data analysis. *Nat. Biotechnol.* 24, 167–175 (2006).