Mitochondrial Fission and Mitophagy Depend on Cofilin-Mediated Actin Depolymerization Activity at the Mitochondrial Fission Site

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Introduction

This study looks into how mitochondrial fission (mitochondria splitting in two) and mitophagy (autophagy of mitochondria) are affected by cofilin and actin.

Conclusions

Mitochondrial fission (larger mitochondria splitting in two) is associated with autophagy.

This mitophagy (mitochondrial autophagy) is proposed to be mediated by a lack of cleavage/degradation of the protein PINK whi ch sticks to mitochondria, enabling the recruitment of the protein ubiquitinase/ligase PARK (Parkin), which is a signal for auto phagy by ubiquitinating (tagging) the mitochondrion for autophagy by LC3.

When stimulated (induced mitochondrial fission), cofilin (an actin depolymerizing protein) moves from the cytosol of the cell to the mitochondria.

Cofilin is necessary for mitochondrial fission, and by extension mitophagy, to occur by interacting with g -actin on filamentous actin around the middle of elongated, fission-ready mitochondrion.

Actin is necessary for mitochondrial fission to occur, as well as mitophagy. Any inability for actin to cycle between free G -actin and its structured F-actin reduces mitochondrial fission and mitophagy.

Amendments

The drugs used throughout this paper have wide reaching effects, beyond those claimed by this paper (for example, CCCP is not just a fission inducer).

Study Design & Additional Information

The researchers focused their efforts on largely measuring protein levels of a series of different functional proteins (described below) within cancer cells (MCF-7 and MDA-MB231), as well as taking microscopy images of fluorescent proteins to determine their localization (where they go in the cell under varying conditions).

Mitophagy (mitochondrial autophagy) is mediated, at least partly, by the lack of cleavage of a protein known as PINK2. Normally, this protein is cut to a shorter, non-functional version, but when the mitochondrion needs to be disposed, PINK is left in its complete form to bind it and then a ubiquitin ligase (a protein that adds ubiquitin tags to things) will attach ubiquitin molecules to the mitochondrion (the name of this ligase is PARK2/Parkin). From there, LC3-I begins the process of autophagy until it matures (now LC3-II) into the vesicle/membrane that envelopes the mitochondrion and degrades it (mitophagy).

Cofilin is a protein that binds polymerized/filamentous actin (actin that is structured together, F-actin) to depolymerize it back to its single form (G-actin). Actin is a protein used for cellular movement and to move things throughout the cell.

Mitochondrial fission is a process wherein a large mitochondrion will split into two smaller mitochondria.

CCCP: Mitochondrial fission inducer/promoter.
ETO & STS: Cell apoptosis inducers/promoters.

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ARTICLE

Mitochondrial fission and mitophagy depend on cofilin-mediated actin depolymerization activity at the mitochondrial fission site

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Abstract
Mitochondria fission and mitophagy are fundamentally crucial to cellular physiology and play important roles in cancer progression. Developing a comprehensive understanding of the molecular mechanism underlying mitochondrial fission and mitophagy will provide novel strategies for cancer prevention and treatment. Actin has been shown to participate in mitochondrial fission and mitophagy regulation. Cofilin is best known as an actin-depolymerizing factor. However, the molecular mechanism by which cofilin regulates mitochondrial fission and mitophagy remains largely unknown. Here we report that knockdown of cofilin attenuates and overexpression of cofilin potentiates mitochondrial fission as well as PINK1/PARK2-dependent mitophagy induced by staurosporine (STS), etoposide (ETO), and carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Cofilin-mediated-PINK1 (PTEN-induced putative kinase 1) accumulation mainly depends on its regulation of mitochondrial proteases, including peptidase mitochondrial processing beta (MPPβ), presenilin-associated rhomboid-like protease (PARL), and ATPase family gene 3-like 2 (AFG3L2), via mitochondrial membrane potential activity. We also found that the interaction and colocalization of G-actin/F-actin with cofilin at mitochondrial fission sites undergo constriction after CCCP treatment. Pretreatment with the actin polymerization inhibitor latrunculin B (LatB) increased and actin-depolymerization inhibitor jasplakinolide (Jas) decreased mitochondrial translocation of actin induced by STS, ETO, and CCCP. Both LatB and Jas abrogated CCCP-mediated mitochondrial fission and mitophagy. Our data suggest that G-actin is the actin form that is translocated to mitochondria, and the actin-depolymerization activity regulated by cofilin at the mitochondrial fission site is crucial for inducing mitochondrial fission and mitophagy.

Introduction

Mitochondria are double membrane-bound organelles that have diverse biological functions such as bioenergy production and regulation of cellular signaling and cell death [1–4]. Mitochondria undergo dynamic processes, including fusion and fission, which are important for the maintenance of mitochondrial functions [5–7]. In mammals, mitofusin1 (Mfn1), mitofusin2 (Mfn2), and optic atrophy 1 (OPA1) are involved in the regulation of mitochondrial fusion [8, 9], and dynamin 1-like (Drp1) participates in regulating mitochondrial fission [10]. A recent study demonstrated that cellular cytoskeleton components such as actin filaments might play a crucial role in mitochondrial fission [11, 12]. There is increasing evidence that actin participates in mitochondrial fission through interaction with Drp1 [13, 14]. It has been reported that the actin polymerization inhibitors cytochalasin D, latrunculin A (LatA), and latrunculin B (LatB) do not affect mitochondrial morphology but abrogate mitochondrial fission induced by a mitochondrial toxin [11, 13]. In one report, LatB also evidently attenuated mitochondrial fission in U2OS cells [15]. In contrast, Beck et al. reported that LatB induced

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mitochondrial fragmentation in cultured neurons [16]. Thus, the underlying mechanism of actin-regulated mitochondrial fission remains unclear.

Mitophagy is the selective process responsible for degradation of damaged mitochondria by autophagy, which plays an important role in cancer progression [17]. In mammals, PINK1 (PTEN-induced putative protein kinase 1) and PARK2 (parkinson protein 2) participate in the regulation of mitophagy [18]. Under normal conditions, PINK1 is rapidly degraded by mitochondrial proteases, including matrix processing peptidase alpha (MPPα), matrix processing peptidase beta (MPPβ), ATPase family gene 3-like 2 (AFG3L2), and presenilin-associated rhomboid-like protease (PARL), to generate a 52 kDa fragment and ultimately degraded by the proteasome [19, 20]. Depolarization of the mitochondrial membrane potential (ΔΨm) mediated by CCCP blocks import and stabilizes PINK1 on the outer mitochondrial membrane, leading to recruitment of PARK2 to the mitochondria, which in turn initiates mitophagy [19]. Mitophagy is preceded by mitochondrial fission [21]. Inhibition of the fission machinery through Drp1 dominant-negative expression reduces the level of mitophagy [22]. A very recent study revealed that actin plays a critical role in the regulation of mitophagy [23]. Actin has been considered to be a regulator of the initial formation of autophagosomes in starved cells [24]. However, it is not clear how actin influences mitochondrial fission and mitophagy.

It is highly likely that other proteins, such as cofilin, are involved in the mechanism of mitochondrial fission through the ability to accelerate both actin assembly and disassembly. Cofilin is an actin-depolymerizing factor that increases actin filament turnover [25]. Increasing evidence has revealed that mitochondrial translocation of cofilin plays a crucial role in apoptosis initiation [26]. Only dephosphorylated cofilin (Ser3) can translocate from the cytosol to the mitochondria [8]. Currently, only a small number of reports point to the role of cofilin in regulating mitochondrial fission. A recent study demonstrated that LIM kinase-2 (a cofilin phosphorylation regulator) promotes programmed necrotic death in neurons through Drp1 mitochondrial translocation-mediated mitochondrial fission [27]. Under certain cellular conditions, cofilin regulates mitochondrial fission by interacting with Drp1 [28]. Downregulation of cofilin resulted in mitochondria elongation. This event is associated with Drp1 mitochondrial translocation [11, 28]. A study by Preau S. et al. also demonstrated that cofilin-actin signaling might affect the balance between mitochondrial fragmentation and mitophagy [29]. However, the molecular mechanism by which cofilin-actin signaling affects mitochondrial fission and mitophagy remains largely unknown.

In the present study, we evaluated the role of cofilin-actin signaling in regulating mitochondrial fission and mitophagy. We found that cofilin-regulated mitochondrial fission and mitophagy depend on the actin-depolymerization activity of cofilin at the mitochondria fission site.

Results

Induction of mitochondrial fission is accompanied by simultaneous induction of mitophagy

First, we investigated the effects of mitochondrial fission-inducing agents, such as the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) and the apoptosis inducers staurosporine (STS) and etoposide (ETO), on the morphology of mitochondria in human breast cancer cells. The mitochondrion-selective probe Mitotracker Red CMXRos was employed to label mitochondria. Treatment of MDA-MB-231 and MCF-7 cells with STS, ETO and CCCP resulted in increases in mitochondrial fission, as revealed by the morphological alterations and significant decreases in the average length of mitochondria (Fig. 1a, Supplementary Fig. 1).

Accumulating evidence indicates that mitochondrial fission is a prerequisite for mitophagy [30, 31], and thus, we next determined whether STS, ETO, and CCCP affect mitophagy. RFP-Mito (Red) and GFP-LC3 (Green) plasmids were transfected into cells to label mitochondria and autophagosomes. As shown in Fig. 1b, c, treatment of MDA-MB-231 cells with STS, ETO, and CCCP caused pronounced colocalization of GFP-LC3 and RFP-Mito. Western blot analysis showed that treating cells with STS, ETO, and CCCP increased the LC3B-II expression in mitochondrial fractions and decreased the levels of several mitochondrial proteins, including heat shock 60 kDa protein 1 (HSP60), translocase of outer mitochondrial membrane 20 homolog (Tom20), and voltage-dependent anion channels (VDAC) (Fig. 1d). Taken together, these findings indicate that induction of mitochondrial fission by STS, ETO, and CCCP was accompanied by simultaneous induction of mitophagy.

Mitochondrial fission-inducing agents trigger PINK1/PARK2-dependent mitophagy

It has been shown that depolarization of mitochondrial membrane potential causes accumulation of full-length PINK1 (64 kDa) on the outer mitochondrial membrane, leading to recruitment of PARK2 to the mitochondria, which in turn initiates mitophagy [32, 33]. We next tested whether STS, ETO, and CCCP affect PINK1 accumulation. Treatment of breast cancer cells with STS, ETO, and CCCP resulted in increases in the expression of full-length PINK1

PINK1 is a kinase (enzyme that adds a phosphate to a protein) that is normally degraded, but if mitophagy (mitochondrial degradation) is needed, PINK1 is allowed to stick to the outer membrane of the mitochondria, which recruits the PARK protein (Parkin) to the mitochondrial membrane, as well. This two part action signals for mitochondria to undergo mitochondria autophagy (mitophagy), but before doing so, mitochondria undergo mitochondrial fission (mitochondrion separating into smaller mitochondria).

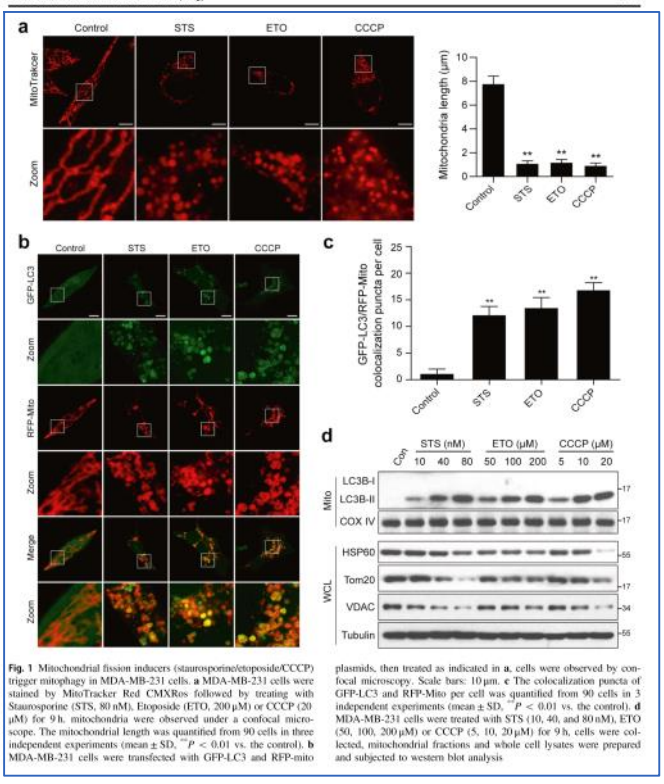

Fig. 1 Mitochondrial fission inducers (staurosporine/etoposide/CCCP) trigger mitophagy in MDA-MB-231 cells. **a** MDA-MB-231 cells were stained by MitoTracker Red CMXRos followed by treating with Staurosporine (STS, 80 nM), Etoposide (ETO, 200 μM) or CCCP (20 μM) for 9 h. mitochondria were observed under a confocal microscope. The mitochondrial length was quantified from 90 cells in three independent experiments (mean ± SD, $^{**}P < 0.01$ vs. the control). **b** MDA-MB-231 cells were transfected with GFP-LC3 and RFP-mito plasmids, then treated as indicated in **a**, cells were observed by confocal microscopy. Scale bars: 10 μm. **c** The colocalization puncta of GFP-LC3 and RFP-Mito per cell was quantified from 90 cells in 3 independent experiments (mean ± SD, $^{**}P < 0.01$ vs. the control). **d** MDA-MB-231 cells were treated with STS (10, 40, and 80 nM), ETO (50, 100, 200 μM) or CCCP (5, 10, 20 μM) for 9 h, cells were collected, mitochondrial fractions and whole cell lysates were prepared and subjected to western blot analysis

(~64 kDa) and decreases in the expression of cleaved PINK1 (~52 kDa) (Fig. 2a, Supplementary Fig. 2a). To further confirm that PINK1 accumulates in depolarized mitochondria in response to STS, ETO, and CCCP treatment, immunofluorescence microscopy was employed to detect the colocalization of PINK1 and mitochondria. As shown in Fig. 2b, PINK1 was precisely colocalized with fragmented mitochondria after treatment with STS, ETO and CCCP.

Figure 1

[1A] The researchers have added a red colored tracker of mitochondria in cells to measure the length/size of mitochondria after the addition of various drugs (STS, ETO, CCCP) that induce mitochondrial fission and apoptosis (cell death).
[1B&C] Here, they have genetically manipulated the cells to express green where the LC3 protein is found (this protein is part of the autophagy system), as well as the previous mito tracker that expresses red for mitochondria and are measuring the co-localization of the two. [1C] is the quantification of that co-localization.

Primary Results

- Mitochondria are smaller upon use of the three drugs mentioned.
- Greater mitochondrial co-localization between mitochondria and autophagy proteins when the three fission inducing drugs are used.

Take Away: Mitochondrial fission (and possibly apoptosis) is associated with autophagy (therefor, assumed mitophagy).

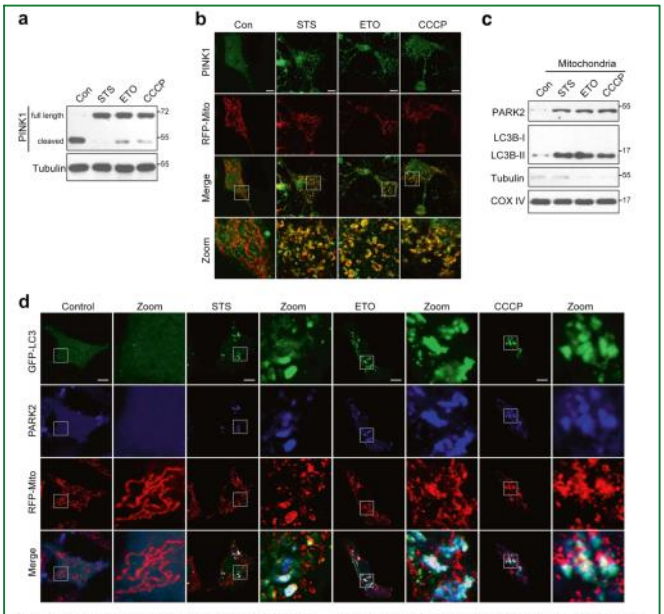

Fig. 2 Staurosporine/etoposide/CCCP induce PINK1/PARK2-dependent mitophagy. **a**, **c** MDA-MB-231 cells were treated with STS (80 nM), ETO (200 μM), or CCCP (20 μM) for 9 h, mitochondrial fractions and whole cell lysates were prepared and subjected to western blot analysis. Tubulin (whole cell lysates) and COX IV (mitochondrial fraction) were used as the loading control. **b** MDA-MB-231 cells were transfected with RFP-mito plasmids for 48 h, then treated as indicated in **a**, the fluorescent signals were detected by confocal microscopy after immunostaining with PINK1 (Alexa Fluor 488, green). **d** Cells were co-transfected with GFP-LC3 and RFP-mito plasmids, treated as indicated in **a**, after immunostaining with PARK2 (Alexa Fluor 647, blue), cells were observed by confocal microscopy. Scale bars: 10 μm

We next examined the effects of STS, ETO, and CCCP on the expression of PARK2 and LC3 in mitochondria. The mitochondrial levels of PARK2 and LC3B-II were obviously elevated in cells treated with STS, ETO, and CCCP (Fig. 2c, Supplementary Fig. 2b). We also observed that the colocalization of PARK2 with LC3 and mitochondria was increased after STS, ETO, and CCCP treatment (Fig. 2d). Taken together, the findings suggest that PINK1 and PARK2 are involved in mitochondrial fission-inducing agent-induced mitophagy.

Mitochondrial translocation of cofilin is involved in mitochondrial fission

Recent evidence indicated that mitochondrial translocation of cofilin plays a crucial role in initiation of mitochondrial

Figure 2

[2A] The researchers are investigating if PINK1 is cleaved (which is normal) or at full length (which is an initial signal for mitophagy (mitochondrial autophagy)) in control (normal) conditions, as well as with the three mitochondrial fission and apoptosis inducing drugs (STS, ETO, CCCP). The darker the smudge, the more there of the protein there is. **[2B]** Here, the researchers are visualizing the localization of PINK (green) and mitochondria (red) and seeing if they localize similarly (overlap between the two is seen as orange). **[2C]** The researchers are finding the quantities of PARK2 (a second signal of mitophagy - Parkin), as well as LC3B (an autophagy protein) without (Con) and with the three fission/apoptosis drugs (STS, ETO, CCCP). The darker the smudge, the more there is. **[2D]** The researchers are again visualizing fluorescent proteins for each of their proteins of interest - LC3 (green), PARK2 (blue), and Mitochondria (red). This is to see the localization and mitochondrial shape between the three with and without the three drug treatment.

Primary Results

- PINK1 remains uncleaved with fission induction.
- PINK1 co-localizes with mitochondria with fission induction.
- PARK2 and LC3 are elevated and localized with mitochondria with fission induction.

Take Away: PINK, PARK, and LC3 are all associated with mitochondria with the induction of mitochondrial fission and possibly apoptosis.

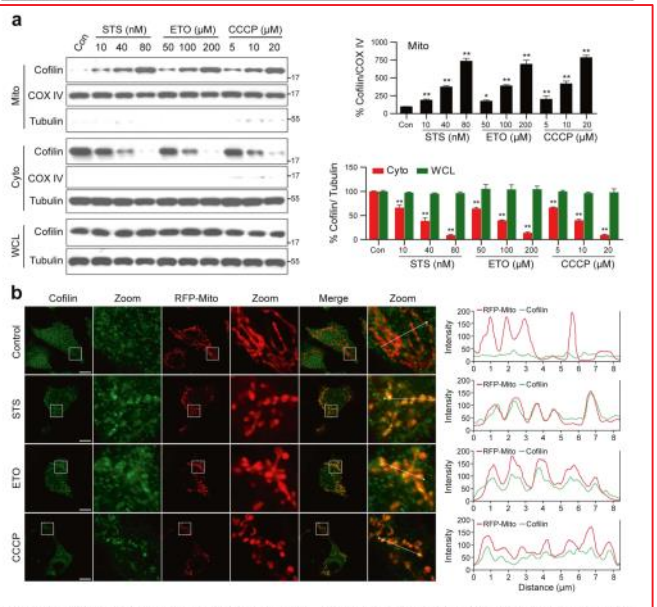

Fig. 3 Staurosporine/etoposide/CCCP induce translocation of cofilin from the cytosol to mitochondria. **a** MDA-MB-231 cells were treated with STS (10, 40, 80 nM), ETO (50, 100, 200 μM) or CCCP (5, 10, 20 μM) for 9 h, mitochondrial (Mito) and cytosolic (Cyto) fractions and whole cell lysates (WCL) were prepared and subjected to western blot analysis. COX IV was used as mitochondrial fractions marker, tubulin was used as cytosolic fractions and whole cell lysates maker. The relative intensities of cofilin were normalized to COX IV or tubulin from three independent experiments, the data were presented as a percentage of control, which was set at 100% (mean ± SD, $^{*}P < 0.05$ or $^{**}P < 0.01$ vs. the control). **b** RFP-mito expressing MDA-MB-231 cells were treated with STS (80 nM), ETO (200 μM) or CCCP (20 μM) for 9 h, after immunostaining with cofilin (Alexa Fluor 488, green), images were captured by confocal microscopy. Scale bars: 10 μm. Intensity line scans of red and green channels along the length of the arrows were measured

fission and apoptosis [26, 28]. Next, using western blot analysis, we determined whether STS, ETO, and CCCP treatment affects mitochondrial translocation of cofilin. Treatment of cells with STS, ETO, and CCCP resulted in a dose-dependent increase in the expression of cofilin in mitochondria and a decrease in the expression of cofilin in the cytosol (Fig. 3a, Supplementary Fig. 3). To determine whether cofilin was colocalized with mitochondria, an immunofluorescence microscopic study was employed. As shown in Fig. 3b, treatment of cells with STS, ETO, and CCCP caused pronounced colocalization of cofilin with fragmented mitochondria. Together, these findings indicate

Figure 3

[3A] The researchers are measuring the amount of cofilin (the other proteins are merely controls) found in each section of the cell with increasing concentrations of the already discussed drugs (STS, ETO, CCCP) - Mito is around the mitochondria, Cyto is in the cytosol exclusively, and WCL is the whole cell. Cofilin is a molecule that binds actin protein pieces that are arranged in a filament and breaks them off the filament to recycle them in a pool of actin that can then be re-used for generating new filaments (more details are listed in the "additional information" section). The graphs are just a visual of the smudges. The darker the smudge, the more of the protein is present. **[3B]** The researchers are visualizing the co-localization of cofilin (green) and mitochondria (red), with close association being orange, in control (unmanipulated) cells, as well as the three drug conditions mentioned previously.

Primary Results

- With administration of drugs, cofilin decreases in the cytosol of the cell, but increases in the mitochondrial compartment, yet shows no differences in whole cell.
- Cofilin co-localizes with mitochondria when fission is initiated.

Take Away: It is likely that cofilin moves from the cytosol to localize around mitochondria.

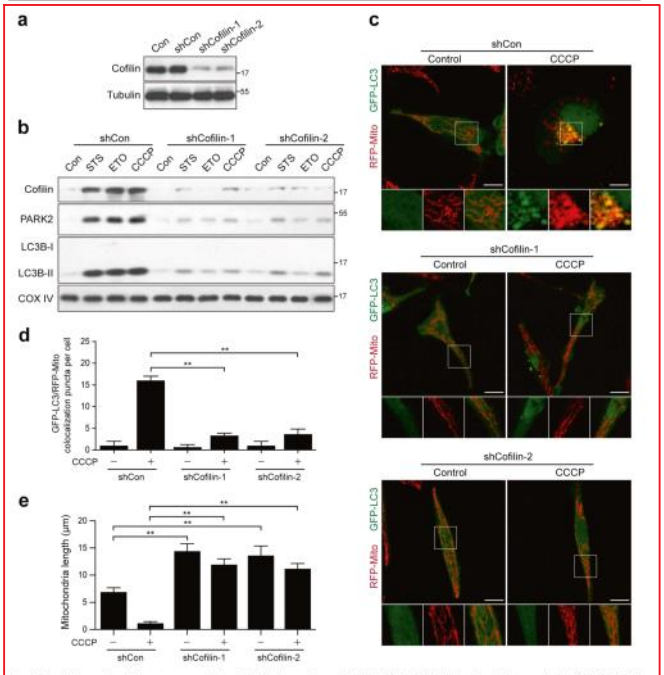

Fig. 4 Knockdown of cofilin attenuates mitochondrial fission and mitophagy induced by staurosporine/etoposide/CCCP. **a**, **b** MDA-MB-231 cells stably expressing Non-Target shRNA (shCon) or cofilin shRNA (shCofilin-1, shCofilin-2) were treated with or without STS (80 nM), ETO (200 μM) or CCCP (20 μM) for 9 h, whole cell lysates and mitochondrial fractions were prepared and detected by immunoblotting. **c** shCon, shCofilin-1 and shCofilin-2 cells were transfected with GFP-LC3 and RFP-mito plasmids, treated with CCCP (20 μM) for 9 h, images were captured by confocal microscopy. Scale bars: 10 μm. **d** The colocalization puncta of GFP-LC3 and RFP-Mito per cell was quantified from 90 cells in 3 independent experiments (mean ± SD, $^{**}P < 0.01$). **e** The mitochondrial length was quantified from 90 cells in three independent experiments (mean ± SD, $^{**}P < 0.01$)

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Figure 4

The researchers are using a silencing RNA (technically *short hairpin* - shRNA) to knockdown the expression of cofilin (the actin depolymerizing protein) and seeing what effects can be seen in a number of conditions. **[4A,B]** The researchers are using these silencing RNA to test if the silencing RNA actually works compared to control (no silencing occurring) in [4A]. In [4B], they are measuring the effect of silencing cofilin on other proteins of importance under the three drug conditions that induce fission/apoptosis (STS, ETO, CCCP) - PARK2, which is a preliminary signal for autophagy; LC3B-II, which is a later signal of autophagy; COX IV is a control that shouldn't change to compare the relative amounts of each protein amount to the unchanging COX IV amount. The darker the smudge, the more protein. **[4C-D]** The researchers are taking images to see if proteins LC3 (autophagy related, green) and mitochondria (red) co-localize (shown in orange if they do), with and without the addition of the drug CCCP with cofilin knocked down (by silencing RNA) - shCon is the control (no silencing of cofilin). The bar graph in [4D] is just the quantification of co-localization. **[4E]** They are showing the length/size of the mitochondria with the knockdown of cofilin; shCon is the control (no knockdown); sh-cofilin-1 is the first knockdown of cofilin; sh-cofilin-2 is the second knockdown of cofilin - with and without CCCP drug added.

Primary Results

- Cofilin knockdown works.
- Cofilin knockdown also knocks down LC3-II and PARK2.
- Cofilin knockdown stops colocalization of LC3 to mitochondria.
- CCCP does not rescue mitochondrial fission when cofilin is knocked down.

Take Away: Cofilin is a necessary protein for mitochondrial fission and autophagy to occur.

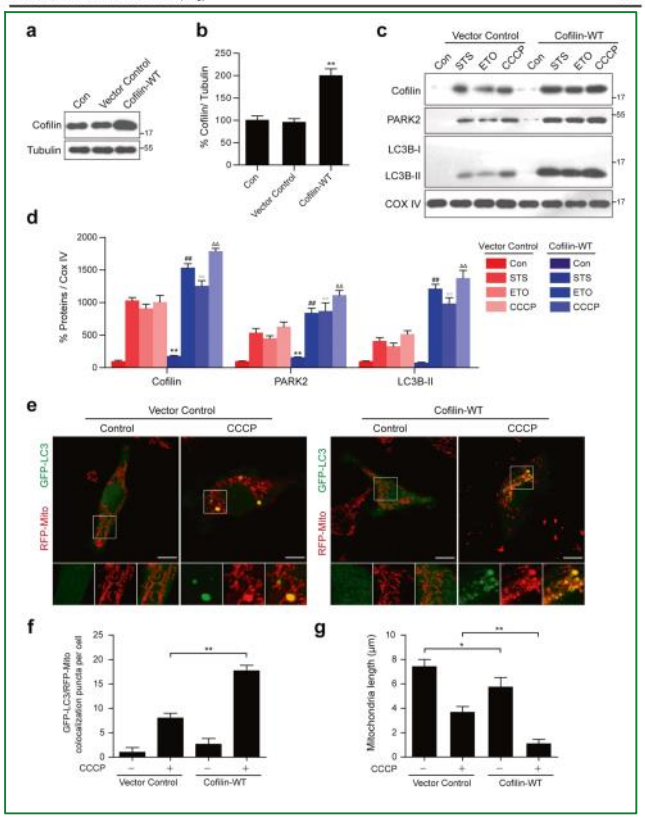

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Figure 5

In the last figure (figure 4), the researchers knocked down cofilin - now, they are overexpressing it (the cell generates more of it). **[5A,B]** The researchers are simply measuring their overexpression (Cofilin-WT) works by measuring the cofilin protein level compared to control (Con and Vector Control) - [5B] is the quantification of the smudge darkness and size (darker and larger is more protein). **[5C,D]** The researchers are comparing the levels of other relevant proteins, with and without overexpression of cofilin, under the three drugs (STS, ETO, CCCP) that induce mitochondrial fission and cell apoptosis - PARK2, which is a preliminary signal for autophagy; LC3B-II, which is a later signal of autophagy; COX IV is a control that shouldn't change to compare the relative amounts of each protein amount to the unchanging COX IV amount. The darker the smudge, the more protein. [5D] is the quantification of [5C]. **[5E-G]** The researchers are taking images to see if proteins LC3 (autophagy related, green) and mitochondria (red) co-localize (shown in orange if they do), with and without the addition of the drug CCCP with cofilin overexpressed (Cofilin-WT). The bar graph in [5F] is just the quantification of co-localization. **[5G]** They are showing the length/size of the mitochondria with the overexpression of cofilin- with and without CCCP drug added.

Primary Results

- Cofilin overexpression increases autophagy markers.
- Cofilin increases co-localization of mitochondria with autophagy markers (LC3-II)
- Cofilin overexpression decreases size of mitochondria, especially when fission is stimulated by CCCP.

Take Away: More cofilin present allows more mitochondrial fission and mitochondrial autophagy (mitophagy) to occur.

◀ **Fig. 5** Overexpression of cofilin increases mitochondrial fission and mitophagy induced by staurosporine/etoposide/CCCP. **a** The expression of cofilin in MDA-MB-231 cells stably expressing vector control or Cofilin-WT plasmids. **b** The relative intensities of cofilin were normalized to tubulin from 3 independent experiments ($^{**}P < 0.01$ compared to vector control group). **c** Cells were treated without or with STS (40 nM), ETO (100 μM) or CCCP (10 μM) for 9 h, mitochondrial fractions were prepared and detected by immunoblotting. **d** The relative intensities of proteins were normalized to COX IV, the data were presented as a percentage of each control group, which was set at 100%. $^{**}P < 0.01$ compared to vector control untreated group, $^{\#\#}P < 0.01$ compared to vector control treated with STS group, $^{\$}P < 0.01$ compared to vector control treated with ETO group, $^{\Delta\Delta}P < 0.01$ compared to vector control treated with CCCP group. **e** Vector control or Cofilin-WT cells were transfected with GFP-LC3 and RFP-mito plasmids, treated with CCCP (10 μM) for 9 h, images were captured by confocal microscopy. Scale bars: 10 μm. **f** The colocalization puncta of GFP-LC3 and RFP-Mito per cell was quantified from 90 cells in 3 independent experiments (mean ± SD, $^{**}P < 0.01$). **g** The mitochondrial length was quantified from 90 cells in three independent experiments (mean ± SD, $^{**}P < 0.01$)

that mitochondrial translocation of cofilin is involved in the mitochondrial fission induced by STS, ETO, and CCCP treatment.

Knockdown of cofilin attenuates, whereas overexpression of cofilin potentiates mitochondrial fission and mitophagy induced by STS, ETO, and CCCP

To further evaluate the functional role of cofilin in STS-, ETO- and CCCP-induced mitochondrial fission and mitophagy, two lentiviruses carrying shRNA (shCofilin-1 and shCofilin-2) were employed to stably knockdown cofilin expression in MDA-MB-231 and MCF-7 cells (Fig. 4a, Supplementary Fig. 4a). Depletion of cofilin observably reduced the mitochondrial translocation of cofilin induced by STS, ETO, and CCCP treatment in both MDA-MB-231 and MCF-7 cells (Fig. 4b, Supplementary Fig. 4b). Because mitochondrial fission is a prerequisite for mitophagy, we next examined whether knockdown of cofilin affects mitophagy. Western blot analysis showed that depletion of cofilin evidently reduced the mitochondrial levels of PARK2 and LC3B-II, induced by STS, ETO, and CCCP treatment in both MDA-MB-231 and MCF-7 cells (Fig. 4b, Supplementary Fig. 4b). In addition, using confocal microscopy, we found that depletion of cofilin significantly reduced the colocalization of mitochondria with LC3 in CCCP-treated cells (Fig. 4c, d). Knockdown of cofilin also reduced mitochondrial fission induced by CCCP treatment (Fig. 4c, e). Together, these findings suggest that knockdown of cofilin attenuates mitochondrial fission and mitophagy induced by STS, ETO, and CCCP.

To further definitively assess the functional significance of cofilin in regulation of mitochondrial fission and mitophagy, a plasmid construct encoding wild-type cofilin (Cofilin-WT) was employed. The marked increased in cofilin at the protein level was observed in both MDA-MB-231 and MCF-7 cells transfected with wild-type cofilin (Cofilin-WT) (Fig. 5a, b, Supplementary Fig. 5a). Overexpression of cofilin increased mitochondrial translocation of cofilin, PARK2 and LC3B-II induced by STS, ETO, and CCCP treatment in both MDA-MB-231 and MCF-7 cells (Fig. 5c, d, Supplementary Fig. 5b). Furthermore, immunofluorescence microscopy revealed that colocalization of mitochondria with LC3 was increased in cofilin-overexpressing cells treated with CCCP (Fig. 5e, f). Finally, overexpression of cofilin remarkably enhanced mitochondrial fission induced by CCCP treatment (Fig. 5e, g). Taken together, the findings suggest that cofilin is an important regulator in mediating mitochondrial fission and mitophagy.

Cofilin-mediated PINK1 accumulation mainly depends on cofilin regulation of protease processing via mitochondrial membrane potential activity

Because the accumulation of active PINK1 on the mitochondrial surface leads to recruitment of PARK2 to the mitochondria, culminating in initiation of mitophagy [21, 22], we next examined the role of cofilin in regulation of PINK1 activation. As shown in Fig. 6a, treatment of MDA-MB-231 cells with STS, ETO and CCCP resulted in a marked increase in the expression of full-length PINK1 (~64 kDa) and a decrease in the expression of cleaved PINK1 (~52 kDa). Overexpression of cofilin further enhanced the increase in the levels of full-length PINK1 and the decrease in the levels of cleaved PINK1 induced by STS, ETO, and CCCP treatment. In contrast, knockdown of cofilin led to decreased expression of full-length PINK1 and increased expression of cleaved PINK1. Similar results were noted in CCCP-treated MCF-7 cells (Supplementary Fig. 6a, b). Together, these findings indicate that cofilin may play a critical role in regulating PINK1 accumulation and function.

Since STS, ETO, and CCCP treatment induced mitochondrial translocation of cofilin and accumulation of PINK1 on the mitochondrial surface, we next examined whether cofilin directly colocalizes with PINK1 at mitochondria using immunofluorescence microscopy. We did not observe colocalization of cofilin and PINK1 in cells after STS, ETO, and CCCP treatment (Supplementary Fig. 7), suggesting that cofilin may indirectly regulate PINK1 accumulation on the mitochondrial surface through other mechanism.

Recent evidence indicates that PINK1 is rapidly degraded by several mitochondrial proteases [19, 20, 32], and

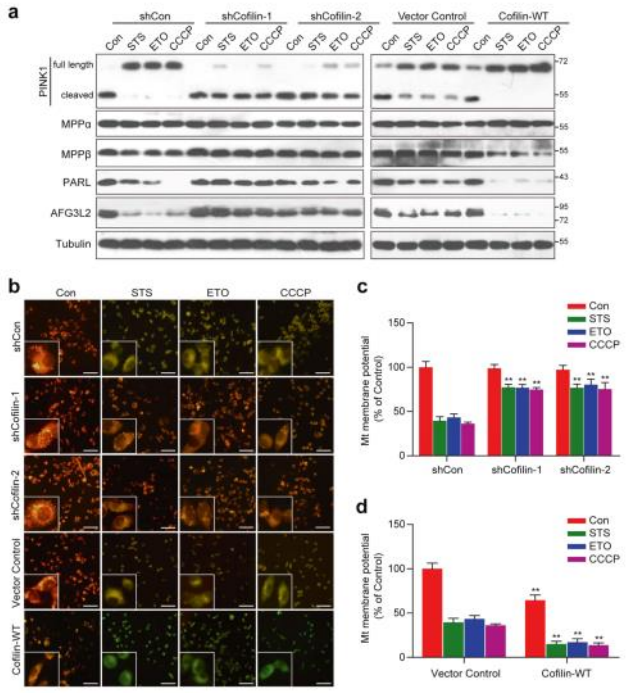
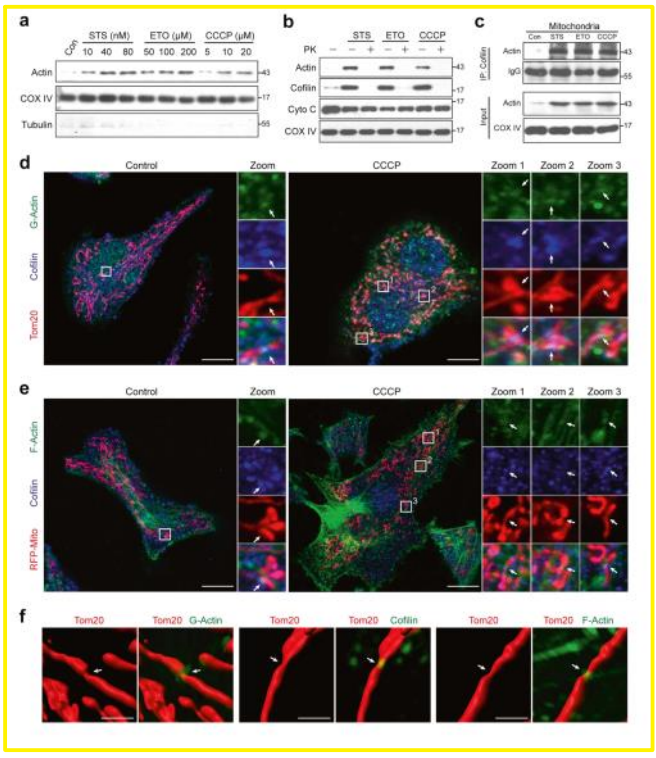


Fig. 6 Mitochondrial membrane potential contributed to cofilin-mediated PINK1-dependent mitophagy. **a** Stably expressing shcon or shCofilin-1 or shCofilin-2 MDA-MB-231 cells were treated with STS (80 nM), ETO (200 μM), or CCCP (20 μM) for 9 h, stably expressing vector control or Cofilin-WT MDA-MB-231 cells were treated with STS (40 nM), ETO (100 μM), or CCCP (10 μM) for 9 h, whole cell lysates were prepared and subjected to western blot analysis. **b–d** Cells were stained by JC-1 and the fluorescent signals were detected by confocal microscopy or microplate reader. Scale bars: 10 μm. Mitochondrial membrane potential was calculated as the fluorescence ratio of aggregates (red) to monomers (green) (mean ± SD, $^{**}P < 0.01$)

therefore, we next examined whether overexpression or knockdown of cofilin regulates the expression of mitochondrial proteases, including MPPα, MPPβ, PARL, and AFG3L2, in response to STS, ETO, and CCCP treatment. Treatment of MDA-MB-231 cells with STS, ETO, and CCCP decreased the levels of MPPβ, PARL and AFG3L2 but did not alter MPPα levels. Overexpression of cofilin evidently enhanced STS-, ETO- and CCCP-mediated decrease in the levels of MPPβ, PARL, and AFG3L2. In contrast, knockdown of cofilin markedly abrogated the



SPRINGER NATURE

Figure 7

[7D] The researchers are now looking at the interaction between cofilin (the actin depolymerizing protein from previous figures) and g-actin (globular, single protein actin; as in, not in a filament anymore), because cofilin binds actin, leading to its dissociation from actin filaments. G-actin is in green. Cofilin is in blue. Tom20 is in red (a marker of the outer mitochondrial membrane - mitochondria, in general). Where you see pink/purple there is overlap between the proteins. This experiment is with or without CCCP (mitochondrial fission drug). **[7E]** The experiment is the exact same as with [7D], but the Tom-20 is replaced by a general mitochondrial marker (RFP-Mito). **[7F]** The researchers are showing the outer membrane of mitochondria (TOM20 in red), as well as single proteins of actin (G-actin in green), as well as the depolymerizing protein (cofilin in green), and chains of actin (F-actin in green) to show the co-localization on the mitochondrion. *Note*: Figure 7A-C are confirmation of 7D and E with more drugs - same pattern seen.

Primary Results

- Cofilin and g-actin are co-localized with mitochondria.
- F-actin is also localized around mitochondria.
- Long mitochondria only have localization of actin (G or F) and cofilin around the middle of the mitochondrion.

Take Away: Cofilin likely interacts with g-actin and f-actin in particular manner that allows mitochondrial fission to occur - in what way exactly is unknown based on this data.

◀ **Fig. 7** Actin interaction and colocalization with cofilin at the mitochondrial fission site. **a** MDA-MB-231 cells were treated with STS (10, 40, 80 nM), ETO (50, 100, 200 μM) or CCCP (5, 10, 20 μM) for 9 h, the expression of actin in mitochondrial fractions were determined by immunoblotting. COX IV was used as mitochondrial fractions marker. **b** Cells were treated without or with STS (80 nM), ETO (200 μM) or CCCP (20 μM) for 9 h, mitochondria were purified and digested by proteinase K (10 ng/ml) on ice for 10 min, followed by centrifugation at 17,000 × *g* for 15 min, pellet was washed twice with Reagent C in Mitochondria Isolation Kit (Pierce, 89874), then lysed for western blot analysis. **c** Immunoprecipitation for cofilin and actin in mitochondrial fractions. **d** MDA-MB-231 cells were treated with CCCP (20 μM) for 9 h, G-actin was stained by Alexa Fluor 594 Conjugate Deoxyribonuclease I, after immunostaining with cofilin (blue) and Tom20 (mitochondrial marker, red), cells were observed by confocal microscopy. Scale bars: 10 μm. **e** RFP-mito-expressing MDA-MB-231 cells were treated with CCCP (20 μM) for 9 h, F-actin was stained by Alexa Fluor 488 Phalloidin, after immunostaining with cofilin (blue), images were captured by confocal microscopy. Scale bars: 10 μm. **f** Cells were treated with CCCP (10 μM) for 9 h, F-actin and G-actin were stained by probes; after immunostaining with cofilin and Tom20, cells were observed by super-resolution microscopy. Arrowheads indicate constriction and/or fission sites. Scale bars: 1 μm

STS-, ETO-, and CCCP-induced decreased expression of MPPβ, PARL, and AFG3L2 (Fig. 6a).

Processing and stability of mitochondrial proteases are regulated by the mitochondrial membrane potential, and loss of mitochondrial membrane potential induced by CCCP resulted in mitochondrial protease degradation [32]. We next examined whether overexpression or knockdown of cofilin regulates the change in mitochondrial membrane potential induced by STS, ETO, and CCCP. Cells were stained with JC-1 and analyzed with confocal microscopy or a microplate reader. Treatment of cells with STS, ETO, and CCCP significantly decreased mitochondrial membrane potential. Overexpression of cofilin markedly enhanced STS-, ETO-, and CCCP-induced decrease in mitochondrial membrane potential. In contrast, knockdown of cofilin abrogated STS-, ETO-, and CCCP-induced decrease in mitochondrial membrane potential (Fig. 6b-d). Taken together, the findings indicate that cofilin plays a critical role in regulating PINK1 accumulation through mitochondrial membrane potential-mediated degradation and instability of mitochondrial proteases.

Actin interacts and is colocalized with cofilin at the mitochondrial fission site

Recent evidence has indicated that the cytoskeleton component actin participates in regulation of mitochondrial fission [15]. We next examined whether STS, ETO, and CCCP induce actin recruitment in mitochondria. Treatment of breast cancer cells with STS, ETO, and CCCP resulted in a dose-dependent increase in the levels of actin in mitochondria (Fig. 7a, Supplementary Fig. 3 and 8). We used proteinase K to digest the outer mitochondrial membrane and examined the accurate mitochondrial location of cofilin and actin as described previously [26, 28]. Immunoblotting analysis showed that both mitochondrial actin and cofilin induced by STS, ETO, and CCCP were completely digested by proteinase K, but the internal mitochondrial marker Cyto C was not digested by proteinase K (Fig. 7b), suggesting that both actin and cofilin are localized at the outer mitochondrial membrane.

Cofilin regulates the dynamics of actin filament assembly/disassembly by binding actin with high affinity [25]. We next tested whether cofilin interacts with actin on the mitochondrial membrane. Immunoprecipitation assays indicated that cofilin was co-precipitated with actin in the mitochondrial fraction after treatment with STS, ETO, and CCCP (Fig. 7c). There are two forms of actin, namely, globular actin (G-actin) and filamentous actin (F-actin) [34]. We then labeled G-actin and F-actin with Alexa Fluor 594-conjugated deoxyribonuclease I and Alexa Fluor 488-conjugated phalloidin, respectively. Immunofluorescence microscopic studies revealed that colocalization of G-actin and cofilin at mitochondrial sites undergo constriction in control cells. Treatment of cells with CCCP resulted in increased colocalization of G-actin and cofilin at mitochondrial fission sites compared to control cells (Fig. 7d). Moreover, F-actin was also colocalized with cofilin at mitochondrial fission sites in control cells, and the increased colocalization of F-actin and cofilin was observed in CCCP-treated cells (Fig. 7e). To more clearly observe the mitochondrial localization of cofilin, F-actin and G-actin, super-resolution 3D microscopy was employed. As shown in Fig. 7f, cofilin, F-actin and G-actin are all localized at the mitochondrial fission site in cells treated with CCCP (constriction sites, arrowheads). Taken together, these results suggest that both F-actin and G-actin may participate in cofilin-regulated mitochondrial fission.

Actin dynamics is required for mitochondrial fission and mitophagy

On the basis of the above results, we hypothesized that cofilin-regulated mitochondrial fission mainly depends on cofilin regulation of dynamic actin activity. To confirm this hypothesis, the actin polymerization inhibitor latrunculin B (LatB) and the depolymerization inhibitor jasplakinolide (Jas) were employed. Pretreatment with LatB evidently enhanced STS-, ETO-, and CCCP-mediated mitochondrial translocation of actin, whereas pretreatment with Jas markedly abrogated mitochondrial translocation of actin induced by these fission inducers (Fig. 8a). These findings indicate that G-actin is the major actin form that is translocated to mitochondria.

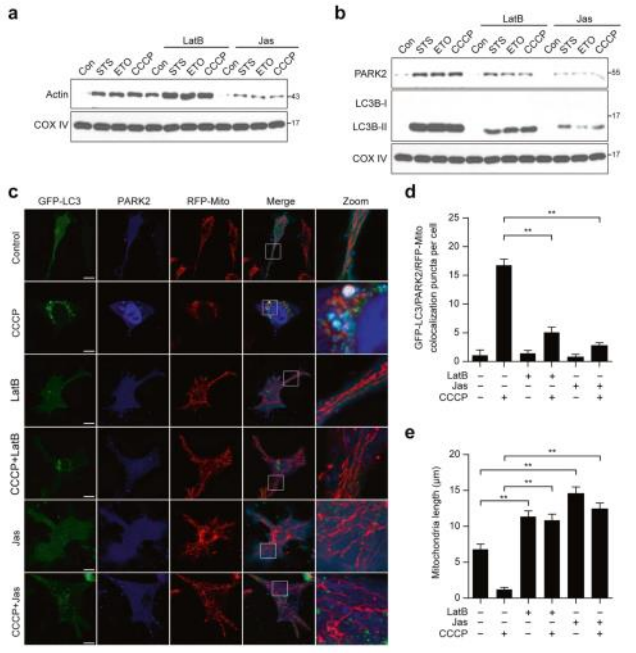


Fig. 8 Actin dynamics is required for mitochondrial fission and mitophagy. **a**, **b** MDA-MB-231 cells were pretreated with 500 nM latrunculin B (LatB, actin-depolymerizing toxin) or 100 nM jasplakinolide (Jas, actin-stabilizing toxin) for 1 h, followed by treating with STS (80 nM), ETO (200 μM) or CCCP (20 μM) for 9 h, mitochondrial fractions were prepared and detected by immunoblotting. **c** GFP-LC3 and RFP-mito-expressing MDA-MB-231 cells were pretreated with 500 nM LatB or 100 nM Jas for 1 h, followed by treating with 20 μM CCCP for 9 h, after immunostaining with PARK2 (blue), cells were observed by confocal microscopy. Scale bars: 10 μm. **d** The colocalization puncta of GFP-LC3, PARK2, and RFP-Mito per cell was quantified from 90 cells in three independent experiments (mean ± SD, $^{**}P < 0.01$). **e** The mitochondrial length was quantified from 90 cells in three independent experiments (mean ± SD, $^{**}P < 0.01$)

We also determined the effects of LatB and Jas on mitochondrial fission and mitophagy in cells treated with STS, ETO, and CCCP. As shown in Fig. 8b, treatment of cells with STS, ETO, and CCCP markedly increased the levels of PARK2 and LC3B-II in the mitochondria. Interestingly, pretreatment with either LatB or Jas evidently decreased the mitochondrial levels of PARK2 and LC3B-II induced by STS, ETO and CCCP treatment. In addition, the inhibitory effect of Jas was better than that of LatB. Furthermore, treating cells with CCCP increased the

Figure 8

[8A,B] The researchers are measuring the amount of protein in the cells with the addition of the apoptotic and mitochondrial fission drugs (STS, ETO, CCCP) with and without two additional treatments - LatB, which stabilizes actin (making it less likely to be degraded), and Jas, which increases the degradation of actin. In [8B], the researchers are again looking at proteins of interest that are related to autophagy (early stage - PARK2 and late stage - LC3B-II). The darker and larger the smudge, the more protein. **[8C,D]** The researchers are again imaging to see where various proteins of interest are localizing in the cell. They are also employing a few treatments to the cell; CCCP, mitochondrial fission inducer; LatB, stabilizes actin; Jas, destabilizes actin. The proteins being investigated are LC3 (late autophagy protein, in green), PARK2 (early autophagy protein in blue), and mitochondrial proteins (generally expresses red for the mitochondria). [8D] is the quantification of the co-localization in [8C]. **[8E]** The researchers are looking at the mitochondrial length/size witnessed in the microscopy images with the various previously described conditions on or off.

Primary Results

- Actin stability and instability both reduce the amount of autophagy related proteins, with instability creating the greatest reduction.
- Actin stability and instability reduce co-localization of autophagy proteins to mitochondria.
- Actin stability and instability increase mitochondrial size.

Take Away: The stability of actin, either too stable or too unstable, reduces the ability for mitochondria to undergo fission (likely) and reduces their capacity to undergo autophagy (mitophagy).

colocalization of PARK2 with LC3 and mitochondria, and pretreatment with either LatB or Jas significantly abrogated the colocalization of PARK2 with LC3 and mitochondria induced by CCCP treatment (Fig. 8c, d). Similarly, pretreatment with either LatB or Jas significantly abrogated CCCP-induced mitochondrial fission (Fig. 8c, e). These findings indicate that actin dynamics play a critical role in regulation of mitochondrial fission and mitophagy.

Discussion

Mitochondria are dynamic double membrane-bound organelles, and their morphology is maintained through a dynamic balance between fusion and fission processes [35]. Many biological functions of mitochondria, beyond energy production, actively contribute to tumorigenesis [6]. Cancer cells regulate mitochondrial dynamics to resist apoptosis and adjust their bioenergetic and biosynthetic needs to support proliferation and therapeutic resistance [36, 37]. A better understanding of processes that control mitochondrial dynamics and their significance in tumor cell development and maintenance would allow considerations of a means to alter mitochondrial dynamics in cancer, thereby providing new possible therapeutic strategies for cancer treatment.

Mitochondrial dynamics are also crucial for selective autophagy of mitochondria, known as mitophagy [18]. Mitophagy promotes mitochondrial turnover and prevents accumulation of dysfunctional mitochondria, which can lead to cellular degeneration [38]. Mitophagy can facilitate survival through adaptation to stress or cell death due to excessive removal of mitochondria [18, 38]. Regulating mitophagy pathways might affect the balance between tumorigenesis and cell death [39]. Therefore, targeting mitophagy, leading to selective elimination of tumor cell death, represents a promising strategy for cancer treatment.

A large body of evidence has revealed that mitochondrial dynamics (fusion and fission) and mitophagy are closely connected with cancer cell fate [36]. Moreover, an imbalance between mitochondrial dynamics and mitophagy alters cellular homeostasis, which may provide the material basis for tumor development. This suggests that there is a complex interplay between mitochondrial dynamics, mitophagy, and tumor development [36]. Cofilin is best known as an actin-depolymerizing factor, and it participates in regulation of mitochondrial dynamics and autophagy [28, 40]. Recent studies have revealed that cofilin is overexpressed in a variety of different cancers and cancer cell lines [41]. Overexpression of cofilin has been linked to the aggressiveness of a variety of cancers, and it is associated with poor outcome, suggesting that cofilin is a promising target that may be used as a biomarker for early diagnosis, monitoring, and therapeutic decision making for cancer treatment [42]. Therefore, an in-depth study of how cofilin regulates mitochondrial dynamics and mitophagy may provide a novel therapeutic strategy for cancer treatment.

In this study, we demonstrate that mitochondrial translocation of cofilin is required for initiation of mitochondrial fission and mitophagy. An increasing amount of evidence has revealed that mitochondrial recruitment of cofilin is an early step in apoptosis induction [26]. In our previous study, we found that cofilin is involved in mitochondrial fission and apoptosis by interacting with Drp1, knockdown of cofilin markedly reduced erucin-mediated mitochondrial fission, thereby inhibiting cell apoptosis [28]. Cofilin also participates in regulating autophagy initiation [40]. Presently, little information is available concerning the functional role of cofilin in regulating mitophagy. In this study, we found that STS, ETO, and CCCP increased mitochondrial translocation of cofilin and that knockdown of cofilin significantly reduced mitochondrial fission and subsequent mitophagy, as well as abrogated the loss of the mitochondrial membrane potential mediated by STS, ETO, and CCCP (Figs. 4 and 6, Supplementary Fig. 4). However, overexpression of cofilin potentiates mitochondrial fission and mitophagy induced by STS, ETO, and CCCP (Fig. 5, Supplementary Fig. 5). Loss of the mitochondrial membrane potential is an early marker of mitochondria-dependent apoptosis [43, 44]. Our study suggests that mitochondrial translocation of cofilin, mitochondrial fission, loss of mitochondrial membrane potential, mitophagy, and apoptosis occur successively.

Recent studies have shown that the PINK1/PARK2 pathway can promote the removal of damaged mitochondria via mitophagy [45, 46]. In this study, we show that cofilin regulates mitophagy through accumulation of PINK1. Knockdown of cofilin-attenuated PINK1 accumulation and the mitophagy induced by STS, ETO, and CCCP treatment (Figs. 4, Fig. 6a and Supplementary Fig. 6a). In contrast, overexpression of cofilin slightly increased PINK1 accumulation and mitophagy induction, which were enhanced by STS/ETO/CCCP treatment (Fig. 5, Fig. 6a and Supplementary Fig. 6b). There are two reasons why mitochondrial fission may be more potent than mitophagy in cofilin-overexpressing cells. First, mitochondrial fission and loss of mitochondrial membrane potential precede mitophagy, as evidenced by the fact that mitophagy can be detected after mitochondrial fission and requires a time interval. Second, mitochondria under fission in cofilin-overexpressing cells are still present as filamentous and are not easily engulfed by autophagosomes. Because mitochondrial translocation of cofilin and accumulation of PINK1 occurred on the mitochondrial surface, we speculate that cofilin may directly interact with PINK1 at mitochondria. However, our study showed that cofilin did not interact with PINK1 at mitochondria, and argues against this notion (Supplementary

Fig. 7). A more likely possibility is that cofilin indirectly regulates the accumulation of PINK1 through another mechanism. Recent evidence has indicated that mitochondrial inner membrane proteases (e.g., MPPα, MPPβ, PARL, and AFG3L2) are involved in PINK1 degradation through modulation of the mitochondrial membrane potential [19, 47, 48]. PINK1 cleavage is inhibited by loss of mitochondrial membrane potential, leading to accumulation of full-length PINK1 (64 kDa) on the outer mitochondrial membrane, recruitment of PARK2 to damaged mitochondria, and initiation of mitophagy [32, 33]. Our data indicate that cofilin indirectly regulates PINK1 accumulation through degradation of mitochondrial proteases mediated by loss of mitochondrial membrane potential, based on the following evidence. First, treatment with STS, ETO, and CCCP modestly decreased the levels of MPPβ, PARL and AFG3L2, and significantly decreased mitochondrial membrane potential (Fig. 6). Second, overexpression of cofilin

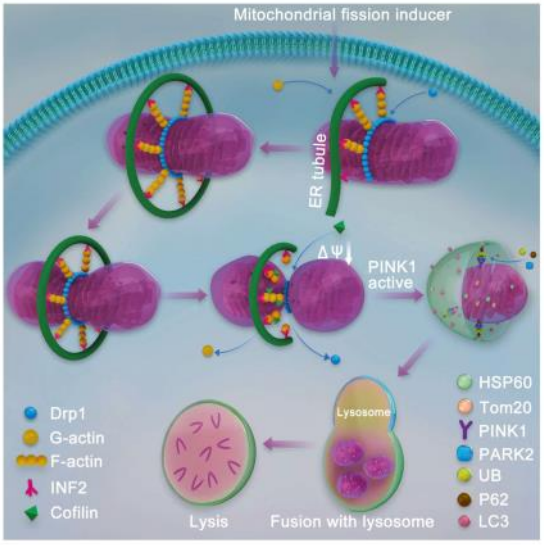


Fig. 9 A proposed model of cofilin-mediated mitochondrial fission and mitophagy.Drp1 translocates from the cytosol to the outer mitochondrial membrane is the initial step for mitochondrial fission (Cell 2016; 166: 555-566). In our study, we found that during the initiation of mitochondrial fission, G-actin also translocated to the outer mitochondrial membrane. Korobova F et al. reported that Endoplasmic reticulum (ER)-mitochondrion interactions activate INF2 to polymerize G-actin to F-actin, and F-actin pointed ends contact Drp1, which results in assembly of a helical ring at constriction sites as well as results in mitochondrial constriction and fission (Science 2013; 339: 464-467). Our reslut showed that post-fission, cofilin is translocated to mitochondria and interacts with F-actin, resulting in depolymerization of F-actin, leading to fission complex segregation. Meanwhile, mitochondrial fission results in mitochondrial membrane potential ($\Delta\Psi$m) dissipation and ultimately triggers PINK1/PARK2-dependent mitophagy

evidently enhanced the degradation of these mitochondrial proteases and the loss of mitochondrial membrane potential induced by STS, ETO, and CCCP treatment (Fig. 6). Third, knockdown of cofilin markedly abrogated the degradation of these mitochondrial proteases and the loss of mitochondrial membrane potential induced by treatment with these agents (Fig. 6). These observations suggest that cofilin might indirectly regulate the accumulation and stability of PINK1 through degradation and instability of mitochondrial proteases mediated by loss of mitochondrial membrane potential.

Accumulating evidence indicates that the actin cytoskeleton and its associated proteins participate in regulation of mitochondrial fission [12, 49]. Recently, it was discovered that INF2 (an ER-associated formin) participates in regulating mitochondrial fission by stimulating actin polymerization at mitochondrial fission sites [15]. The possible direct involvement of actin dynamics in mitochondrial fission has implications for other observed connections between the actin cytoskeleton and its associated proteins [11, 12, 50]. It has been shown that the dynamics of actin assembly and disassembly on mitochondria are involved in mitochondrial fission mediated by Drp1 [11]. This report also revealed that knockdown of cofilin led to mitochondrial translocation of Drp1, resulting in mitochondrial elongation [11]. However, the molecular mechanism by which cofilin-actin signaling might affect mitochondrial fragmentation and mitophagy remains unclear. In this study, we showed that cofilin can interact with actin on the outer mitochondrial membrane. We found that cofilin is colocalized with G-actin at mitochondria sites undergoing constriction (Fig. 7d), Similarly, F-actin was also found to colocalize with cofilin at mitochondrial fission sites (Fig. 7e). The super-resolution 3D microscopy images revealed that cofilin, F-actin and G-actin are all localized at the mitochondrial fission site (Fig. 7f). Further in-depth study showed that pretreatment with latrunculin B (LatB), an actin polymerization inhibitor, enhanced the mitochondrial translocation of actin induced by STS, ETO and CCCP treatment (Fig. 8a). In contrast, pretreatment with jasplakinolide (Jas), a depolymerization inhibitor, markedly abrogated mitochondrial translocation of actin induced by these agents (Fig. 8a). These findings suggest that G-actin is the major actin form that is translocated to mitochondria in response to STS, ETO and CCCP treatment.

It has recently been shown that cofilin regulates transient assembly of F-actin on the outer mitochondrial membrane, leading to mitochondrial fission [11]. It is possible that cofilin-mediated mitochondrial accumulation of F-actin contributes to mitochondrial fission. Another report showed that F-actin enhanced mitochondrial size, whereas an increase in G-actin resulted in mitochondrial fragmentation [16]. In this study, we showed that the dynamic transformation of G-actin and F-actin plays a crucial role in regulating mitochondrial fission and mitophagy, based on several lines of evidence. First, pretreatment with either LatB or Jas significantly abrogated CCCP-induced mitochondrial fission (Fig. 8c, e). Second, pretreatment with either LatB or Jas decreased the mitochondrial levels of PARK2 and LC3B-II induced by STS, ETO and CCCP treatment (Fig. 8b). Third, pretreatment with either LatB or Jas markedly abrogated the colocalization of PARK2 with LC3 and mitochondria induced by CCCP treatment (Fig. 8c, d). Due to cofilin regulation of the dynamics of actin filament assembly/disassembly by increasing the rate of actin-depolymerization, our study suggests that cofilin-regulated mitochondrial fission and mitophagy primarily depend on the actin-depolymerization activity of cofilin at the mitochondrial fission site.

After combining literature reports and our findings, we propose a model of cofilin-mediated mitochondrial fission and mitophagy. Step 1: Drp1 and G-actin are translocated from the cytosol to the outer mitochondrial membrane during initiation of mitochondrial fission. Step 2: Endoplasmic reticulum (ER)-mitochondrion interactions activate INF2 to polymerize G-actin to F-actin, and F-actin pointed ends contact Drp1, which results in assembly of a helical ring at constriction sites as well as mitochondrial constriction and fission. Step 3: Post-fission, cofilin is translocated to mitochondria and interacts with F-actin, resulting in depolymerization of F-actin, leading to fission complex segregation. Meanwhile, mitochondrial fission results in mitochondrial membrane potential ($\Delta\Psi m$) dissipation and ultimately triggers PINK1/PARK2-dependent mitophagy (Fig. 9). Our study reveals the molecular mechanisms by which cofilin regulates mitochondrial fission and mitophagy, which may provide an important target for cancer therapy.

Materials and methods

Reagents

Staurosporine (STS, S1421) was purchased from Selleckchem; Etoposide (ETO, E1383) and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, C2759) were purchased from Sigma; Latrunculin B (LatB, sc-203318) and Jasplakinolide (Jas, sc-202191) were obtained from Santa Cruz Biotechnology.

Cell culture

MDA-MB-231 cells (HTB-26, ATCC) were grown in Dulbecco's modified Eagle medium (DMEM, 11995065,

Gibco) containing penicillin/streptomycin (1%) (15070-063, Gibco) and fetal bovine serum (10%) (10100-147, Gibco). Cell line was verified to be mycoplasma negative monthly.

Plasmids and generation of stable cell lines

GFP-LC3 and RFP-mito plasmids were obtained from GeneChem Biotechnology, pcDNA3.1 Cofilin-WT (50853), GFP-UB (11928) and RFP-LC3 (21075) plasmids were purchased from Addgene, vector control plasmid (pcDNA3.1, V790-20) was purchased from Invitrogen. Cells were transfected with Cofilin-WT or vector control plasmids using Lipofectamine 3000 (Invitrogen, L3000015) for 48 h, cells were selected by 500 μg/ml G418 (Sigma, A1720) for one week to establish stable cofilin-overexpressing or vector control cell lines. The target sequence of cofilin shRNA were as follows: shCofilin-1: 5′-CCGGCTATGAGACCAAGGAGAGCAACTCGAGTTG CTCTCCTTGGTCTCATAGTTTTTG-3′; shCofilin-2: 5′-CCGGAAGGTGTTCAATGACATGAAACTCGAGTTTC ATGTCATTGAACACCTTTTTTTG-3′. In order to generate cofilin knockdown stable cell lines, a lentiviral system was employed as our previously described [28].

Mitochondrial membrane potential

Cells were seeded on coverslips or 96-well plates and cultured overnight, and then incubated with 5 μg/ml JC-1 (T-3168, Molecular Probes) in DMEM for 20 min in cell incubator. After washing, the green and red fluorescence were detected using a microplate reader (Safire II, Tecan) or Zeiss confocal microscope (LSM780NLO). The mitochondrial membrane potential was expressed as relative aggregates (red) to monomers (green) fluorescence intensity ratio.

Immunoprecipitation, western blot

Immunoprecipitation and western blot analysis were performed as our previously described [28, 51]. The primary antibodies and their dilutions were as follows: HSP60 (12165, 1:1000), COX IV (4850, 1:5000), Tubulin (2125, 1:5000) and Ubiquitin (3936, 1:1000) were purchased from Cell Signaling Technology; LC3B (L7543, 1:5000) was purchased from Sigma. Tom20 (sc-11415, 1:2000), VDAC (sc-8828, 1:1000), PARK2 (sc-32282, 1:500), AFG3L2 (sc-84687, 1:1000), PARL (sc-133884, 1:500), MPPα (sc-390471, 1:1000), MPPβ (sc-160672, 1:1000), Cyto C (sc-13156, 1:5000) and Actin (sc-1616, 1:1000) were from Santa Cruz Biotechnology; PINK1 (BC100-494, 1:2000) was from Novus; Cofilin (ab42824, 1:2000) was from Abcam. The relative intensity of bands was measured by Quantity One software (Bio-Rad).

Immunofluorescence

Cells were cultured on cover-slips to 70% confluency, and then transfected with plasmids for 48 h; after treatment with drugs, cells were prepared for immunostaining by incubation with primary antibodies, including LAMP1 (1:100), PARK2 (1:50), Tom20 (1:50), PINK1 (1:50), Cofilin (1:50) at 4 °C overnight. Cells were then incubated with secondary antibodies conjugated with Alexa Fluor 405 (A31553, 1:300), Alexa Fluor 488 (A11001, 1:300), Alexa Fluor 647 (A31573, 1:300) (Molecular Probes) for 1 h at 37 °C. F-actin, G-actin, and mitochondria were stained as our previously described [28, 51]. Images were captured using a Zeiss confocal microscope (LSM780NLO). All experiments were performed in triplicate. Mitochondrial length and GFP-LC3 puncta (diameter greater than four pixels were calculated) from randomly selected 30 cells in each experiment were measured using Image J software. The proteins colocalization was analyzed by the plots of fluorescence intensities scanned along a line using Zeiss LSM Image Examiner software.

Super-resolution microscopy

Cells were imaged by super-resolution microscopy (Deltavision OMX V4 Blaze, GE Healthcare). The serial Z-stacks images were carried out at 100 nm intervals and processed with softWoRx v.6.0 to reconstruct 3D images. Mitochondria was rendered by Imaris 7.4.2. software using the add surfaces function.

Statistical analysis

Results are expressed as mean ± SD. The comparisons were performed using One-way analysis of variance (ANOVA). $^{*}P < 0.05$ and $^{**}P < 0.01$ were regarded as significant difference.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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