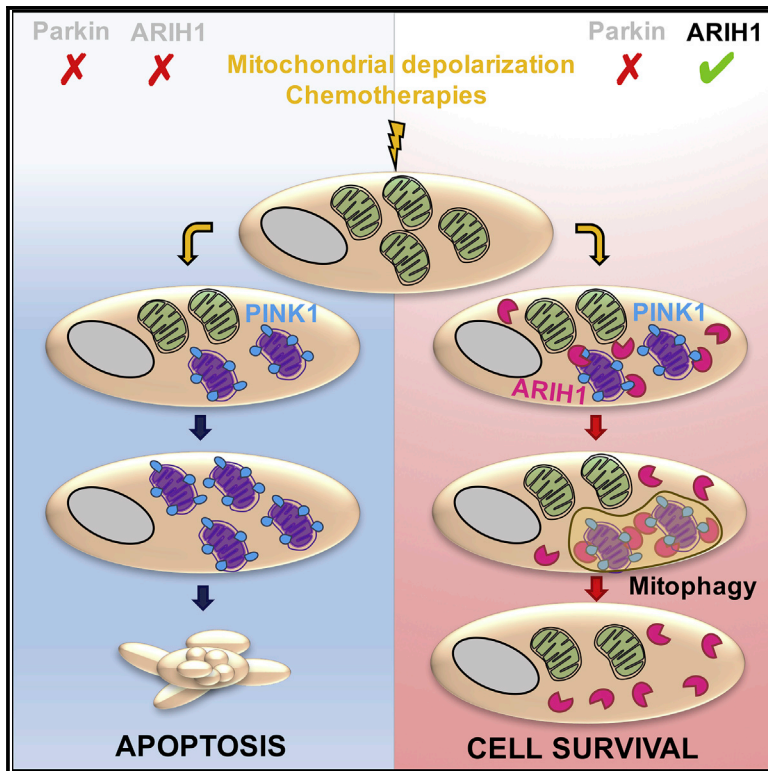# Parkin-Independent Mitophagy Controls Chemotherapeutic Response in Cancer Cells

Graphical Abstract

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In Brief

Clearance of damaged mitochondria (mitophagy) is involved in the resistance to chemotherapeutic-induced death, but the main known regulators of mitophagy are not expressed in cancer cells. Villa et al. show that the RBR E3 ligase ARIH1 is expressed in several cancer cell types. ARIH1 controls PINK1-dependent mitophagy and sensitivity to chemotherapies.

Highlights

- Parkin (RBR E3 ligase) is absent in most cancer cells when mitophagy is functional

- ARIH1, an E3 ligase belonging to the RBR family, is expressed in cancer cells

- ARIH1 controls mitophagy of damaged mitochondrial in a PINK1-dependent manner

- ARIH1's control of mitophagy protects cancer cells from chemotherapy-induced death

Parkin-Independent Mitophagy Controls Chemotherapeutic Response in Cancer Cells

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http://dx.doi.org/10.1016/j.celrep.2017.08.087

SUMMARY

Mitophagy is an evolutionarily conserved process that selectively targets impaired mitochondria for degradation. Defects in mitophagy are often associated with diverse pathologies, including cancer. Because the main known regulators of mitophagy are frequently inactivated in cancer cells, the mechanisms that regulate mitophagy in cancer cells are not fully understood. Here, we identified an E3 ubiquitin ligase (ARIH1/HHARI) that triggers mitophagy in cancer cells in a PINK1-dependent manner. We found that ARIH1/HHARI polyubiquitinates damaged mitochondria, leading to their removal via autophagy. Importantly, ARIH1 is widely expressed in cancer cells, notably in breast and lung adenocarcinomas; ARIH1 expression protects against chemotherapy-induced death. These data challenge the view that the main regulators of mitophagy are tumor suppressors, arguing instead that ARIH1-mediated mitophagy promotes therapeutic resistance.

INTRODUCTION

Mitochondria are essential for energy production, reactive oxygen species (ROS) production, calcium buffering, and regulation of several forms of cell death (Villa and Ricci, 2016; Wallace, 2005). Over time, or in response to various stresses, mitochondria will accumulate damage. Therefore, cells have adopted several quality-control processes, including cycles of mitochondrial fusion and fission and the selective elimination of dysfunctional mitochondria by mitophagy, an organelle-specific type of macroautophagy, to maintain a functional network of healthy mitochondria (Wei et al., 2015).

The ubiquitin (Ub) E3 ligase Parkin, which is mutated in recessive familial forms of Parkinson's disease, is a key mediator of mitochondrial quality control processes (Kitada et al., 1998; Shimura et al., 2000). Phosphatase and tensin homolog deleted on chromosome 10-induced kinase 1 (PINK1) is a serine/threonine kinase that shuttles between the cytosol and mitochondria in healthy cells. Normally, it is rapidly degraded by mitochondrial proteases, but PINK1 can stabilize on the outer membrane of

depolarized mitochondria and recruit Parkin, which is initially inactive (Clark et al., 2006; Narendra et al., 2008; Park et al., 2006). PINK1 will phosphorylate Parkin on the Ub-like (UBL) domain on the Ser^{65} in a $\Delta\Psi m$-dependent process, resulting in an increase of its Ub ligase activity and the formation of polyubiquitin chains on the surface of depolarized mitochondrial membranes. PINK1 will also phosphorylate the conserved Ser^{65} site of Ub molecule (Kane et al., 2014; Kazlauskaite et al., 2015; Koyano et al., 2014; Wauer et al., 2015). It has been proposed that the phosphorylated Ub could act as a Parkin activator by overcoming the autoinhibitory mechanism of Parkin. Both events are needed to fully activate Parkin, which will, in turn, polyubiquitinate numerous mitochondrial outer membrane proteins, leading to the recruitment of the Ub- and LC3-binding adaptor p62 to these damaged organelles (Herhaus and Dikic, 2015). While p62 was initially shown to be critical for removing depolarized mitochondria by transporting them to autophagosomes, later studies suggested a possible redundancy with the related Ub- and Atg8/LC3II-binding protein NBR1 (Narendra et al., 2010; Okatsu et al., 2010), NDP52 (nuclear domain 10 protein 52, also known as CALCOCO2), or optineurin (Lazarou et al., 2015). In particular, NDP52 and optineurin recognize phospho-Ub, leading to the recruitment of the autophagy machinery to initiate mitophagy (Lazarou et al., 2015).

Loss of either PINK1 or Parkin leads to accumulation of damaged mitochondria in several models (fly, mouse, and human), further supporting their central and conserved role in mitochondrial quality-control pathways (Herhaus and Dikic, 2015).

A growing body of evidence has shown the involvement of somatic Parkin inactivation in a broad panel of human cancers. Indeed, Parkin has been shown to be downregulated in multiple cancer cell lines and primary tumors (Gong et al., 2014; Veeriah et al., 2010a, 2010b). Parkin-deficient mice show increased susceptibility to tumorigenesis, while ectopic Parkin expression reduces the in vitro or in vivo growth of cancer cells of various origins, strongly suggesting a tumor-suppressive role for Parkin (for review, see Xu et al., 2014). Therefore, as Parkin is often downregulated in tumors, the molecular events that promote mitophagy in these cells remain to be determined.

Ub and Ub-like modifications occur in a three-step enzymatic process. E1 is an activating enzyme that forms a thioester bond with the Ub protein. Then, the charged Ub monomer is transferred to an E2 enzyme that conjugates the Ub molecule to its target protein, with the help of an E3 Ub ligase (Kerscher

et al., 2006; Nagy and Dikic, 2010). While there are few E1 and E2 ligases, there are many diverse E3 Ub ligases that control substrate specificity and are responsible for the enormous diversity of the Ub system. Several different classes of E3 Ub ligases have been identified. The RING ubiquitinases function as a scaffold between the E2 ligase and the substrate, allowing the transfer of the Ub moiety to the target protein. In contrast, homologous to the E6-AP carboxyl terminus (HECT) ubiquitinases play a direct role in substrate ubiquitination by forming a catalytic intermediate thioester between the C-lobe cysteine residue and the C terminus of Ub (Spratt et al., 2014). Recently, E3 ligases from the Parkin family were classified as hybrids between RING and HECT and were therefore referred to as RING-between-RING (RBR) E3 ligases (Wenzel et al., 2011). They are composed of a canonical RING domain, an in-between ring fingers (IBR) domain, and a RING2 domain. This family has 14 members, including Parkin and Ariadne RBR E3 Ub protein ligase 1 (ARIH1), also known as HHARI. For these ligases, the first RING domain of the RBR module does not directly transfer an E2-bound Ub onto a substrate but instead transfers it to a Cys residue in the RING2 domain (Kulathu and Komander, 2012).

Here, we explored the mechanism controlling mitophagy in cancer cells. As Parkin is not expressed in most cancer cells, we investigated how mitophagy could occur in these cells.

RESULTS

ARIH1 Expression Promotes Elimination of Depolarized Mitochondria

Parkin is a member of the RBR family of E3 ligases that is composed of 14 complex multidomain enzymes. As it is frequently downregulated in cancer cells, we investigated whether other E3 ligases could control mitophagy in these cells. We hypothesized that another member of the RBR family could possibly fulfill this function. A survey of the different family members led us to focus on ARIH1, as it shares the same E2 ligase as Parkin (UbcH7, also known as UBE2L3) (Wenzel et al., 2011), and because an elegant study using a small interfering RNA (siRNA)-based screen recently determined that this E3 ligase is involved in the protection of cancer cells against genotoxic stress (von Stechow et al., 2015).

Depolarization of the mitochondria in HeLa cells using the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) promoted mitophagy of damaged mitochondria only upon expression of Parkin (Figure 1A). Importantly, removal of damaged mitochondria was also observed following ARIH1 overexpression (Figure 1A). Indeed, after 6 hr of CCCP treatment, we observed mitochondrial network collapse around the perinuclear region in HeLa cells expressing ARIH1, as determined by TOM20 staining (Figure S1A), while after 24 hr of CCCP treatment, we observed a complete loss of the mitochondrial marker TOM20 (Figures 1A–1C) and a strong reduction in COX IV, succinate dehydrogenase iron-sulfur subunit (SDHB), and NDUFB8 expression, mitochondrial proteins that are typically degraded during mitophagy (Figures 1D and 1E). We observed that ARIH1-dependent mitophagy occurred to the same extent as

Parkin-dependent mitophagy (Figures 1D and 1E). Importantly, as described for Parkin, ARIH1 was recruited to mitochondria upon CCCP treatment (Figure S1A). Thus, we concluded that ARIH1 overexpression led to the removal of depolarized mitochondria.

ARIH1-Mediated Removal of Damaged Mitochondria Occurs via Mitophagy

We addressed whether the ARIH1-mediated removal of damaged mitochondria involved mitophagy. To establish this point, we measured mitophagy using m-Keima fluorophore, a biosensor of mitochondrial degradation by the lysosomes (Katayama et al., 2011). m-Keima is a variant of RFP that is targeted to the mitochondrial matrix. This cellular biomarker changes its fluorescence profile in response to pH and is resistant to degradation within lysosomes. As presented in Figures 2A and 2B, we measured m-Keima conversion from green (488 nm) to red (561 nm) fluorescence during treatment with several mitochondria-damaging agents, such as CCCP, oligomycin/antimycin (O/A), and valinomycin, using fluorescence-activated cell sorting (FACS) (Lazarou et al., 2015). We observed that ARIH1 could mediate mitophagy upon mitochondrial damage to the same extent as Parkin overexpression. ARIH1-mediated mitophagy upon mitochondrial damage was confirmed by the strong reduction of several mitochondrial markers, such as SDHB and NDUFB8, at the protein level (Figure 2C). Interestingly, we observed that basal mitophagy that removes damaged mitochondria produced over time was also dependent on ARIH1 expression (Figures S1B and S1C).

To further characterize ARIH1-dependent mitophagy induction, we measured autophagic flux in ARIH1- and Parkin-overexpressing HeLa cells following CCCP treatment. We observed an increase of the autophagic flux as determined by the increased conversion of LC3B-I to LC3B-II and increased degradation of the autophagy receptor SQSTM1/p62 and the mitochondrial protein COX IV (Figures S2B and S2C) in the presence of ARIH1 or Parkin overexpression. Importantly, treatment with the lysosomal inhibitor bafilomycin A1 (BafA1) further increased LC3B-II accumulation and reduced p62 and COX IV degradation, indicating a complete autophagic response (Figures S2B and S2C). We verified that CCCP had no effect on the expression of those proteins in the absence of ARIH1 or Parkin expression (Figure S2A). We also observed that LC3, ARIH1 (or Parkin used as a positive control), and the mitochondrial marker cytochrome *c* were co-localized on mitochondria upon CCCP treatment (Figures S2D and S2E), suggesting that ARIH1 mediates mitophagy upon mitochondrial damage. To strengthen this point, we observed that in HeLa cells transfected with an empty vector, LC3 was not co-localized on mitochondria upon CCCP treatment (Figure S2F), confirming that HeLa cells cannot perform mitophagy to a significant extent in the absence of one of those E3 ligases.

To further investigate the role macroautophagy in ARIH1-dependent mitophagy, we used ATG7 knockout (KO) mouse embryonic fibroblasts (MEFs) and KO MEFs reconstituted with ATG7-GFP (R-ATG7 MEFs; Figures 2D, 2E, and S3) (Taherbhoy et al., 2011). Importantly, MEFs (KO and reconstituted) expressed equivalent endogenous levels of ARIH1 but did not
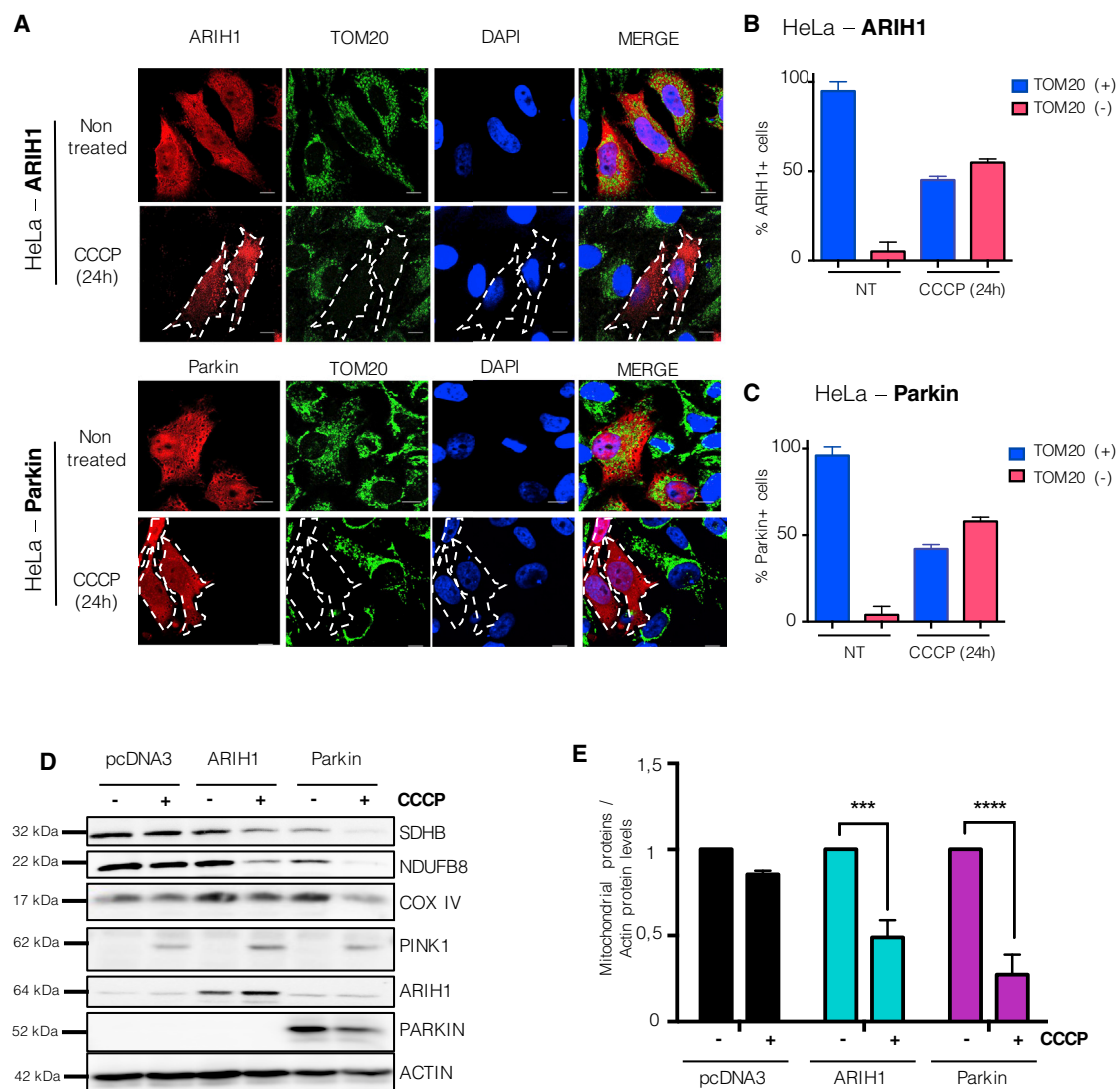
Figure 1. ARIH1 Promotes the Removal of Damaged Mitochondria

HeLa cells were transfected to transiently overexpress the control vector (pcDNA$_3$), ARIH1, or Parkin and then treated with the mitochondrial uncoupling agent CCCP (10 μM) for 24 hr.

(A–C) Mitochondria were immunostained for TOM20 (green), and the absence of the mitochondrial marker TOM20 was assessed in ARIH1$^+$ cells (B) or in Parkin$^+$ cells (C) using confocal microscopy (scale bar, 10 μm). Quantification of mitophagy was estimated by counting a minimum of 100 cells for each condition. Data are shown as the mean of 3 independent experiments.

(D) Whole-cell lysates were analyzed for indicated protein expression by immunoblotting (actin was used as a loading control).

(E) Data are shown as the mean ± SEM of 3 independent experiments.

***p < 0.001 and ****p < 0.0001 according to a two-way ANOVA.

express Parkin (Figures 2D and S3). We observed that endogenous expression of ARIH1 in MEFs was sufficient to decrease SDHB expression (Figures 2D and 2E) following CCCP treatment in reconstituted MEFs. However, this effect was not observed in ATG7 KO MEFs. We also verified that knockdown of endogenous ARIH1 expression using siRNA prevented ARIH1-dependent mitophagy upon CCCP treatment in R-ATG7 MEFs.

Collectively, these data demonstrate that ARIH1 mediates the removal of depolarized mitochondria through mitophagy.

ARIH1-Mediated Mitophagy Is Dependent on Its Ub Ligase Activity and PINK1 Stabilization

To further investigate the molecular mechanisms underlying ARIH1-mediated autophagy, we evaluated the contribution of PINK1. To accomplish this, we first knocked down PINK1 expression using siRNAs in ARIH1-overexpressing HeLa cells. As shown in Figures 3A and 3B, silencing of PINK1 prevented the COX IV decrease following CCCP treatment in HeLa cells overexpressing ARIH1. The involvement of PINK1 was further tested by knocking out PINK1 using CRISPR/Cas9 (Figures 3C
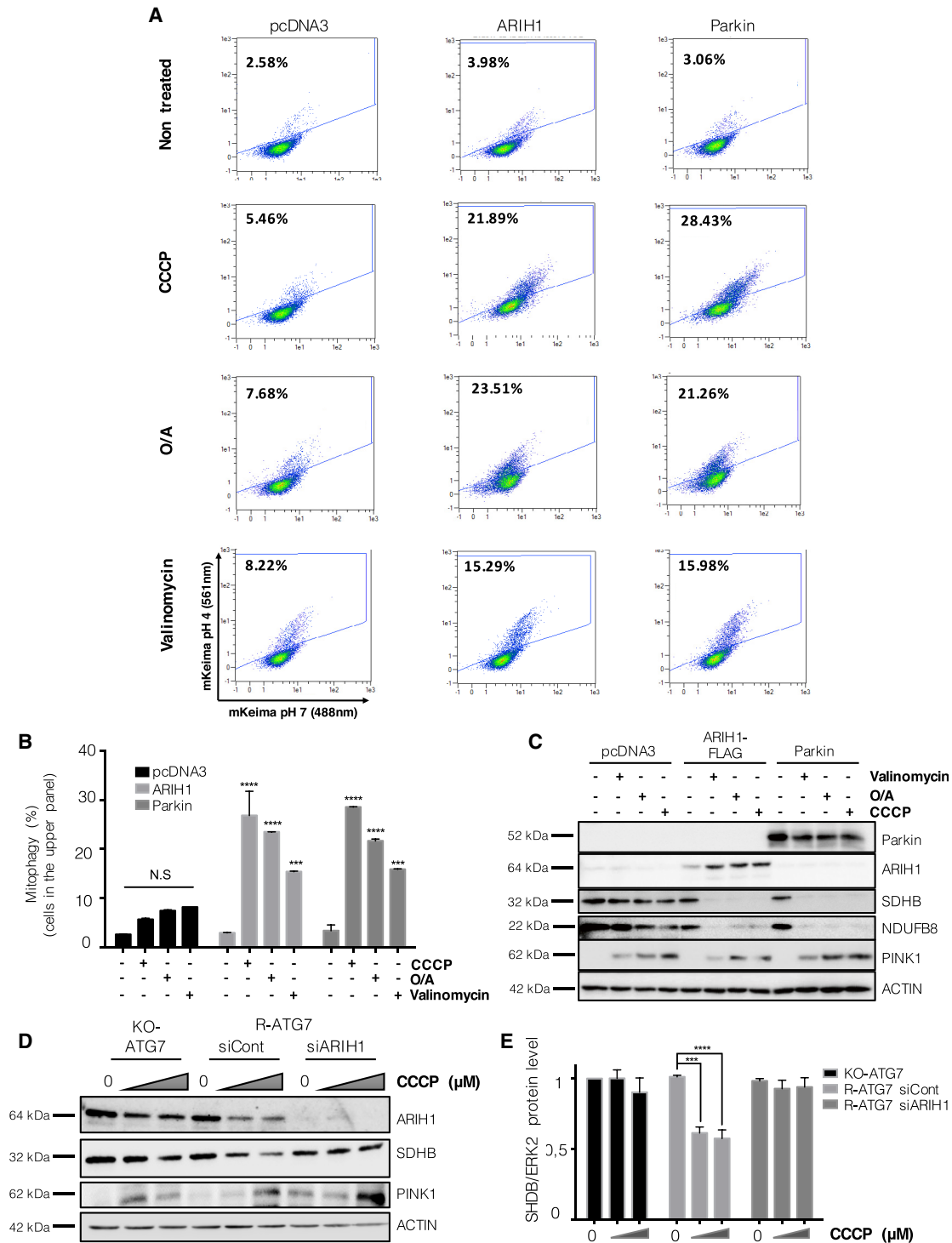
Figure 2. ARIH1 Removes Damaged Mitochondria via Autophagy

(A) HeLa cells overexpressing pcDNA3, ARIH1, or Parkin were transfected with m-Keima; treated with CCCP (10 μM), oligomycin/antimycin A (O/A; 25 nM and 250 nM, respectively), or valinomycin (10 nM) for 24 hr; and analyzed by flow cytometry. Green fluorescence of m-Keima reflects mitochondria in the cytosol (FL_{mito}, green), while red fluorescence reflects mitochondria in lysosomes (FL_{lyso}, red). The ratio of mitophagy is reflected by the percentage of cells in the top panel.

(B) Data are shown as the mean ± SEM of 3 independent experiments performed as in (A).

(legend continued on next page)

and 3E) in HeLa cells overexpressing ARIH1. Indeed, while ARIH1 decreased COX IV (Figures 3C and 3D) and increased conversion from green to red m-Keima following CCCP treatment (Figure 3E), both effects were blunted upon PINK1 KO. Equivalent results were obtained upon treatment with other mitochondria-damaging agents (Figure 3E).

It has been shown that during mitophagy, PINK1 phosphorylates Parkin and Ub on Ser[65]. We established here that in response to CCCP treatment, ARIH1 was phosphorylated on a Ser/Thr residue (Figure 3F), suggesting that phosphorylation by PINK1 is the first step in ARIH1-mediated mitophagy. Thus, ARIH1-dependent mitophagy requires PINK1 expression.

We then verified that the mitochondria of ARIH1-expressing HeLa cells were polyubiquitinated upon CCCP treatment as determined by the increase in Ub staining that co-localized with TOM20 staining on the mitochondria (Figure 4A). We also established that upon relocalization to the mitochondria, ARIH1 expression leads to TOM20 and MFN2 (mitofusin 2) degradation in a proteasomal-dependent manner, as MG132 could prevent it (Figure 4B).

We then used a mutant of ARIH1 with deletions in the RING type 1, IBR type, and RING type 2 domain (referred to as ΔARIH1). As previously shown, overexpression of full-length ARIH1 or Parkin resulted in mitophagy upon CCCP treatment, as determined by the conversion of m-Keima from green to red (Figure 4C) and the decrease in COX IV and SDHB expression (Figures 4D and 4E). In contrast, while ΔARIH1 was overexpressed to the same extent as full-length ARIH1, it did not lead to mitophagy (Figures 4C–4E) upon CCCP treatment.

We could therefore conclude that ARIH1 induces mitophagy by polyubiquitination of the damaged mitochondria, leading to its removal by the autophagic machinery.

ARIH1 Is Overexpressed in Cancer Cells and Is the Main Regulator of Mitophagy in These Cells

After establishing ARIH1 as a regulator of mitophagy, we assessed its expression in various cell lines and tissues (Figures 5A, 5B, and S4). ARIH1 mRNA was widely expressed in human cancer tissues and cancer cell lines, with strong expression in lung adenocarcinoma samples (Figures S4A and S4B). We then investigated ARIH1 protein expression in a panel of 9 different human cancer cell lines (Figure 5A). In contrast to Parkin, which was not expressed in any of the tumor cell lines tested (either at the protein or mRNA level; Figures 5A, 5B, and S4C), ARIH1 was endogenously expressed in several of them (Figures 5A and 5B), with the highest expression in lung cancer cell lines (A549 and H1975).

We then determined whether endogenous expression of ARIH1 could activate mitophagy upon mitochondrial depolarization. To accomplish this, A549 and H1975 cells were incubated

with increasing amounts of a decoupling agent, and mitophagy was assessed by measuring COX IV expression. As shown in Figures 5C and 5D, COX IV expression was reduced upon decoupling of the mitochondria. This removal of damaged mitochondria was associated with an increase in autophagic markers (LC3 conversion and decrease in p62 expression; Figure 5E), altogether indicating that endogenous ARIH1 expression in lung cancer cell lines induced the removal of damaged mitochondria through mitophagy.

Importantly, we then demonstrated using three independent siRNAs that ARIH1 knockdown was sufficient to prevent CCCP-induced mitophagy as determined by the absence of the decrease in COX IV expression (Figure 6A). Equivalent results were obtained in H1975 cells following CCCP treatment (Figure S5A). To further support our observations, we performed a rescue experiment by knocking down endogenous ARIH1 expression in A549 cells, and we re-expressed an siRNA-resistant ARIH1-FLAG. As presented in Figures 6B, 6C, and S5B, mitophagy was not observed upon ARIH1 knockdown in the presence of CCCP or O/A, while ARIH1-FLAG expression restored it. We then measured mitophagy using m-Keima (as done previously) using a mitochondrial-damaging treatment (O/A). Mitophagy was blunted upon ARIH1 knockdown (Figure 6C), while it was observed when ARIH1-FLAG was expressed (Figure 6D).

We then deleted PINK1 expression using a CRISPR/Cas9 interference technique to validate the implication of this kinase in ARIH1-mediated mitophagy upon endogenous expression of this E3 ligase (Figures 6E and 6F). While mitochondrial network collapse around the perinuclear region and PINK1 induction (two early signs of mitophagy) could be observed in control cells, these markers were absent in A549 cells lacking PINK1. Similarly, COX IV reduction following CCCP treatment was not observed in cells lacking PINK1 (Figure 6F). We confirmed that the removal of damaged mitochondria in A549 cells was indeed mediated through mitophagy, as a cellular invalidation of ATG12 or ATG7 using the CRISPR/Cas9 technique (Wang et al., 2014) prevented CCCP-induced COX IV degradation and m-Keima conversion (Figures 6G–6I).

It was recently suggested that optineurin and NDP52 are key cargo adaptors for Parkin-mediated mitophagy (Heo et al., 2015; Lazarou et al., 2015; Richter et al., 2016). We therefore knocked down their expression using specific siRNA (Figures S5C and S5D) to evaluate their implication in ARIH1-mediated mitophagy. While respective protein expression was massively reduced in A549 cells, it did not prevent the COX IV decrease observed upon CCCP treatment, altogether indicating that neither optineurin nor NDP52 acts as a cargo adaptor for ARIH1-mediated mitophagy. MFN2 was recently suggested to be a mitochondrial receptor for Parkin that is required for mitophagy (Chen and Dorn, 2013). Using two independent

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(C) Whole-cell lysates were treated as in (A) and analyzed for SDHB, NDUFB8, PINK1, Parkin, and ARIH1 expression by immunoblotting (actin was used as a loading control).

(D) Mitophagy was analyzed in MEF KO ATG7 cells and MEF KO cells reconstituted with ATG7-GFP (R-ATG7 MEFs) transfected with an siRNA control or an siRNA targeting ARIH1 by immunoblotting for SDHB or PINK1.

(E) Data are shown as the mean ± SEM of 3 independent experiments.

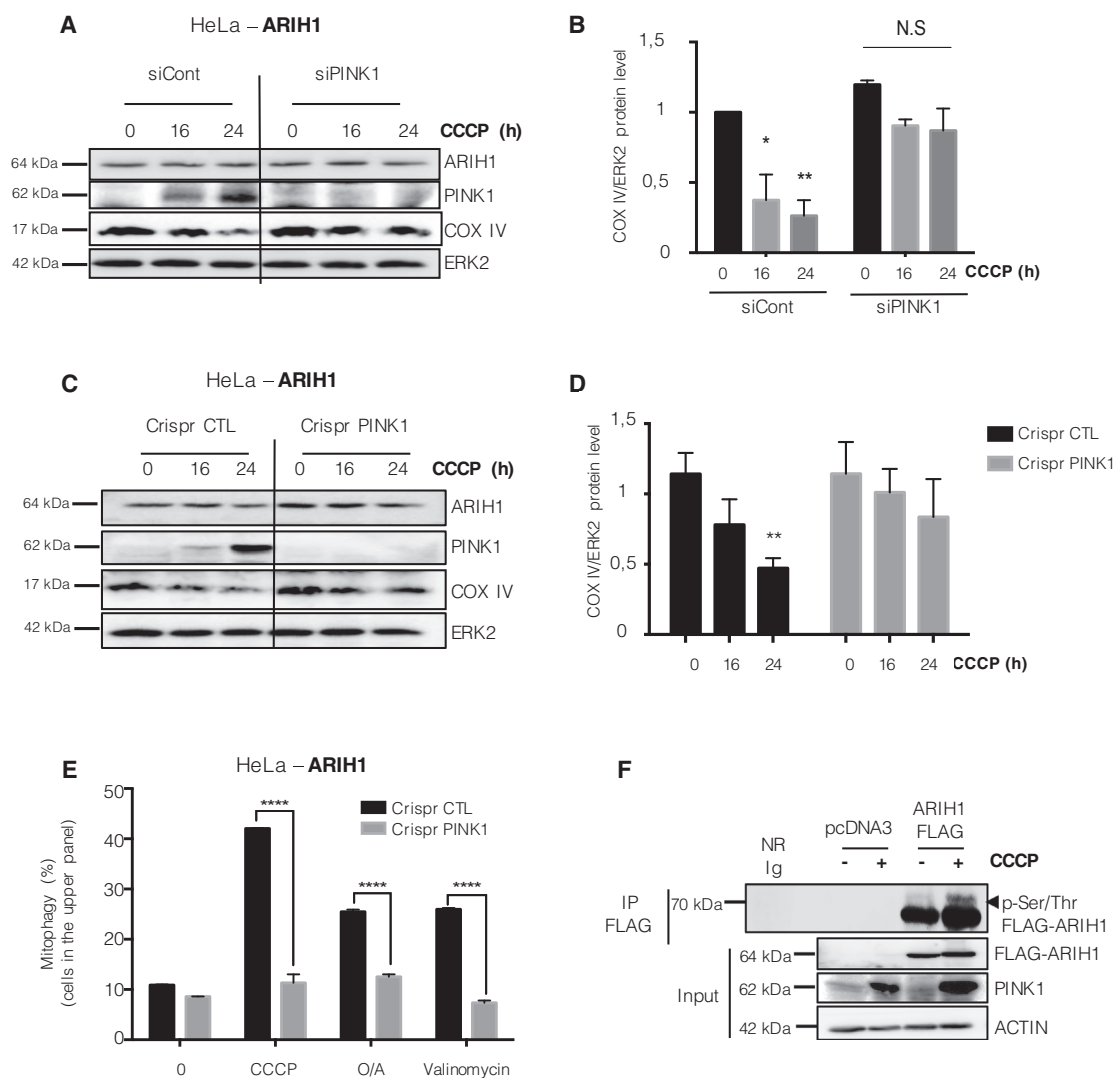$^{***}p < 0.001$, $^{****}p < 0.0001$ according to a two-way ANOVA.

Figure 3. ARIH1-Dependent Mitophagy Requires PINK1

HeLa cells overexpressing ARIH1 were transfected with an siRNA control or an siRNA targeting PINK1 and treated with CCCP (10 μM) for the indicated times.

(A) Whole-cell lysates were analyzed for COX IV, PINK1, and ARIH1 expression by immunoblotting (ERK2 was used as a loading control).

(B) Data are shown as the mean ± SEM of 3 independent experiments.

(C–E) HeLa-expressing ARIH1 were transfected with a control construct (CRISPR CTL) or CRISPR/Cas9 construct in order to delete PINK1 and then treated with CCCP (10 μM) for the indicated times.

(C) Whole-cell lysates were analyzed for COX IV, PINK1, and ARIH1 expression by immunoblotting (ERK2 was used as a loading control).

(D) Data are shown as the mean ± SEM of 3 independent experiments.

(E) HeLa cells CRISPR CTL or CRISPR PINK1 ARIH1 were transfected with m-Keima; treated with CCCP (10 μM), oligomycin/antimycin A (O/A; 25 nM and 250 nM, respectively), or valinomycin (10 nM) for 24 hr; and analyzed by flow cytometry as in Figures 2A and 2B. Data are shown as the mean ± SEM of 3 independent experiments.

(F) HeLa cells were transfected with pcDNA3 or FLAG-ARIH1 plasmids. Cells were left untreated (left) or treated with CCCP (10 μM) for 4 hr (right). ARIH1 was immunoprecipitated (IP) with an anti-FLAG antibody followed by immunoblotting for phospho-Ser/Thr antibody. Actin was used as a loading control.

*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 according to a two-way ANOVA. N.S: non-significant.

siRNAs, we established that MNF2 knockdown does not impair ARIH1-mediated mitophagy or sensitivity to cisplatin (Figures S5E–S5H).

Overall, we detected endogenous expression of ARIH1 in several cancer cell lines, including lung cancer cells, and established that ARIH1 is the main regulator of PINK1-dependent mitophagy upon mitochondrial damage.

ARIH1-Mediated Mitophagy Controls the Sensitivity of Lung Cancer Cells to Chemotherapy-Induced Death

After we identified ARIH1 as an endogenous regulator of mitophagy in lung cancer cells, we then decided to investigate its role in cell sensitivity or resistance to chemotherapy. We first investigated whether modulation of ARIH1 expression had an impact on cell survival. To accomplish this, HeLa cells expressing
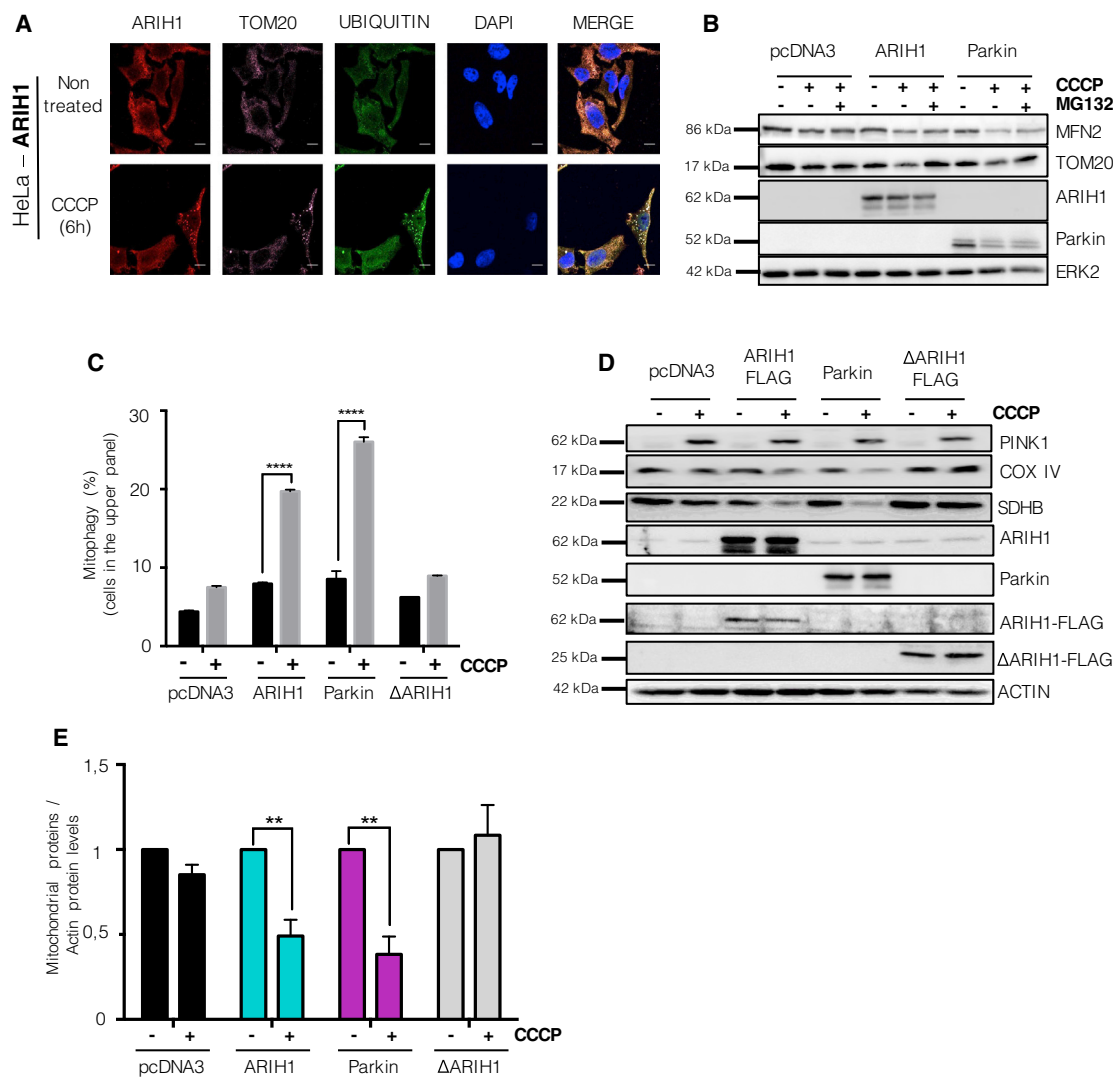
Figure 4. ARIH1-Dependent Mitophagy Requires Its Ub Ligase Activity

(A) ARIH1-overexpressing HeLa cells were treated with CCCP (10 μM) and co-immunostained for TOM20 (pink), Ub (green), and ARIH1 (red). Co-localization was analyzed by confocal microscopy (scale bar, 10 μm).

(B) HeLa cells were transiently transfected with a control vector (pcDNA₃), ARIH1, or Parkin and were treated with the mitochondrial uncoupling agent CCCP (10 μM) for 24 hr alone or with MG132 (10 μM). Whole-cell lysates were analyzed for TOM20, MFN2, PINK1, ARIH1, and Parkin expression by immunoblotting (ERK2 was used as a loading control).

(C) HeLa cells overexpressing pcDNA3, ARIH1, Parkin, or a truncated form of ARIH1 (ΔARIH1-FLAG) were transfected with m-Keima and treated with CCCP (10 μM) for 24 hr and analyzed by flow cytometry as in Figures 2A and 2B. Data are shown as the mean ± SEM of 3 independent experiments.

(D and E) HeLa cells were transiently transfected with a control vector (pcDNA₃), ARIH1-FLAG, Parkin, or a truncated form of ARIH1 (ΔARIH1-FLAG) and treated with the mitochondrial uncoupling agent CCCP (10 μM) for 24 hr.

(D) Whole-cell lysates were analyzed for COX IV, SDHB, PINK1, ARIH1, Parkin, and FLAG expression by immunoblotting (actin was used as a loading control).

(E) Data are shown as the mean ± SEM of 3 independent experiments.

**p < 0.01 and ****p < 0.0001 according to a two-way ANOVA.

ARIH1 were treated with CCCP, and we monitored their ability to grow as clones (clonogenic test). We observed that while only a few control HeLa cells could grow after CCCP treatment, the number of clones was significantly increased in cells expressing ARIH1 (Figure 7A), suggesting a protective effect of ARIH1 following mitochondrial damage.

We then verified that ARIH1 knockdown in A549 cells did not affect CCCP-induced mitochondrial depolarization (Figure S6A)

or the ability of these cells to form colonies (Figure 7B). In contrast, knockdown of endogenous ARIH1 was sufficient to reduce the ability of A549 cells to survive following treatment with CCCP (Figure 7B). Indeed, two independent siRNAs targeting ARIH1 sensitized A549 cells to CCCP-induced apoptosis, as shown by an increase in PARP cleavage (Figure 7C), an increase in DEVDase activity (Figure 7D), and an increase in sub-G1 DNA content (Figure 7H), typical hallmarks of apoptosis. This
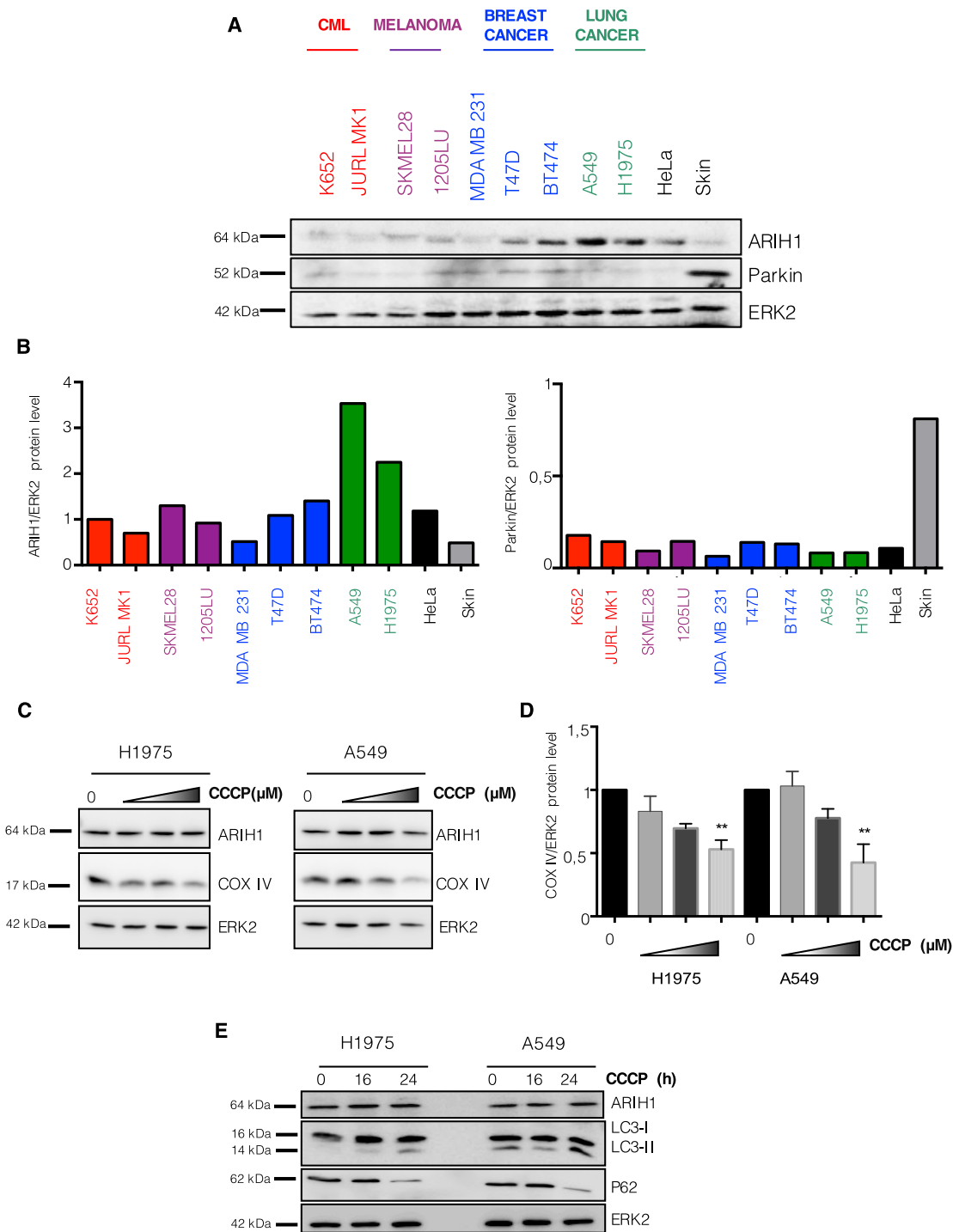
Figure 5. ARIH1 Is Overexpressed in Breast and Lung Cancer Cells

(A) Immunoblots of ARIH1 and Parkin in the indicated cancer cell lines and healthy skin sample (used as a positive control for endogenous Parkin expression). ERK2 was used as a loading control.

(B) The ratio of ARIH1 or Parkin to ERK2 expression (average of 2 independent experiments).

(C) A549 and H1975 lung cancer cell lines were treated with CCCP (from 1 to 10 μM) for 24 hr. The decrease in mitochondrial mass was analyzed by immunoblotting COX IV.

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sensitization to CCCP-induced apoptosis following ARIH1 knockdown was also observed in H1975 cells (Figures S6B and S6C).

The alkylating agent cisplatin is a standard treatment for several cancers, including lung carcinomas. We therefore investigated whether endogenous ARIH1 expression could affect cisplatin-induced cell death. We selected a dose of cisplatin with a limited ability to prevent control A549 cell growth (transfected with a scramble siRNA). Strikingly, the same treatment substantially impaired the growth of ARIH1 knockdown A549 cells (Figure 7E) and sensitized these cells to apoptosis, as shown by the increase in PARP cleavage (Figure 7F), DEVDase activity (Figure 7G), and sub-G1 DNA content (Figure 7H). We confirmed that the decrease in mitochondrial potential ($\Delta\Psi$m) upon cisplatin treatment was not altered in ARIH1 knockdown cells, indicating that this E3 ligase acts downstream of the mitochondrial dysfunction (Figure S6D).

We demonstrated that ARIH1 functions both in mitophagy and in resistance to cisplatin. To determine whether ARIH1 promotes resistance to chemotherapy through mitophagy, we knocked out the molecular actors implicated in ARIH1-mediated mitophagy (PINK1, ATG7, and ATG12) in A549 cells. KO of those key proteins sensitized cells to various types of chemotherapy-induced death (Figures 7I, 7J, and S7A), suggesting that lung cancer cells use mitophagy as a defense mechanism against chemotherapy-induced cell death.

Finally, Parkin was recently suggested to regulate Bax levels and promote resistance to apoptosis independently of mitophagy (Cakir et al., 2017; Johnson et al., 2012). We did not observe any regulation of Bax or Bak levels upon ARIH1 modulation or PINK1 KO, suggesting that ARIH1-dependent control of cell death was not mediated by the regulation of the level of expression of those Bcl-2 members (Figures S6E–S6G). These results suggest that ARIH1-dependent mitophagy is protective in cancer cells.

DISCUSSION

The removal of dysfunctional mitochondria is required to maintain a healthy mitochondrial network and promote cell survival in response to certain stresses. How mitophagy promotes the turnover of damaged mitochondria that would otherwise injure the cell has not been fully elucidated. The most extensively characterized mitophagy regulators are Parkin/PINK1, BNIP3, and NIX (known as BNIP3L), which have non-overlapping roles in promoting autophagy (for review, see Chourasia et al., 2015). Importantly, in most cancers, BNIP3, NIX, and Parkin expression has been shown to be downregulated, indicating their role as tumor suppressors. Indeed, significant deletions of the BNIP3 locus at 10q26.3 were observed in half of the human tumor types, including lung carcinomas (Beroukhim et al., 2010). In addition, epigenetic silencing of BNIP3 expression as tumors progress to invasion and metastasis has been reported (Calvisi

et al., 2007; Erkan et al., 2005). Similarly, Parkin (PARK2) maps to a common fragile site on human chromosome 6q25-q26 that is frequently deleted in cancers (Cesari et al., 2003). Therefore, until now the main regulators of mitophagy were considered as tumor suppressors and, therefore, the vast majority of the studies suggesting that the removal of damaged mitochondria could play a role in the survival of cancer cells following chemotherapeutic treatment could only be obtained after ectopic expression of those genes.

In sharp contrast, we identify here that the E3 ligase ARIH1 is regulator of PINK1-dependent mitophagy (Figures 1, 2, 3, and 4) that is overexpressed in several cancers, including lung adenocarcinomas (Figures 5, 6, and S4). We established that ARIH1-dependent control of mitophagy was indeed dependent on its Ub ligase activity (Figure 4). Importantly, we showed that ARIH1 overexpression is associated with resistance to chemotherapeutic-induced apoptosis (Figure 7). We also demonstrated that removal of ARIH1 or of key mitophagy or autophagy regulators sensitized tumor cells to chemotherapy-induced death (Figure 7), suggesting that mitophagy is protective in those cells, although we cannot formally exclude at this stage that other cellular functions of those proteins (which remain to be identified) are partially involved in the described effect.

Our results also suggest that ARIH1, as opposed to Parkin, BNIP3, or NIX, could be a predictive marker of chemotherapy. This notion is supported by the observation that lung adenocarcinoma patients with high levels of ARIH1 showed decreased survival after treatment (Figure S7B).

ARIH1 is a 557-amino-acid protein (64 kDa) distributed in the cytoplasm and the nuclei of cells (Figure 1A; Elmehdawi et al., 2013) that shares many structural and functional properties with Parkin (Parelkar et al., 2012). It is highly conserved, sharing 72% and 98% amino acid sequence identity with the Drosophila and mouse genes, respectively (Tan et al., 2000). Despite the widespread distribution of ARIH1 transcripts (Moynihan et al., 1999) and the lethality of KOs (Aguilera et al., 2000), its cellular functions are not well characterized. It was previously reported that ARIH1 levels were higher in cancer tissues than in normal tissues of the same origin (Elmehdawi et al., 2013), as opposed to the other known regulators of mitophagy. The same study reported that increased ARIH1 expression was associated with enhanced cell proliferation (Elmehdawi et al., 2013). Mechanistically, a yeast two-hybrid screen suggested an interaction between ARIH1 and the protein translation initiation factor eIF4E2 (Tan et al., 2003), suggesting a role in protein translation or RNA processing. It was later shown that this interaction with eIF4E2 was required for the protection of embryonic stem cells (ESCs) from DNA damage (von Stechow et al., 2015). However, whether this ARIH1-dependent control of mRNA translation arrest is required for the control of mitophagy in cancer cells is not known and will be the subject of further studies.

At the molecular level, it will be important to uncover how PINK1 activates ARIH1. Recently, ARIH1 was shown to be a

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(D) Data are shown as the mean ± SEM of 3 independent experiments.
(E) A549 and H1975 lung cancer cell lines were treated with 10 μM CCCP for the indicated times, and autophagy induction was assessed by immunoblotting LC3 and P62. ERK2 was used as a loading control.
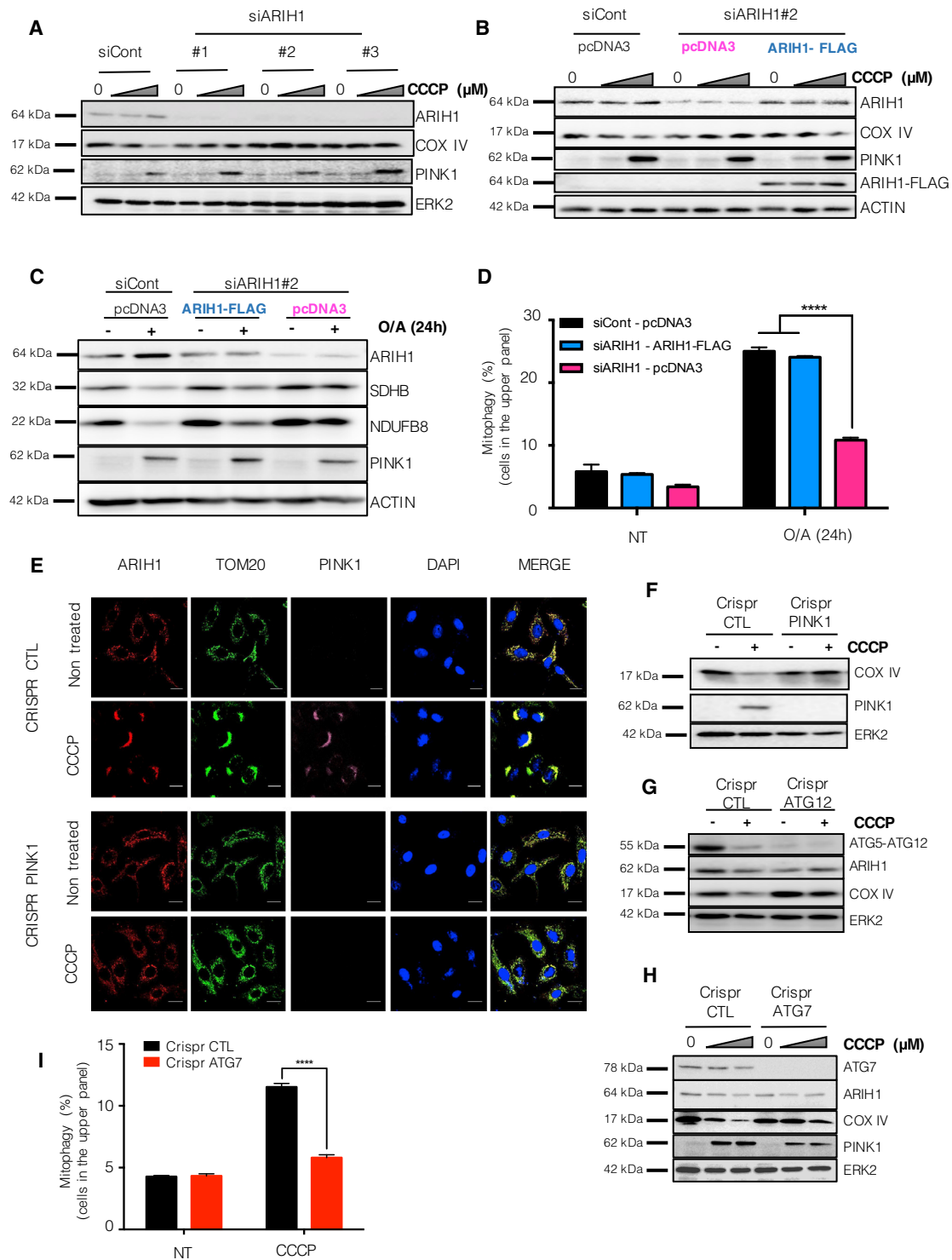**p < 0.01 according to a two-way ANOVA.

Figure 6. ARIH1 Induces Mitophagy in Lung Cancer Cells

A549 cells were transfected with the indicated siRNAs and treated with increasing amounts of CCCP (from 1 to 10 μM) for 24 hr.

(A) Whole-cell lysates were analyzed for COX IV, PINK1, and ARIH1 expression by immunoblotting (ERK2 was used as a loading control).

(B and C) A549 cells were transfected with the indicated siRNAs and then transfected with either an empty vector or FLAG-ARIH1 in order to rescue the knockdown of ARIH1 expression.

(B) Cells were treated with increasing amounts of CCCP (from 1 to 10 μM). Whole-cell lysates were analyzed for SDHB, NDUFB8, PINK1, and ARIH1 expression by immunoblotting (actin was used as a loading control).

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new component of the cullin-RING E3 ligases (CRLs), which specifically mediate monoubiquitylation of several substrates (Scott et al., 2016). We therefore speculate that this novel role of ARIH1 could at least in part be involved in PINK1-mediated mitophagy, possibly through direct interaction with PINK1. PINK1 is a Ser/Thr kinase stabilized at the outer membrane of depolarized mitochondria, and it can phosphorylate Parkin in its UBL domain in order to overcome its autoinhibitory mechanism. Here, we show that ARIH1, after a brief exposure to CCCP, can also be phosphorylated on a Ser/Thr residue (Figure 3F). It is important to note that, like Parkin, ARIH1 also has an inhibitory (Ariadne) domain masking the RING type 2 domain containing catalytic activity. Furthermore, this Ariadne domain contains eight serine residues and two threonine residues that could potentially be phosphorylated by PINK1, leading to the unmasking of the ARIH1 catalytic site. Also, we established that ARIH1-mediated mitophagy and protection from chemotherapy-induced death was dependent on PINK1 expression, but not on NDP52, optineurin, MFN2, Bax, or Bak expression (Figure S5), suggesting that ARIH1 has a different set of targets than Parkin that remains to be identified.

Another open question is how ARIH1 is overexpressed in cancer cells. A screen of different public databases indicated that ARIH1 mRNA expression is upregulated in a wide variety of cancer tissues. As an example, a survey of Tumorscape (Broad Institute, Cambridge, MA, USA) (Beroukhim et al., 2010) indicated that ARIH1 was overexpressed in colorectal and medulloblastoma as well as in 21 out of 40 lung squamous carcinoma samples (tissues/cell lines) present in the database. These data suggest that transcriptional regulation of ARIH1 may be involved. However, the transcription factors involved have not been identified. Therefore, in addition to gene expression, the regulation of ARIH1 protein stability cannot be excluded at this stage.

The role of Parkin in the regulation of cell death is debated. While it is widely assumed to inhibit cell death, it was recently shown that several anti-apoptotic members of the Bcl-2 family could prevent Parkin translocation to the depolarized mitochondria and therefore regulate the onset of Parkin-dependent mitochondrial clearance (Hollville et al., 2014). It was then suggested that Parkin activation in response to mitochondrial dysfunction resulted in apoptosis by promoting the degradation of Mcl-1, a pro-survival gene of the Bcl-2 family (Carroll et al., 2014). These results suggest a close link between Parkin and the Bcl-2 family.

However, as Parkin is rarely expressed in cancer cells, it would be interesting to investigate whether ARIH1 can interact with and control these pro-survival factors, as (1) ARIH1 was reported to limit protein translation, and (2) Mcl-1 has a short half-life and is strongly affected by a block in translation (Meynet et al., 2012, 2013; Pradelli et al., 2010).

Finally, it was recently suggested that PINK1 could induce mitophagy directly through phospho-Ub-mediated recruitment of autophagy receptors (Lazarou et al., 2015). While this interesting observation might be relevant to cells that do not express significant levels of Parkin or ARIH1, these results should be interpreted with caution in cells expressing ARIH1 (such as breast or lung cancer cell lines).

In conclusion, we show here that ARIH1 is a regulator of mitophagy in cancer cells that is involved in the protection of these cells from chemotherapy-induced death. This report challenges the view that the main regulators of mitophagy are tumor suppressors and suggests that ARIH1 may facilitate the removal of damaged mitochondria to promote tumor resistance to chemotherapy. While the association between Parkin genotype and cancer susceptibility is still under debate (Alcalay et al., 2012), our work indicates that ARIH1 is a potential therapeutic target and potentially a predictive marker of lung cancer sensitivity to chemotherapy.

EXPERIMENTAL PROCEDURES

Cell Death Measurement

To induce cell death, cells were treated with CCCP, cisplatin, or etoposide or irradiated with a UV lamp (254 nm) with the indicated doses. Cell death was analyzed either by DEVDase activity or DAPI staining. To assess DEVDase activity, cells were lysed in buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% NP40, 10 μg/mL aprotinin, 1 mM PMSF, and 10 μM leupeptin) 16 hr after treatment. Lysates were standardized for protein content and loaded into a black 96-well plate (CellStar) in the presence of 0.2 mmol/L of the caspase-3 substrate Ac-DEVD-AMC diluted in the following buffer: 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 20 mmol/L EDTA, and 10 mmol/L DTT. Caspase activity was determined both with and without the presence of 1 μmol/L Ac-DEVD-CHO using a fluoroscan at 460 nm, and specific activity was expressed as the change in absorbance per minute per milligram protein.

In parallel, cell viability of the treated cells was assessed by looking at the plasma membrane permeabilization using DAPI staining and then analyzed by flow cytometry (Miltenyi Biotec).

For the cell cycle experiments, cells treated for 16 hr were permeabilized in 70% ethanol overnight at $-20°C$ and washed with PBS. Cells were incubated with PBS, RNase (20 μg/mL), and propidium iodide (50 μg /mL, Sigma Aldrich) for 30 min at 4°C and then analyzed by flow cytometry (Miltenyi Biotec).

(C) Cells were treated with oligomycin/antimycin A (O/A; 25 nM and 250 nM, respectively) for 24 hr. Whole-cell lysates were analyzed for SDHB, NDUFB8, PINK1, and ARIH1 expression by immunoblotting (actin was used as a loading control).

(D) A549 were transfected with the indicated siRNAs and m-Keima, treated with O/A (25 nM and 250 nM, respectively) for 24 hr, and then analyzed by flow cytometry as in Figures 2A and 2B. Data are shown as the mean ± SEM of 3 independent experiments.

(E) A549 control cells (CRISPR CTL) and A549 cells invalidated for PINK1 (CRISPR PINK1) by the CRISPR/Cas9 technique were treated for 6 hr with CCCP (10 μM) and co-immunostained for TOM20 (green), PINK1 (pink), and ARIH1 (red). Co-localization was analyzed by confocal microscopy (scale bar, 10 μm).

(F) A549 CRISPR CTL and CRISPR PINK1 cells were treated for 24 hr with CCCP (10 μM). Whole-cell lysates were analyzed for COXIV, PINK1, and ARIH1 expression by immunoblotting (ERK2 was used as a loading control).

(G) A549 CRISPR CTL and CRISPR ATG12 cells were treated as described in (F).

(H) A549 CRISPR CTL and CRISPR ATG7 cells were treated for 24 hr with increasing amounts of CCCP (from 1 to 10 μM). Whole-cell lysates were analyzed for COX IV, PINK1, ATG7, and ARIH1 expression by immunoblotting (ERK2 was used as a loading control).

(I) A549 CRISPR CTL and CRISPR ATG7 were transfected with m-Keima, treated with CCCP (10 μM) for 24 hr, and analyzed by flow cytometry as in Figures 2A and 2B. Data are shown as the mean ± SEM of 3 independent experiments.
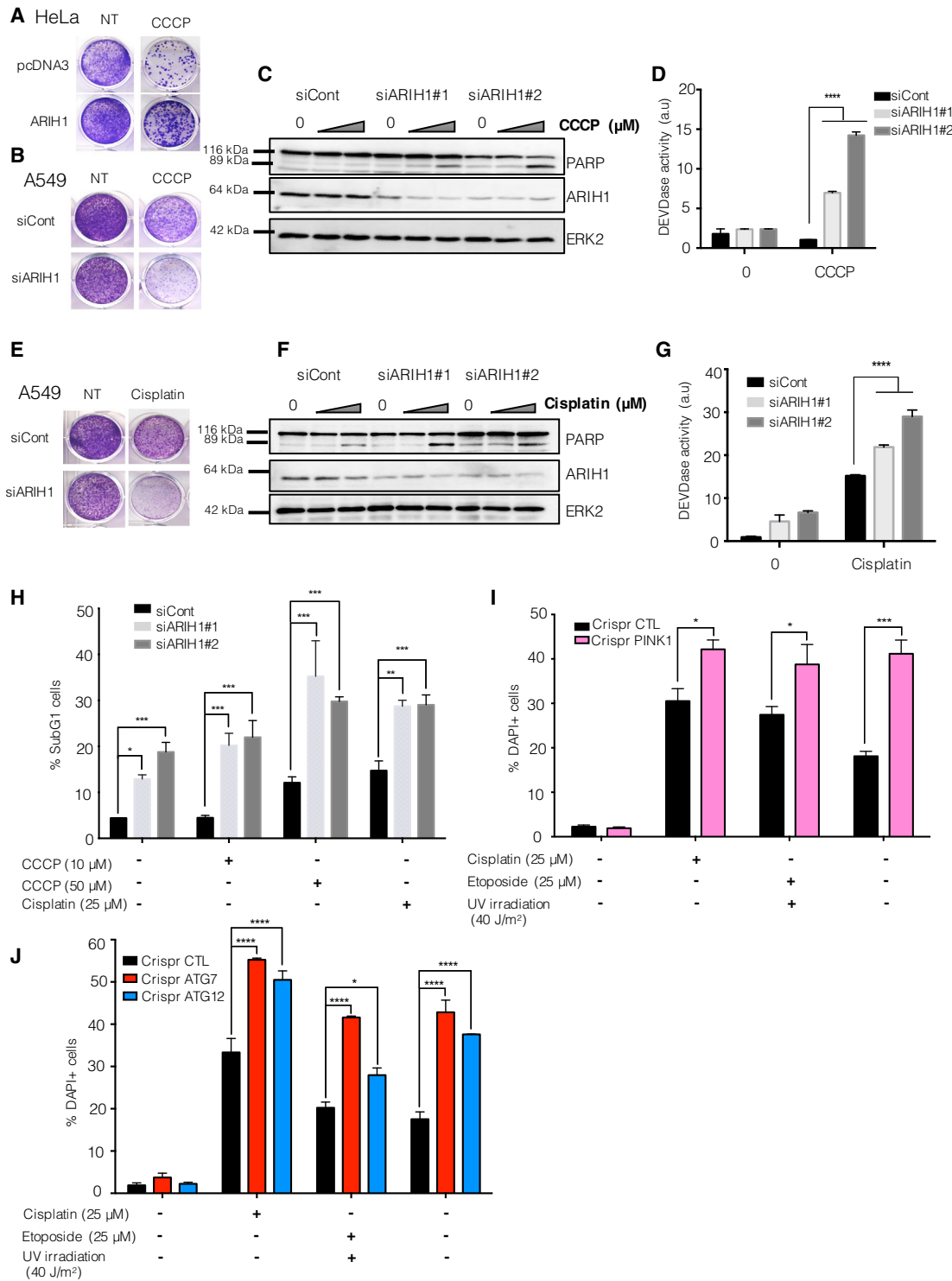
***p < 0.001 according to a two-way ANOVA.

Figure 7. ARIH1 Protects Lung Cancer Cells from Cell Death

(A) Clonogenic assay of HeLa control cells (pcDNA₃) or cells overexpressing ARIH1, which were treated with CCCP (10 μM) for 6 hr. Pictures were taken 10 days after treatment.

(B) Clonogenic assay of A549 control cells (siCont) or ARIH1-silenced cells (siARIH1), which were treated with CCCP (10 μM) for 6 hr. Pictures were taken 5 days after treatment.

(C and D) A549 cells silenced for ARIH1 with two different siRNAs were treated with increasing concentrations of CCCP (from 1 to 10 μM). Apoptosis was analyzed by immunoblotting for PARP cleavage (C) and DEVDase activity (D).

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Mito Keima Mitophagy Analysis

HeLa cells and A549 cells were transfected with m-Keima and then analyzed by flow cytometry (Miltenyi Biotec) as previously reported (Lazarou et al., 2015). Excitation 458 nm and emission >650 nm were used to detect m-Keima in mitochondria in the cytosol (FL_{mito}, green). Excitation 561 nm and emission >650 nm were used to detect mitochondria in lysosomes (FL_{lyso}, red).

Statistical Methods

Data are expressed as mean ± SEM. Differences in the calculated means between groups were assessed by two-way ANOVA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.087.

AUTHOR CONTRIBUTIONS

E.V. performed most experiments. E.P., C.R.-P., B.Z., J.P.B., R.M.R, J.C., and L.M. performed experiments. S.O., J.S.R., S.M., E.V., and S.W.G.T. contributed reagents and scientific input. E.V. and J.E.-R. designed research and interpreted data. J.E.-R. wrote the manuscript.

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(E) Clonogenic assay of A549 control cells (siCont) or cells silenced for ARIH1 (siARIH1) and treated with cisplatin (25 μM) for 6 hr. Pictures were taken 5 days after treatment.

(F and G) A549 cells silenced for ARIH1 with two different siRNAs were treated with increasing concentrations of cisplatin. Apoptosis was analyzed by immunoblotting for PARP cleavage (F) and DEVDase activity (G).

(H) A549 cells silenced for ARIH1 were treated for 16 hr with the indicated amounts of CCCP or cisplatin (25 μM), and apoptosis was assessed using propidium iodide staining and flow cytometry measuring sub-G1 DNA fragmentation. Data are shown as the mean ± SEM of 3 independent experiments.

(I) A549 CRISPR CTL and CRISPR PINK1 cells were treated for 48 hr with cisplatin (25 μM) or etoposide (25 μM) or irradiated with UV (40 J/m²). Plasma membrane permeabilization (i.e., cell death) was assessed using DAPI staining and analyzed by flow cytometry. Data are shown as the mean ± SEM of 3 independent experiments.

(J) A549 CRISPR CTL, A549 CRISPR ATG7, and A549 CRISPR ATG12 were treated as described in (I).

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