Ultrastructure and dynamics of the actin–myosin II cytoskeleton during mitochondrial fission

Changsong Yang and Tatyana M. Svitkina

Mitochondrial fission involves the preconstriction of an organelle followed by scission by dynamin-related protein Drp1. Preconstriction is facilitated by actin and non-muscle myosin II through a mechanism that remains unclear, largely due to the unknown cytoskeletal ultrastructure at mitochondrial constrictions. Here, using platinum replica electron microscopy, we show that mitochondria in cells are embedded in an interstitial cytoskeletal network that contains abundant unbranched actin filaments. Both spontaneous and induced mitochondrial constrictions typically associate with a criss-cross array of long actin filaments that comprise part of this interstitial network. Non-muscle myosin II is found adjacent to mitochondria but is not specifically enriched at the constriction sites. During ionomycin-induced mitochondrial fission, F-actin clouds colocalize with mitochondrial constriction sites, whereas dynamic myosin II clouds are present in the vicinity of constrictions. We propose that myosin II promotes mitochondrial constriction by inducing stochastic deformations of the interstitial actin network, which applies pressure on the mitochondrial surface and thus initiates curvature-sensing mechanisms that complete mitochondrial constriction.
exposure of the cytoskeleton, fixation and critical point drying of the sample. Although critical point drying can lead to some shrinkage of the cell, the relative arrangement of the intracellular structures remains largely preserved, as evidenced by correlative light microscopy and PREM. However, detergent extraction would dissolve membrane organelles. Alternatively, the cell interior can be exposed by mechanical ‘unroofing’ of the cells, but most common unroofing protocols lead to a loss of most intracellular organelles, including mitochondria.

Here, we developed protocols to expose the cell interior, while preserving both membrane organelles and the associated cytoskeleton, and used PREM to reveal the organization of the actin–NMII cytoskeleton at mitochondrial constriction sites. Our observations are not consistent with either the polymerization-mediated pushing or contractile ring models. Instead, they suggest that NMII-dependent stochastic contractions of the interstitial cytoskeletal network induce deformations of mitochondria, which eventually lead to mitochondrial constriction and fission.

**Results**

**Visualization of membrane organelles and the cytoskeleton by PREM.** To reveal the cytoskeletal organization at mitochondrial constriction sites using PREM, we tested various approaches that remove the apical plasma membrane but preserve the organelles and the associated cytoskeleton. Our two most effective approaches were (1) extraction with a mild detergent, saponin, in cold conditions (Fig. 1a–d and Supplementary Fig. 1a–d) and (2) ripping off the apical membrane using a lightly attached nitrocellulose membrane (Fig. 1e–f and Supplementary Fig. 1e–f; see Methods). Both methods produced openings in the apical surface through which a nearly intact cell interior with membrane organelles could be visualized.

Mitochondria and the ER were recognized by their distinctive morphology in successfully exposed interiors of glial cells in mixed cultures of dissociated rat hippocampal cells (Fig. 1 and Supplementary Fig. 1). Mitochondria often had an elongated tubular shape with smooth outlines and were characterized by their relatively high electron density, probably due to dense contents. Although PREM primarily reveals surface topography, the biological material retained underneath the metal layer creates additional contrast, especially after fixation with uranyl acetate. The ER was more polymorphic and consisted of relatively translucent membrane structures, such as thin anastomosing tubules with irregular contours, as well as larger sacs and sheets. The shapes and dimensions of the ER components varied broadly, both within a cell and among different cells, so that in some cases the ER could not be definitively identified. The two complementary methods of opening up the cell—chemical extraction and mechanical rupture—produced similar results, suggesting that they faithfully report the overall organization of membrane organelles and the cytoskeleton in the cell interior. In subsequent experiments, we predominantly employed nitrocellulose-based unroofing, because it was faster and more efficient across different cell types.

To unambiguously identify the mitochondria and ER in unroofed PREM samples, we performed correlative light and EM (CLEM) on COS-7 cells transfected with the mitochondrial marker Mito—blue fluorescent protein (BFP), either alone (Fig. 2a–d) or in combination with the ER marker mCherry—Sec63β (Fig. 2e–h). Under our culture conditions, most mitochondria in COS-7 cells—either unroofed (Fig. 2) or living (Fig. 3a)—were short and only a few of them had a typical elongated shape. CLEM showed that morphologically identifiable mitochondria colocalized with the Mito–BFP-labelled structures visualized by confocal microscopy. Similarly, a network of thin electron-translucent tubules visible in the PREM images closely matched the fluorescently labelled ER network in confocal images of the same cells (Fig. 2). These results validate our morphology-based identification of mitochondria and the ER in PREM samples.

**Structural organization of the cytoskeleton and ER around mitochondria.** We observed different modes of ER—mitochondria interaction in glial cells (Fig. 1), which were more apparent in 3D views of PREM stereo pairs (Supplementary Fig. 1b–d,f). ER tubules could cross a mitochondrion at different angles to form underpass
or overpass intersections and could end at the mitochondrion or wrap around it. We defined constrictions as sites at which the width of a mitochondrion decreased by ≥20%. Most ER−mitochondrion interactions (95%, 355 sites in 92 cells from 6 independent experiments) occurred at unconstricted mitochondrial sites, while some ER tubules contacted a mitochondrion close to a constriction site (Fig. 1b,c, white arrows). On the other hand, ~75% (n = 111) of mitochondrial constrictions were intersected by ER tubules, consistent with reported light microscopy data11. The average length of mitochondrial constrictions, measured between half-maximal widths on each side of the constriction, was 701 ± 21 nm (mean ± s.e.m.; n = 113 constrictions from 10 cells), when estimated from fluorescence images, and 763 ± 24 nm (n = 132 constrictions from 37 cells), when measured using PREM images. The similarity of these values (P = 0.11, two-tailed nonparametric Mann–Whitney test) further supports the proper preservation of the mitochondrial shape in the course of PREM preparation.

Both the mitochondria and the ER were entangled with the cytoskeleton, which formed a loose 3D network in the cell interior. This network predominantly consisted of actin filaments, but also included microtubules and intermediate filaments that could be recognized by their distinct thicknesses. Some actin filaments that comprised this interstitial network seemed to emanate from the ER and could extend to mitochondria or another ER structure (Fig. 1d,f, green). Actin filaments appeared to originate at the mitochondrial surface less frequently (Fig. 1f, red). Most actin filaments in the interstitial network appeared unbranched (no detectable branches over the visible length), although occasional branched actin filaments could be detected (Fig. 1f, blue). Importantly, we did not encounter the stereotypical bush-like networks of short branched filaments, which are characteristic of sites of intense Arp2/3 complex-dependent nucleation23,32, at the mitochondrial surface. The average length of the actin filaments in the interstitial network, measured over distances that allowed us to clearly...
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visualize individual filaments between their overlaps with other structures, was 203 ± 91 nm (mean ± s.d.; n = 535 filaments in 12 cells from 2 independent experiments). This result is close to the observed ~270 nm length of actin filaments assembled in vitro in the presence of constitutively active INF2 and profilin33. We cannot exclude the possibility that very short filaments that were not incorporated into larger cytoskeletal systems could be lost as a result of opening up the cell interior. However, such filaments, if they exist, are poorly suited to force generation because they would be unable to gain traction without associating with more stable structures.

Mitochondrial constriction sites were not frequent in glial cells, consistent with relatively rare fission events at the steady state40. However, the density of actin filaments seemed to be greater at mitochondrial constrictions, defined as described above (20.8 ± 1.8 μm μm⁻²; mean ± s.e.m.; n = 12 constrictions with a total area of 0.88 μm² in 6 cells from 3 independent experiments), than at adjacent regions on both sides of the constriction (5.2 ± 0.9 μm μm⁻²; n = 24 regions with a total area of 1.8 μm²; P < 0.0001; paired Student’s t-test; Fig. 1b,c,f). Notably, these actin filaments exhibited neither end-on abutting nor circumferential orientation, which would be predicted by the pushing-based and contractile ring-based models, respectively. Instead, the actin filaments from the interstitial network intersected the mitochondrial constriction sites at a variety of angles, but mostly diagonally (Fig. 1b,c,f). Another conspicuous feature of mitochondria–cytoskeleton interactions was that some mitochondrial ends (9%, n = 355 ends) were tightly associated with an array of actin filaments that was denser than at the adjacent mitochondrial regions (Fig. 1b, white arrowhead).

Together, these results do not support the existence of mitochondrion-associated actin arrays that would be specifically dedicated to generating mitochondrial constriction. Instead, our observations raise the possibility that actin-dependent promotion of mitochondrial constriction could be mediated by actin filaments from the interstitial cytoskeletal network.

Organization of the cytoskeleton at mitochondrial constriction sites after the inhibition of Drp1-mediated scission. The low intrinsic frequency of mitochondrial fission is a challenge for our PREM analysis, because of a low probability that two rare events—mitochondrial fission and unroofing—would occur at the same location. This problem also means that we might miss constriction-specific actin arrays. Therefore, we used a dominant negative Drp1 mutant, Drp1-K38A34, to inhibit mitochondrial fission, as reported previously20,21,22, without affecting cytoskeleton-dependent preconstriction mechanisms; the frequency of mitochondrial constrictions should be increased in such cells.

We used a construct that simultaneously expresses short-hairpin RNA against endogenous Drp1 and short-hairpin RNA-insensitive green fluorescent protein (GFP)–Drp1-K38A. Expression of GFP–Drp1-K38A in COS-7 cells, which normally have short mitochondria, resulted in the formation of many elongated mitochondria (Fig. 3a,b), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission. PREM analysis of these cells revealed multiple constrictions along the length of elongated mitochondria (Fig. 3c–h), probably due to stalled fission.

Highly constricted sites tended to be associated with diagonal actin filaments, which criss-crossed the constriction site above and below the mitochondrion (Fig. 3g,h and Supplementary Fig. 4; Supplementary Video 1). We sometimes observed mitochondria that formed a prominent bulge near the constriction site (Fig. 3c and Supplementary Fig. 4), as if experiencing longitudinal pressure from the constriction area. More shallow mitochondrial constrictions had a less distinct criss-cross orientation of actin filaments at the constriction site (Fig. 3e,f). We again observed a fraction of mitochondrial ends associating with arrays of relatively dense actin filaments that contain both unbranched and branched actin filaments...
Constitutively active INF2-CAAX induces abundant actin filaments at the ER surface. Frequent instances of actin filaments emanating from the ER, including those extending towards mitochondria, support the idea that actin filaments involved in mitochondrial constriction are nucleated, at least in part, by ER-associated INF2-CAAX (ref. 13). A constitutively active form of INF2-CAAX, INF2-A149D-CAAX, induces actin assembly at the ER in U2OS cells67 and stimulates mitochondrial fission10. To determine the precise localization and ultrastructural organization of actin filaments induced by INF2-A149D-CAAX, we used COS-7 cells transfected with INF2-A149D-CAAX for confocal and PREM analyses (Supplementary Fig. 3).

In COS-7 cells coexpressing GFP−INF2-A149D-CAAX, mCherry−Sec61β and BFP-Mito and stained with Alexa 680−phalloidin to detect F-actin, GFP−INF2-A149D-CAAX colocalized with a subset of ER structures, usually with abnormally thick ER tubules. F-actin was enriched in these cells, specifically at the GFP−INF2-A149D-CAAX-positive ER components, but also extended from these regions into the interstitial space and could reach mitochondria (Supplementary Fig. 3a–c).

Using CLEM on COS-7 cells transfected with mCherry−INF2-A149D-CAAX, we found that the mCherry−INF2-A149D-CAAX-positive ER-like structures were densely covered with actin filaments (Supplementary Fig. 3d–h). As expected for formin-mediated nucleation, most of these filaments were long and unbranched. They typically ran along the ER tubule as a loose bundle (Supplementary Fig. 3b), although randomly oriented actin filaments extending from the ER into adjacent areas and towards mitochondria were also observed (Supplementary Figs. 3h and 4).

Expression of INF2-A149D-CAAX stimulates mitochondrial fission in U2OS cells14. As the mitochondria in COS-7 cells are already short, the mitochondrial constriction sites in mCherry−INF2-A149D-CAAX-expressing cells were undetectable by PREM. Therefore, we expressed mCherry−INF2-A149D-CAAX in COS-7 cells that had been transfected with GFP−K38A-Drp1 3 d earlier to inhibit mitochondrial fission (Fig. 4). Confocal microscopy of double-expressing cells (Fig. 4a) showed an ER-like distribution of mCherry−INF2-A149D-CAAX, as well as GFP−K38A-Drp1 aggregates16 and faint puncta.

As in cells with single INF2-A149D-CAAX transfection (Supplementary Fig. 4), CLEM of double-transfected cells showed numerous long actin filaments associated with mCherry−INF2-A149D-CAAX-positive ER structures. At a low magnification, mitochondria in these cells appeared short and round (Fig. 4c). However, high-magnification views showed that they were connected by thin bridges, some of which were marked by faint GFP−K38A-Drp1 puncta (Fig. 4d,e). The presence of long mitochondria with ‘bead-on-a-string’ morphology due to multiple constrictions allowed us to identify double-transfected cells even by non-correlative PREM (Fig. 4f,g). The mitochondrial constrictions in double-transfected cells were significantly deeper (Fig. 4), compared with cells expressing K38A-Drp1 alone (Fig. 3), suggesting that extensive actin polymerization induced by constitutively active INF2-CAAX enhances mitochondrial constriction. These narrow mitochondrial constrictions were crossed by unbranched actin filaments, some of which could be traced back to ER structures, whereas other filaments had no obvious connection with the ER. The population of actin filaments that extend from the ER towards a mitochondrial constriction represented only a small fraction of the ER-associated filaments (Fig. 4d–f). The mixed origin of constriction-associated actin filaments suggests that INF2-A149D-CAAX can enhance mitochondrial constriction by producing more interstitial actin filaments, which can then be used stochastically for mitochondrial fission.

Organization of the cytoskeleton at mitochondrial constriction sites after drug-induced mitochondrial fission. Several drugs have previously been used to stimulate mitochondrial fission, including the electron transport chain complex I inhibitor rotenone16,39 and the calcium ionophore ionomycin40,41. We analysed rotenone-treated glial cells by confocal microscopy and PREM.
Fluorescence microscopy of mitochondrial marker Tom20–mCherry revealed that mitochondrial constriction and fission were efficiently induced by rotenone treatment (Fig. 5a). PREM showed that the mitochondria in cells that were unroofed 8–20 min after rotenone application contained many constriction sites that were often associated with a criss-cross array of actin filaments extending from the surrounding interstitial network and sometimes attached to putative ER components located nearby (Fig. 5b–c). Actin filaments could also intersect a mitochondrion at other places, whereas mitochondrial ends were often associated with relatively dense actin filaments, some of which extended from adjacent ER segments (Fig. 5b,d). Overall, the cytoskeletal organization at the rotenone-induced mitochondrial constrictions was similar to that observed at the less-frequent constrictions in untreated cells, although mitochondria in rotenone-treated cells appeared to be more extensively intertwined with the cytoskeleton.

As PREM images suggested a more dense interstitial actin network after rotenone treatment relative to basal conditions (see Fig. 1), we monitored rotenone-induced changes in F-actin density in live HeLa cells expressing an F-actin reporter, GFP–F-tractin, and Mito–BFP. Imaging was performed at the cell midplane along the z axis to specifically observe F-actin in the cytosol away from abundant cortical F-actin. Rotenone treatment induced F-actin assembly in the cytoplasm and progressive mitochondrial fission (Fig. 5f–h, Supplementary Video 2). Moreover, the newly formed F-actin structures exhibited substantial overlap with mitochondria (Fig. 5g), raising the intriguing possibility that rotenone induces actin polymerization off mitochondria. The rotenone-induced F-actin signal probably corresponds to the interstitial actin network observed by PREM in the cell interior, which suggests that drug-induced actin accumulation plays a role in enhanced mitochondrial fission.

Dynamics of cytoplasmic F-actin and non-muscle myosin IIA (NMIIA) at mitochondrial fission sites. The diagonal criss-cross pattern of unbranched actin filaments that was often observed at mitochondrial constriction sites could form effective tracks for myosin motors. As NMIIA stimulates mitochondrial fission, we examined the localization and dynamics of NMIIA during ionomycin-induced mitochondrial fission.

For live-cell imaging, HeLa cells were triple-transfected with Mito–BFP, mCherry–NMIIA and GFP–F-tractin (Fig. 6). Ionomycin treatment has been shown to stimulate F-actin assembly in the cytosol due to INF2-CAAX activation at the ER. Accordingly, we observed significant accumulation of F-actin in the cytoplasm after ionomycin application (Fig. 6a,b) and partial colocalization of this cytoplasmic F-actin with the ER (Supplementary Fig. 5a, Supplementary Video 3). Ionomycin treatment also induced a significant increase in the fluorescence intensity of NMIIA in the cytoplasm, which slightly lagged behind the increase in F-actin (Fig. 6a,b). The mitochondrial lengths decreased in parallel with the accumulation of F-actin and NMIIA in the same cells (Fig. 6b), supporting the idea that increased density of the interstitial actin–NMII network correlated with mitochondrial fission.

Detailed analysis of the time-lapse sequences showed that initial mitochondrial constrictions were usually accompanied by the arrival of an F-actin cloud that traversed the mitochondrion at the constriction site. Although these clouds exhibited dynamic behaviour, they usually remained at the constriction site until fission occurred (Fig. 6c, Supplementary Fig. 5b and Supplementary Videos 4 and 5). These F-actin clouds probably corresponded to the criss-cross arrays of actin filaments at mitochondrial constriction sites that were revealed by PREM. Clouds of NMIIA also fluctuated around the mitochondria during their constriction and fission and could occasionally overlap with the constriction site. However, most of the time, the NMII clouds flanked the mitochondrion rather than overlapping with it (Fig. 6c, Supplementary Fig. 5b and Supplementary Videos 4 and 5).

We also analysed the dynamics of another NMII isoform, NMIIB, during mitochondrial fission in COS-7 cells, which have NMIIA as their major NMII isoform. In COS-7 cells expressing GFP–NMIIA, mCherry–F-tractin and Mito–BFP, ionomycin treatment induced F-actin assembly and mitochondrial fission (Supplementary Fig. 6 and Supplementary Video 6). The observed behaviour of NMIIB and F-actin clouds at mitochondrial fission sites in these cells was similar to those described for HeLa cells and NMIIA (Fig. 6 and Supplementary Fig. 5).

These data show that NMII does not exhibit specific enrichments at mitochondrial constrictions during ionomycin-induced mitochondrial fission, as would be expected for contractile ring-mediated force generation. Instead, the NMII signals fluctuate in the vicinity of the mitochondrion suggesting that NMII exerts force from the mitochondrial neighbourhood to stimulate constriction, but does not specifically accumulate at the constriction sites.

Functions and ultrastructural organization of NMII at mitochondrial constriction sites. Inhibition of NMIIA activity in U2OS cells was previously shown to result in the elongation of mitochondria, suggesting impaired fission. We evaluated the roles of NMII in mitochondrial constriction using NMII inhibitors (Fig. 7a–c).

Untreated or DMSO-treated COS-7 cells expressing GFP–K38A- Drp1 exhibited long mitochondria with multiple constrictions (Fig. 7a, left). Treatment with an inhibitor of NMII ATPase, blebbistatin (Fig. 7a, middle), or an inhibitor of myosin light chain kinase ML-7 (Fig. 7a, right) resulted in the dramatic relaxation of constrictions, as validated by the quantification of constriction depths (Fig. 7b). Live-cell imaging of COS-7 cells expressing GFP–K38A-Drp1 also demonstrated rapid relaxation of pre-existing constrictions after ML-7 treatment (Fig. 7c).

To determine NMII localization at the mitochondrial constriction sites at high resolution, we performed immunogold PREM with an NMIIA antibody of unroofed ionomycin-treated HeLa cells (Fig. 7d–f, and Supplementary Video 7). As expected, the NMIIA immunogold particles were associated with actin filaments throughout the cell. Although some gold particles could have been present at the exact site of mitochondrial constriction, we did not detect an enrichment of NMIIA at mitochondrial constrictions. In fact, the average density of gold particles at mitochondrial constrictions (103 ± 40 per µm²; mean ± s.d.; n = 20 regions with a total area of 1.2±2 µm² in 7 cells from 2 independent experiments) was slightly lower than the average density of gold particles associated with actin networks surrounding mitochondria (116 ± 42 per µm²; n = 20 regions with a total area of 3.9±6 µm² from 7 cells; P = 0.0083, Wilcoxon paired two-tailed test). When a constriction site was cross by actin filaments, NMIIA immunogold tended to concentrate on these intersecting actin filaments, usually on one or both sides of the mitochondrial constriction and only occasionally overlapping with the constriction (Fig. 7e). Similarly, immunogold staining of COS-7 cells with NMIIA antibody did not show an enrichment of NMIIA at mitochondrial constriction sites (Supplementary Fig. 6c), although the overall density of NMIIA staining was significantly lower. The specificity of NMIIA immunogold staining was validated previously. As additional controls, we stained COS-7 cells with only the secondary antibody, or with NMIIA antibody, which should not recognize any endogenous proteins in this cell type. These control samples contained 1.4 ± 0.2 (mean ± s.e.m.; n = 25 cells) and 1.7 ± 0.2 (n = 11 cells) particles per µm², respectively, which were not significantly different from each other (P = 0.73, Games–Howell’s posthoc multiple comparisons test), but dramatically lower than in HeLa cells stained with NMIIA antibody (100 ± 8 particles per µm²; n = 44 cells; P < 0.0001; Welch’s analysis of variance test), demonstrating a high specificity of staining. These data suggest that in both HeLa cells and COS-7 cells, NMIIA and NMIIB are not specifically enriched at mitochondrial constrictions.
Fig. 5 | Rotenone treatment induces mitochondrial fission and actin reorganization in cells. **a.** The time course of rotenone-induced mitochondrial constriction (yellow arrows) and fission (red arrows) in a glial cell expressing mitochondrial marker Tom20−mCherry and exposed to rotenone at time 0. Left: an overview of the cell after 2 min 21 s. Right: time-lapse sequence of four time points (mins after rotenone addition) for the boxed region. **b–d.** PREM images of constricted mitochondria (purple) in unroofed glial cells treated with rotenone for 8 min. Membranous structures that probably represent the ER are shaded yellow. Actin filaments from the relatively dense surrounding network intersect the constriction sites (boxed regions) or enwrap the mitochondrial ends (arrowheads). **c,e.** Enlarged views of the boxed regions in **b** and **d,** respectively. **f,g.** Confocal microscopy images of HeLa cells coexpressing GFP−F-tractin (green) and Mito−BFP (magenta) before (**f**) and 2 min 32 s after (**g**) rotenone treatment. Left, midplane confocal slices of the F-tractin fluorescence images show an increase in cytosolic F-actin after rotenone treatment. Right, enlarged views showing partial colocalization of mitochondria and F-actin. Mitochondrial fission after rotenone treatment is also evident. **h.** Relative mean fluorescence intensity of GFP−F-tractin (green, left y axis) and the average mitochondrial length (purple, right y axis) over time in rotenone-treated HeLa cells. Rotenone was added at time 0. Average fluorescence intensity of GFP−F-tractin from ten cells was normalized against the pretreatment values for individual cells. Mitochondrial lengths were measured for ~100 to ~400 mitochondria per time point from 10 cells. Error bars show the s.e.m. Scale bars: 5 μm (**a, left**), 1 μm (**a, right**), 200 nm (**b−e**), 5 μm (**f,g, left**) and 2 μm (**f,g, right**). The images are representative of n = 2 independent experiments, which gave similar results.
and COS-7 cells NMII contributes to mitochondrial constriction without the formation of a contractile ring, by acting as part of the surrounding interstitial network. NMII probably creates tension in the interstitial actin filaments that cross the mitochondrion.

**Discussion**

Insufficient structural information on actin organization at mitochondrial constriction sites precluded the formulation of an explicit model for actin-stimulated mitochondrial constriction. To overcome this bottleneck, we developed an optimized PREM protocol to visualize both the membrane organelles and the cytoskeleton in an apparently intact state. Although we cannot exclude the possibility of occasional ruptures, the similarity of the intracellular organization after chemical (saponin extraction) and mechanical (nitrocellulose sheet) exposure suggests that the extent of potential damage is relatively minor.

The two major models of actin-dependent mitochondrial constriction predict the specific organization of actin filaments at mitochondrial constrictions. However, our PREM data are not consistent with these predictions. We predominantly observe diagonally oriented unbranched actin filaments that intersect the mitochondrion near constriction sites. At relatively deep mitochondrial constrictions, the diagonal actin filaments appeared straight and taut, as if they were under tension, and were often associated with NMII. Branched actin filaments, although present, were not abundant at the mitochondrial surface, consistent with the reported lack of effect of Arp2/3 inhibition on mitochondrial length\(^{14}\). The reported positive roles of the Arp2/3 complex in mitochondrial fission\(^{16}\) can be potentially explained by the contribution of Arp2/3-dependent nucleation to the formation of the interstitial actin network.

Our data suggest the following mechanistic model for actin-mediated mitochondrial constriction (Fig. 7d). In the cytoplasm,
Fig. 7 | NMII associated with the interstitial actin network stimulates mitochondrial constrictions. 

**a–c**, Inhibition of NMII activity causes the relaxation of mitochondrial constrictions in K38A-Drp1-expressing COS-7 cells. **a**, COS-7 cells expressing GFP–K38A-Drp1 and Mito–BFP were treated for 1 h with DMSO, 50 μM blebbistatin or 50 μM ML-7. The images are representative of n = 2 independent experiments with similar results. **b**, The quantification of the constriction index (a degree of invagination) for individual constrictions in the conditions shown in **a**. Thick horizontal lines and error bars represent the mean and s.d., respectively. n = 491 constrictions from 16 DMSO-treated cells, 567 constrictions from 10 blebbistatin-treated cells and 448 constrictions from 10 ML-7-treated cells (n = 2 independent experiments with similar results). P values were determined by the Kruskal–Wallis test with a posthoc Dunn’s test; normality was determined by the Kolmogorov–Smirnov test. **c**, Time frames of COS-7 cells expressing GFP–K38A-Drp1, labelled with MitoTracker DeepRed in the course of treatment with 75 μM ML-7. Time (in min) is shown relative to ML-7 addition. Magenta arrowheads and arrows of different lengths mark individual constrictions that relax over time. The images are representative of n = 2 independent experiments with similar results.

**d–f**, Immunogold PREM images of NMIIA localization at mitochondrial constrictions in HeLa cells treated with ionomycin for 2 min. Immunogold particles (12 nm) are marked by yellow dots. Some NMIIA enrichment is seen on actin filaments intersecting the constriction (arrows in **e** and boxed region in **f**). The inset in **f** shows an enlarged image of the boxed region with uncoloured gold particles. Scale bars: 2 μm (**a, c**), 200 nm (**d,e,f**) and 50 nm (**f, inset**). The images are representative of n = 2 independent experiments with similar results. **g**, A model of actin–NMII-dependent mitochondrial constriction. The mitochondria and ER are embedded in the interstitial actin network, which includes many actin filaments nucleated at the ER by INF2-CAAX. NMII filaments in the interstitial network cause stochastic distortions of the network, thereby applying pressure to mitochondria trapped in the network. Indentations on the mitochondrial surface that result from mechanical activity of the actin–NMII interstitial network eventually develop into constrictions in a mitochondrial fission factor (Mff)-dependent manner and lead to Drp1-mediated fission. See text for details.
mitochondria are suspended in the interstitial cytoskeletal network, which is formed primarily of unbranched actin filaments, but also includes NMII filaments, microtubules and intermediate filaments. We suggest that the contractile activity of NMII causes stochastic inhomogeneous deformations of this interstitial network, such as stretching, bundling and twisting. A mitochondrion trapped in this mechanically active network can be locally squeezed between two actin filaments, which would cause local invaginations in the mitochondrial surface that probably act as the pivotal point for initiating mitochondrial constriction. This idea is supported by recent findings that mitochondrial fission can be induced by force application in the absence of the ER or a functional actin network. The underlying mechanism was proposed to include the accumulation of the curvature-sensing Mff at the initial invagination and subsequent propagation of the invagination into a constriction via curvature-inducing Mff properties. As Mff is a mitochondrial receptor for Drp1, the constriction- enriched Mff then recruits Drp1 to execute scission.

In the context of our model, NMII can pull on actin filaments that intersect the mitochondrion from a distance. This process can be loosely compared to how pulling on corset strings can cause the constriction of a waistline. NMII filaments are probably more efficient if they localize in the mitochondrial neighbourhood. Otherwise, the force would be absorbed by network deformations in the intervening space. Accordingly, we often see fluctuating NMII clouds in the vicinity of constricting mitochondria. A possible correlation between the accumulation of cytoplasmic F-actin induced by ionomycin10,11 or rotenone (this study) and the drug-induced frequency of mitochondrial fission10,11,12 can be explained by the greater probability of a mitochondrion being deformed by a denser interstitial actin–NMII network. The origin of interstitial actin filaments may not be critically important. For example, ionomycin induces actin polymerization through ER-associated INF2-CAAX13, whereas rotenone-induced F-actin assembly may potentially involve the regulation of mitochondrion-anchored Spire1C12.

The stochastic nature of our model does not exclude some degree of specificity for the location of constriction sites. For example, cross-talk between INF2-CAAX and Spire1C at ER–mitochondria juxtapositions10,11,12 can induce local network densities and increase the chance of constriction. The internal organization of the mitochondrion can also be a contributing factor. Thus, mitochondrial fission often occurs near a nucleoid14, probably because nucleoid-containing segments of mitochondria are denser—as can be inferred from nucleoid recovery from the bottom fractions of density gradients15,16—and therefore probably stiffer. If so, then tense actin filaments could ‘slip’ to the adjacent softer regions, where they could induce deformation. Similarly, in cases when the inner mitochondrial membrane is constricted before the outer membrane16,49, the separation of two mitochondrial membranes may locally soften the mitochondrion and make this site more susceptible to deformation.

In conclusion, using a newly developed PREM protocol, we revealed an interior structure of the cell that incorporates both membrane organelles and the cytoskeleton. Our data suggest a mechanism of actin-dependent mitochondrial constriction, in which stochastic deformations of the mechanically active interstitial actin network generate the force required for this process.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at [https://doi.org/10.1038/s41556-019-0313-6](https://doi.org/10.1038/s41556-019-0313-6).

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**Author contributions**

C.Y. performed all the experimental work and data analyses. C.Y. and T.S. prepared the figures and wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Correspondence and requests for materials should be addressed to T.M.S.

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Methods

Cell culture. Primary dissociated cell cultures from embryonic rat hippocampus, isolated as described previously, were obtained from the MINS Neurocon 8875 (Sigma) was used at 250 ng ml⁻¹ on glass coverslips at a concentration of 150,000 cells per 35 mm dish in 1.5 ml of neurobasal medium (Gibco) with 2% B27 supplement and cultured for 7–14 d. Only giant glial cells were used for the experiments. COS-7 and HeLa cells were cultured in DMEM medium supplemented with GlutaMAX (10-569-010, Gibco), 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin at 37°C and 5% CO₂. For the drug treatment, rotenone (R8875, Sigma) was used at 250 ng ml⁻¹ for glial cells and 750 ng ml⁻¹ for HeLa cells. Ionomycin (I0634, Sigma) was used at 4 μM.

Exposure of the cell to cisplatin. For saponin-mediated extraction, cells on glass coverslips were transferred into a solution (prechilled to 4°C) of 0.1% saponin (47036, Sigma) in PEM buffer (100 mM PIPES−KOH, pH 6.9, 1 mM MgCl₂, and 1 mM EGTA) that contained 2% unlabeled phalloidin (P2141, Sigma), protease inhibitor cocktail (37786, Sigma) and 300 mM sucrose. After incubation on ice for 7 min, the cells were fixed with 2% glutaraldehyde (01909, Polysciences) in 0.1 M Na-cacodylate buffer (pH 7.3) at room temperature for 20 min.

For mechanical unroofing, COS-7 and HeLa cells were seeded on coverslips coated with poly-o-lysine (0.2 mg ml⁻¹) or HistoGrip (Thermo Fisher Scientific, 080050) and cultured for 1–3 d. Neuronal cells were cultured as described above. A piece of nitrocellulose filter membrane (0.8 µm pore size; AABPC5000, EMD Millipore) was soaked in PEM buffer containing 2% phallolidin and 10 µM taxol. The excess buffer was removed by touching a piece of filter paper to the coverslip edges, and the coverslip was placed cell-side down on the nitrocellulose membrane for about 10 s. Gentle pressure was then applied to one corner of the coverslip to generate a gradient of nitrocellulose−cell adhesion and thus produce properly unroofed cells somewhere along this gradient. The coverslip was then lifted from the nitrocellulose membrane and immediately placed in 0.2% glutaraldehyde solution in 0.1 M Na-cacodylate buffer for fixation. After evaluation of the unroofing quality by phase contrast microscopy, the samples were postfixed with 2% glutaraldehyde.

As a control for the potential damage caused by our unroofing procedure, we confirmed that the incubation of living cells in cytoskeletal buffer for more than 5 min—much longer than the ~30 s required for nitrocellulose application and detachment—does not cause any apparent aberrations in the organization and dynamics of mitochondria and the actin cytoskeleton (Supplementary Fig. 2a). We also found that the morphology of the fluorescently labelled ER and mitochondria was similar in unroofed and non-unroofed cells from the same CLEM preparations and comparable to control cells not subjected to the unroofing procedure (Supplementary Fig. 2b).

PREM and CLEM. Sample processing for regular and immunogold PREM and for CLEM was performed as described previously. In brief, glutaraldehyde-fixed cells were postfixed by sequential treatment with 0.1% tannic acid and 0.2% glutaraldehyde. After quenching with 2 mg ml⁻¹ NaBH₄ in PBS and permeabilization with 0.2% glutaraldehyde, quenched 3 times with NaBH₄ in PBS and permeabilized with 0.1% saponin in PBS for 5 min. Stained samples were mounted with ProLong Diamond Antifade Mountant (P36961, Thermo Fisher Scientific) for imaging.

PREM is closely related to confocal microscopy and comparable to control cells not subjected to the unroofing procedure. The specificity of NMII antibodies was previously validated using INF2-A149D-CAAX construct was prepared by releasing INF2-A149D-CAAX from the GFP−INF2-A149D-CAAX construct (a gift from M. Higgs) in the mCherry−C1 vector (Chrom Chem). For transient transfection, mCherry−INF2-A149D-CAAX construct was prepared by releasing INF2-A149D-CAAX from the GFP−INF2-A149D-CAAX construct (a gift from M. Higgs) in the mCherry−C1 vector (Chrom Chem). For double transfection with GFP−K38A-Drp1 and mCherry−INF2-A149D-CAAX, cells were first transfected with GFP−K38A-Drp1 and cells were then re-plated onto coverslips and, 6 h after plating, transfected with mCherry−INF2-A149D-CAAX for 16 h before unroofing.

Fluorescence microscopy. For the staining of F-actin with Alexa Fluor 680 phalloidin (A22286, Thermo Fisher Scientific, 1:150 in PBS), cells were fixed with 0.2% glutaraldehyde, quenched 3 times with NaBH₄ in PBS and permeabilized with 0.1% saponin in PBS for 5 min. Stained samples were mounted with ProLong Diamond Antifade Mountant (P36961, Thermo Fisher Scientific). For the live-cell imaging, cells were cultured in glass-bottomed MatTek dishes in phenol red-free Leibovitz’s L-15 medium (21083027, Gibco) supplemented with 1% FBS and maintained during observation at 37°C in a humidified atmosphere using a UNO stage-top incubator (Okolab). For the treatment with ionomycin or rotenone during live-cell imaging, the drugs were first diluted in 200 μl of prewarmed medium to 10-fold the final concentration and added to the dish with cells, which contained 1.8 ml of medium.

Epifluorescence microscopy was performed using an Eclipse TE2000-U inverted microscope (Nikon) equipped with a PLANAPO ×100 1.3 NA objective and Cascade 512B CCD camera (Photometrics) driven by Metamorph imaging software (Molecular Devices). Spinning disc confocal microscopy was performed using an Eclipse Ti inverted microscope (Nikon) equipped with a CSU-X1 spinning disc (Yokogawa Electric Corporation), a CF60P Affochromat TIRF ×100 1.49 NA oil objective, a QuantEM 512SC digital camera (Photometrics) and a perfect focus system driven by NIS-Elements Advanced Research software (Nikon; version 4.5). The 405 nm laser and ET455/50 M filter were used for GFP; the 488 nm laser and 525/36 filter for RFP; the 561 nm laser and 605/70 filter for RFP; and the 640 nm laser and 700/75 filter for Alexa Fluor 680. Time-lapse sequences were acquired at rates that ranged from 10 s to 2 min per frame. To minimize photobleaching, a low laser power, short exposure times and a digital gain were used. Three-dimensional reconstructions of confocal stacks were generated from 3–5 consecutive z slices with a thickness of 0.2–0.3 μm using the alpha blending option in the NIS-Elements software.

To determine the effect of the inhibition of NMII activity on mitochondrial constriction, COS-7 cells expressing GFP−K38A-Drp1 and Mito−BFP were treated with DMSO (D2650, Sigma) as a control, 50 μM blebbistatin (B592500, Toronto Research Chemicals) or 50 μM ML-7 (I715000, Toronto Research Chemicals) for 16 h. Both treated and untreated cells were fixed in PEM buffer and imaged using epifluorescence microscopy.

The extent of mitochondrial constriction was determined using Mito−BFP fluorescence intensity profiles generated using the Plot Profile tool in ImageJ software after subtracting the background. Profiles of average fluorescence intensity were generated using lines drawn along the mitochondrial long axis and spanning the mitochondrial membrane. The mitochondrial constriction index (MCI) was calculated using the following equation:

\[
MCI = \frac{1}{2} \left( \frac{I_{max} - I_{min}}{I_{max} + I_{min}} \right)
\]

where \(I_{min}\) is the local minimum of fluorescence intensity and \(I_{max}\) and \(I_{local}\) are local fluorescence intensity maxima within 0.4–2 μm on each side of the local minimum. The MCI is close to 100% for minimally constricted regions and decreases progressively with the extent of constriction.

Data analyses. For the quantification of the localization of mitochondria−ER overlaps relative to mitochondrial constrictions in PREM images, a distance...
of ≤100 nm between the narrowest part of the mitochondrion and the closest edge of the ER was considered to represent a colocalization of the ER with the mitochondrial constriction. To measure the lengths of actin filaments in the interstitial cytoskeletal network, individual filaments that had a diameter of ~10 nm (including the platinum coating)—which correspond to actin filaments—were traced with a line tool in ImageJ for as long as they could be distinguished from the neighbouring or overlapping filaments. Very short filaments (<50 nm) were ignored for this quantification. For the evaluation of actin filament densities at mitochondrial constriction sites, an area of mitochondrial constriction was defined as extending from the narrowest region of the mitochondrion to the half-maximal width of the adjacent unconstructed regions on both sides of the constriction, whereas mitochondrial regions beyond this boundary were selected for the determination of actin filament densities at adjacent unconstructed mitochondrial regions. The total length of all actin filaments within the selected regions was measured using the ImageJ line tool and normalized to the area of the region of interest. For the quantification of NMII immunogold densities at mitochondrial constriction sites, an area of mitochondrial constriction was defined in the same way. The regions of the surrounding interstitial network were selected near to the constricted mitochondrial, but not overlapping with it and excluding other membrane structures in the region. The number of immunogold particles was counted and normalized to the area of the region of interest.

The time course of F-actin or NMIIA accumulation following drug treatment was assessed on the basis of the cumulative fluorescence intensities of respective markers (F-tractin or NMIIA) in confocal slices with a total width of 0.9–1 μm, taken from the midplane of the cells. Three regions of interest located away from the nucleus and the cortical region were selected for each cell. Average fluorescence intensities of these regions were determined after the background was subtracted for each time point, and then normalized against an average fluorescence intensity before drug treatment for each individual cell. The changes in the mitochondrial lengths after ionomycin treatment were monitored for individual cells. Only easily distinguishable mitochondria longer than 0.5 μm were measured.

Statistics and reproducibility. Considering the large variations in the structural organization of mitochondria and the cytoskeleton among cells, we used very large sample sizes despite substantial technical challenges. At least two independent experiments were performed to confirm the consistency of the results. All the experiments gave consistent results when repeated. Numerical datasets were first evaluated for normality by the Kolmogorov–Smirnov test. For the normally distributed values, a two-tailed t-test was used to compare two datasets and Welch’s analysis of variance test with a posthoc Games–Howell test was used for multiple comparisons. For not-normally distributed values, the Mann–Whitney test was used to compare two unpaired datasets, the Wilcoxon test was used to compare two paired datasets and the Kruskal–Wallis test with a posthoc Dunn test was used for multiple comparisons.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Source data for Figs. 3b, 6b and 7b and the statistical results presented in the text have been provided as Supplementary Table 1. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Software and code

Policy information about availability of computer code

**Data collection**

For PREM samples, images were acquired by an ORIUS 832.10W CCD camera (Gatan, Warrendale, PA) driven by Gatan Digital Micrograph software (Version 1.8.4). The original files in dm3 format were converted into tiff files using the same software. Light microscopy images were acquired using either Cascade 512B CCD camera (Photometrics) driven by Metamorph imaging software (Molecular Devices) (Version 6.2r6) or using QuantEM 512SC digital camera (Photometrics) driven by Nikon (NIS)-Elements Advanced Research software (Version 4.50). Subsequent image processing and figure preparation was done by Adobe Photoshop software (Version CS5.1 or version CS4).

**Data analysis**

Nikon (NIS)-Elements Advanced Research software (Version 4.50) and ImageJ software (Version 1.51v, NIH) were used for quantification of fluorescence images. 3D reconstructions of confocal stacks were generated using the alpha blending option in Nikon (NIS)-Elements Advanced Research software (Version 4.50). Morphometry of electron microscopy data was done using ImageJ software (Version 1.51v, NIH) with common tools, such as length and area measurements and intensity profiles. Statistical analyses were done using GraphPad Prism (Version 8.0.1).

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**Sample size**
No statistical method was used to predetermine sample size because no human or animal subjects were used in this study. Sample size was chosen based on previous experience and standards in the field. Considering large variations in structural organization of mitochondria and the cytoskeleton among cells, we used large sample sizes that were often well above what is standard in the field. We have been able to do so despite significant technical challenges. Sample sizes are indicated in the main text and figure legends, as well as in the supplementary table 1 (“Statistics Source Data”). Extremely high significance values indicate that these sample sizes were more than sufficient.

**Data exclusions**
No data were excluded from analyses in light microscopy experiments or in technically successful PREM experiments involving unroofing. However, if the unroofing procedure resulted in unsatisfactory exposure of the cell interior, thus precluding visualization structures of interest, such experiments were discarded. These exclusion criteria were pre-established.

**Replication**
Repetitive experiments (at least two independent experiments) have been done to confirm consistency of results. All repetitive experiments gave consistent results without exceptions. Also, different approaches have been used to induce mitochondrial constrictions to confirm the mechanism. Hundreds of individual examples from many cells and experiments have been examined by PREM to account for individual biological variations. Only one experiment was performed in this study as a negative control to validate specificity of immunogold staining, but many such controls were done for our previous projects.

**Randomization**
No randomization was applied as there were no human or animal subjects used in this study. The samples were organized according to cell types and treatment conditions.

**Blinding**
Not applicable as human or animal subjects were not used in this study.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| [x] | Unique biological materials |
| [ ] | Antibodies |
| [ ] | Eukaryotic cell lines |
| [ ] | Palaeontology |
| [ ] | Animals and other organisms |
| [ ] | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| [x] | ChIP-seq |
| [ ] | Flow cytometry |
| [x] | MRI-based neuroimaging |

Antibodies

| Antibodies used |
|-----------------|
| NMIIA antibody was from Biomedical Technologies (IBT-567) and NMIIIB was from Cell Signaling (#3404). They were diluted 1:20 in the PEM buffer. |
Validation

Rabbit anti-NMIIA antibody (#BT-567) has been previously validated by the Biomedical Technologies (more information is available at https://fnkprddata.blob.core.windows.net/domestic/data/datasheet/BTI/BT-567.pdf). It was also validated by RNAi-mediated knockdown assay (Rai et al., 2017 JBC, 292(8): 3099-3111). In addition, we further validated its specificity by staining NMIIA-negative Cos-7 cells in this study.

Rabbit anti-NMIIB antibody (#3404) has been validated by the Cell Signaling (more information is available at https://media.cellsignal.com/pdf/3404.pdf). We have previously validated this antibody by RNAi-mediated knockdown and Western blotting (Shutova et al., 2017, JCB, 216(9) 2877-2889).

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**Eukaryotic cell lines**

| Policy information about cell lines |  |
|------------------------------------|---|
| **Cell line source(s)**            | COS-7 cells were from ATCC. Hela cells were from Dr. Giorgio Scita (Istituto FIRC di Oncologia Molecolare), Italy (Disanza et al., 2006, Nat Cell Bio 8:1337-1347). |
| **Authentication**                 | Cell lines were not authenticated by ourselves. |
| **Mycoplasma contamination**       | Cell lines were regularly tested for mycoplasma contamination by DAPI staining and were found negative. |
| **Commonly misidentified lines**   | No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample. (See ICLAC register) |