

Homeostatic mitochondrial lipid peroxidation governs mitochondrial fission site selection and daughter mitochondrial fate

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Summary

Mitochondrial fission is essential for cellular homeostasis, yet signals that license specific sites for division and link fission to subsequent fate remain largely elusive. Here, we identify homeostatic inner mitochondrial membrane lipid peroxidation (IMM LPO) as an upstream signal coordinating these two fundamental processes. We show, during homeostatic conditions, a focal IMM LPO increase is necessary for division, marking mitochondria-ER contacts (MERCs) for fission. IMM LPO and MERCs formation can precede one another, engaging in a positive feedback loop: focal IMM LPO promotes ER recruitment and subsequent mitochondrial Ca^{2+} influx, which in turn accelerates IMM LPO, creating a robust commitment for division. Critically, the magnitude of the IMM LPO signal at the prospective fission site dictates daughter mitochondrial fate. Modest LPO generates healthy daughter mitochondria, while severe LPO targets depolarized daughters for mitophagy. This IMM LPO-mediated fission is a general mechanism, exhibiting hyperactivation in prostate cancer. Our study defines a fundamental initiation event for mitochondrial fission, establishing homeostatic IMM LPO as a signal that integrates fission site selection with mitochondrial fate.

Keywords: Mitochondrial fission; Fission site selection; Lipid peroxidation; Mitochondria-ER contacts; Mitochondrial fate; Mitophagy

Main

Mitochondrial fission is a fundamental process essential for mitochondrial homeostasis, quality control, and cellular adaptation^{1, 2}. While the downstream scission machinery, involving the formation of mitochondria-endoplasmic reticulum contact sites (MERCs)^{3, 4} and subsequent recruitment of the fission effector DRP1^{5, 6}, is well-characterized, the upstream signals that determine where fission occurs remain largely elusive. This knowledge gap is underscored by the notable observation that, although fission occurs predominantly at MERCs, only a minute fraction (~1-4%) of these sites proceed to active scission⁷. This discrepancy raises a critical question: what early molecular signal is required for licensing a specific mitochondrial site for division? Although mtDNA replication has been implicated as a potential signal⁷, it likely represents a concurrent event rather than a determinant⁸. Therefore, identifying the earlier, instructive signal that defines a nascent fission site remains a central question in the field⁸.

Mitochondrial fission is essential for both mitochondrial biogenesis and mitophagy⁹⁻¹¹; however, it remains a critical puzzle how the act of fission can be coupled to these distinct and opposing fates of the resulting daughter mitochondria. It is documented that fission location correlates with daughter mitochondrial fate; division at the mitochondrial periphery tends to segregate damaged material for mitophagy, whereas midzone fission supports proliferation¹². However, this observation of spatial bias does not provide the molecular logic linking fission to daughter mitochondrial fate. Consequently, the mechanisms by which fission governs these divergent outcomes of daughter mitochondria remain poorly understood.

Here, we resolve these long-standing questions by identifying homeostatic inner mitochondrial membrane (IMM) lipid peroxidation (LPO) as a molecular signal not only determining the fission site but also quantitatively encoding information that pre-determines the fate of the daughter mitochondria. We demonstrate that a focal increase in IMM LPO serves as the primary spatial landmark licensing the fission site. Furthermore, we uncover a self-amplifying feedback loop wherein IMM LPO promotes MERCs formation and Ca²⁺ influx, which in turn accelerates IMM LPO, thereby creating a robust trigger for fission. Crucially, we reveal that the magnitude of this IMM LPO signal at the fission sites predicts mitochondrial fate. This IMM LPO-mediated fission is a general mechanism, which is pathologically hyperactivated in malignant prostate cancer to drive mitochondrial fragmentation, representing a potential selective vulnerability. Our study

establishes IMM LPO as the initiating event determining the mitochondrial fission site and identifies it as a critical regulator that integrates site selection with mitochondrial fate determination.

Results

The unexpected requirement of homeostatic IMM LPO for mitochondrial fission

While investigating the impact of IMM LPO on mitochondrial function using a doxycycline (Dox)-inducible system to express mitochondrial-targeted glutathione peroxidase 4 (mitoGPX4)¹³, a specific scavenger of lipid peroxides¹⁴ (Fig. 1a, Extended Data Fig. 1a), we unexpectedly discovered a surprising role for homeostatic IMM LPO in facilitating mitochondrial fission (Fig. 1b). Specifically, mitoGPX4 induction in U-2 OS cells significantly reduced basal IMM LPO, as evidenced by decreased fluorescence of the LPO-specific dye MitoPeDPP¹⁵ (Extended Data Fig. 1b). The probe's specificity for the inner membrane was confirmed by its colocalization with the IMM marker PK Mito Deep Red and its distinct separation from the outer mitochondrial membrane (OMM) marker TOMM20 (Extended Data Fig. 1c). Critically, mitoGPX4 expression resulted in an approximately 50% reduction in the mitochondrial fission rate (Fig. 1b). These findings indicate that homeostatic IMM LPO is required for mitochondrial fission.

Consistent with inhibited fission, scavenging IMM LPO resulted in pronounced mitochondrial elongation. The mean mitochondrial branch length increased from a baseline of 2.32 μm in control cells to 3.01 μm in mitoGPX4-expressing cells (Fig. 1c, d), a hyperfused phenotype comparable to the 3.33 μm length observed upon expression of the dominant-negative DRP1(K38A) mutant, which inhibits fission⁵ (Extended Data Fig. 1d, e). This effect, comparable to that of DRP1, supports that IMM LPO is a major requirement for mitochondrial fission. Furthermore, the anti-fission effect of mitoGPX4 was strictly dependent on its catalysis of LPO, as expression of the inactive mutants mitoGPX4(U73C) and mitoGPX4(N164A) failed to induce mitochondrial elongation¹⁶ (Fig. 1c, d). Further supporting the requirement of IMM LPO for mitochondrial fission, mitoGPX4 over-expression reduced DRP1 activity for fission, evidenced by decreased phosphorylation at the pro-fission site DRP1-S616¹⁷ and increased phosphorylation at the anti-fission site DRP1-S637¹⁸ (Fig. 1e, f). Additionally, mitoGPX4 overexpression did not alter mitochondrial content, indicating that its effect on decreasing fission is not due to a reduction in

total mitochondrial abundance (Extended Data Fig. 1f). Collectively, these data establish that IMM LPO is required for mitochondrial fission under homeostatic conditions.

IMM LPO levels are focally elevated at future mitochondrial fission sites and are sufficient to induce mitochondrial fission.

We next sought to determine if IMM LPO spatially marks future division sites. Live-cell imaging revealed focal surges in IMM LPO, labelled with MitoPeDPP, at the prospective mitochondrial division sites (Fig. 1g, Supplementary Video 1). Quantification of 109 fission events across 27 cells stained with MitoPeDPP showed that 76.15% were marked by a preceding, localized increase in IMM LPO (Fig. 1h). Furthermore, using another independent IMM LPO probe MitoPerOx (Extended Data Fig. 1g), that shifts its fluorescence emission from red (reduced state) to green (peroxidized state) upon lipid peroxidation¹⁹, we validated this surge of IMM LPO at future fission sites (Extended Data Fig. 1h, i, Supplementary Video 2). Quantification of fission events in cells stained with MitoPerOx showed that 71.875% were marked by a preceding, localized increase in IMM LPO as well (Extended Data Fig. 1i). These data establish that a focal increase in IMM LPO occurs at the prospective fission sites.

Furthermore, we investigated whether a localized increase in IMM LPO is sufficient to induce mitochondrial fission. As previously described, we employed a focal photo-stimulation to induce localized peroxidation of the IMM²⁰, evidenced by a red-to-green shift in MitoPerOx staining¹⁹ (Fig. 1i). We found that the targeted stimulation site with focal increase in IMM LPO showed obvious mitochondrial constriction and subsequent fission (Fig. 1j, Supplementary Video 3). Quantitative analysis revealed that among all stimulation sites exhibiting IMM LPO induction, 71.05% progressed to constriction, with 77.78% of these undergoing complete fission within the observation period (Fig. 1k). These findings provide direct evidence that a localized increase in IMM LPO is able to initiate mitochondrial fission. Collectively, these data indicate that homeostatic IMM LPO marks mitochondrial fission sites and is both necessary and sufficient for mitochondrial division.

Elevated IMM LPO enhances the propensity of MERCS for fission

MERCS is the known early signal marking fission site³, thus we investigated whether IMM LPO marks the fission-competent MERCS subset. Firstly, we found that ER-associated mitochondrial constriction sites displayed focal IMM LPO enrichment (Fig. 2a, b). Strikingly,

quantification of 1001 MERCS showed that 83.33% of fission-competent MERCS displayed local IMM LPO elevation (Fig. 2c), supporting that fission primarily occurs at MERCS with increased IMM LPO. Furthermore, we evaluated the percentage of MERCS exhibiting a focal increase in IMM LPO that underwent fission. We found that among all MERCS, approximately 16.79% underwent constriction, with only 4.80% proceeding to complete division (Fig.2d). In contrast, among MERCS exhibiting local IMM LPO elevation, these proportions increased to 32.36% for constriction and 10.61% for fission (Fig.2d). Therefore, the majority of fission events occur at MERCS exhibiting a focal increase in IMM LPO, but not all such MERCS undergo fission. One possible explanation for this observation is that the combination of MERCS and homeostatic IMM LPO initiates the fission cascade, with only those sites exceeding a specific threshold establishing a robust fission locus, given that strong IMM LPO from light stimulation is sufficient to induce fission (Fig. 1i-k). Altogether, mitochondrial fission predominantly occurs at the MERCS with increased IMM LPO, and local IMM LPO enrichment is required for the competence of MERCS to facilitate mitochondrial constriction and division.

IMM LPO could occur before mitochondria-ER contact during mitochondrial fission

We then sought to elucidate the temporal and functional interplay between IMM LPO elevation and ER recruitment during mitochondrial fission. Live-cell imaging revealed that IMM LPO and ER recruitment can occur reciprocally, with either preceding the other. Specifically, among 15 fission events exhibiting discernible temporal ordering between IMM LPO and MERCS, six exhibited local IMM LPO elevation prior to ER recruitment, whereas nine displayed ER recruitment preceding local IMM LPO elevation (Fig. 2e). Detailed time-lapse imaging analyses showed that focal IMM LPO elevation can precede ER recruitment to mitochondria, followed by IMM constriction and subsequent mitochondrial division (Fig. 2f). Conversely, ER recruitment could also precede local IMM LPO elevation before subsequent IMM constriction and mitochondrial division (Extended Data Fig. 2a). This either-preceding-the-other phenomenon suggests that IMM LPO and ER recruitment may reciprocally promote each other.

We next used a dimerization-dependent fluorescent protein (ddFP) reporter system that quantify the contacts between ER and mitochondria via reversible fluorescence reconstitution²¹ (Fig. 2g, Extended Data Fig. 2b), to investigate whether elevated IMM LPO drives ER recruitment. Consistent with above mentioned results, the majority (76.09%) of fission-competent ddFP-

labeled MERCS exhibited elevated IMM LPO at fission sites prior to division (Extended Data Fig. 2c). Using this system to determine the potential role of IMM LPO in facilitating ER and mitochondrial contact, we knocked down DHODH (Extended Data Fig. 2d, e), an enzyme known for maintaining mitochondrial reductive capacity and suppressing IMM LPO²². Indeed, DHODH knockdown significantly increased the average MERCS frequency marked by ddFP system from ~68 to ~85 per cell (Fig 2h, i). Critically, overexpressing mitoGPX4 reversed this shDHODH-induced increase in MERCS, reducing the number of ddFP-marked MERCS from ~85 to ~60 per cell (Fig 2h, i). These data indicate that elevated IMM LPO is able to promote MERCS formation.

IMM LPO elevation and MERCS-mediated mitochondrial Ca²⁺ influx form a positive feedback loop promoting mitochondrial fission

Given that ER-mitochondria contact can occur both before and after IMM LPO increase, we next investigated whether MERCS may function reciprocally to promote IMM LPO. As MERCS facilitate fission primarily via mediating mitochondrial Ca²⁺ influx⁴, we tested this potential reciprocal relationship by testing whether IMM LPO and MERCS-mediated mitochondrial Ca²⁺ influx could promote each other. Indeed, we first observed that under homeostatic conditions, focal IMM LPO surges strongly colocalize with peaks in mitochondrial matrix Ca²⁺ detected by the sensor mito-R-GECO1 at the region with constriction of mitochondrial inner compartments (CoMIC)²³, a crucial step during mitochondrial fission (Fig. 3a, b). Furthermore, our data indicated a role of IMM LPO in promoting mitochondrial Ca²⁺, as evidenced by scavenging basal IMM LPO with mitoGPX4 overexpression reduced homeostatic mitochondrial Ca²⁺ levels by approximately 30% (Fig. 3c, d), thereby in consistent with the aforementioned role of IMM LPO in sustaining MERCS. Next, we tested whether increased mitochondrial Ca²⁺ reciprocally amplifies IMM LPO and fission. Crucially, increasing mitochondrial matrix Ca²⁺ using the Ca²⁺ ionophore A23187 increased mitochondrial Ca²⁺²⁴ (Fig. 3e, f), robustly elevated IMM LPO (Fig. 3e, g) and reduced mitochondrial branch length (Fig. 3e, h). Notably, mitoGPX4 overexpression attenuated the A23187-induced increases in mitochondrial Ca²⁺ and IMM LPO and significantly reversed mitochondrial shortening (Fig. 3e-h), further confirming that IMM LPO and mitochondrial Ca²⁺ influx act within a positive feedback loop to facilitate mitochondrial division.

To verify whether the homeostatic surges of IMM LPO are sustained by MERCS-mediated mitochondrial Ca²⁺ influx, we tested the critical involvement of ER-localized Ca²⁺ channel IP3R

²⁵ and mitochondrial calcium uniporter (MCU) ²⁶. We inhibited Ca²⁺ transfer from the ER to mitochondria by either pharmacologically blocking IP3R with 2-APB ²⁷ (Fig. 3i, j) or employing shRNA-mediated knockdown of MCU ²⁸ (Fig. 3l, m; Extended Data Fig. 3a, b). Both interventions significantly decreased basal IMM LPO levels (Fig. 3 i, k, l, n), demonstrating that MERCS-mediated Ca²⁺ influx is required to sustain homeostatic IMM LPO. Further supporting this observation, in fission events where MERCS formation (Extended Data Fig. 2a, 0 s) precedes the elevation of IMM LPO, a progressive increase in IMM LPO is evident following MERCS assembly (Extended Data Fig. 2a, 5-20 s). Collectively, these results define a self-amplifying positive feedback loop critical for fission site selection, wherein focal IMM LPO and MERCS-mediated Ca²⁺ influx mutually reinforce one another, thereby committing specific mitochondrial sites to fission (Fig. 3o).

The magnitude of IMM LPO at the fission site predicts the fate of daughter mitochondrion

We next addressed another central question in mitochondrial biology: how the act of fission is coupled to daughter mitochondrial fate. Given the correlation of fission location with the fate of daughter mitochondria ¹², we tested whether the magnitude of IMM LPO encodes information about the fission mode (midzone vs. peripheral) and subsequent mitochondrial state. We performed live-cell imaging of mitochondrial fission dynamics in U-2 OS cells to analyze the distribution of IMM LPO intensity at prospective fission sites. Changes in fission-site IMM LPO intensity were quantified as the ratio F_a/F_{ref} , defined as the MitoPeDPP fluorescence at the prospective fission site (F_a) relative to the reference fluorescence of the corresponding mother mitochondrion (F_{ref}) (Fig. 4a). A clear distinction in the ratio F_a/F_{ref} emerged between fission types (Fig. 4a). Strikingly, the fold change of IMM LPO intensity at midzone division sites was uniformly less than twofold, with an average of approximately 1.48. In contrast, IMM LPO intensity at peripheral division sites exhibited a significantly higher mean increase of approximately 2.26-fold change. Based on this distribution, we established an empirical threshold of $F_a/F_{ref} = 2$ to distinguish 'modest' (< 2) from 'severe' (≥ 2) IMM LPO. Using this classification, we found that midzone division is characterized by modest IMM LPO, whereas severe LPO was a feature exclusive to peripheral divisions (Fig. 4a).

We then evaluated how this IMM LPO signal is partitioned and inherited. IMM LPO intensity changes in daughter mitochondria during U-2 OS mitochondrial fission were quantified by the

ratio F_b/F_{ref} , defined as MitoPeDPP values at the division end of the daughter mitochondria (F_b) relative to the background MitoPeDPP signal across the mother mitochondrion (F_{ref}). Following midzone division, IMM LPO was partitioned symmetrically, with both daughters inheriting modest LPO levels (< 2 -fold) (Fig. 4b, c). In contrast, peripheral division often resulted in asymmetric partitioning, where the smaller daughter inherited significantly higher LPO than its larger counterpart (≥ 2 -fold) (Fig. 4d, e). Crucially, this asymmetric inheritance of IMM LPO was dependent on the initial LPO magnitude at the fission site (Fig. 4d). Only peripheral divisions originating from a site of severe IMM LPO (≥ 2 -fold) produced a high-LPO daughter (Fig. 4d; upper); if the initial peripheral fission event was modest (< 2 -fold), both resulting daughters also maintained modest LPO (Fig. 4d; lower).

This key observation, that not all peripheral divisions yield a high-LPO daughter, prompted us to test whether the inherited IMM LPO level itself, rather than fission location alone, is the primary determinant of mitochondrial fate, particularly given that many shorter daughter mitochondria resulting from peripheral division escape mitophagy^{9, 10, 12}. Indeed, applying this twofold increase in IMM LPO as a classifier across all daughter mitochondria precisely reflected their physiological state and fate. Time-lapse imaging indicated that daughter mitochondria retaining elevated MitoPeDPP signals displayed reduced TMRE signals (Extended Data Fig. 4). Further quantitative analyses validated that this IMM LPO-based classification directly predicts the subsequent state and fate of daughter mitochondria (Fig. 4f, g). Live-cell imaging tracking of 284 mitochondria revealed that 94.78% of severe-LPO (≥ 2 -fold) daughters were depolarized (TMRE-low) (Fig. 4f-h), whereas 86.98% of modest-LPO (< 2 -fold) daughters maintained their membrane potential (TMRE-high) (Fig. 4h). This divergence in IMM LPO state strongly correlates with distinct destinies of these daughter mitochondria: examination of 520 mitochondria showed that 80.87% of severe-LPO mitochondria were targeted for mitophagy as indicated by the association with LC3B-positive autophagosomes (Fig. 4i), while 97.04% of their low-LPO counterparts escaped this fate (Fig. 4i). Collectively, these data identify the magnitude of IMM LPO as the quantitative signal that directly couples the initiation of fission to subsequent quality control decisions, thereby determining the fate of daughter mitochondria.

IMM LPO-mediated fission is pathologically hyperactivated in prostate cancer

We subsequently investigated whether IMM LPO-mediated fission occurs in disease contexts, focusing on prostate cancer, a malignancy characterized by profound metabolic reprogramming, particularly altered mitochondrial function²⁹⁻³¹. First, we examined mitochondrial shape in human prostate cancer specimens from three patients by transmission electron microscopy (TEM) analyses. To quantify mitochondrial morphological changes, we performed major-minor axis morphometry on TEM images of paired tumor and adjacent non-tumor prostate epithelial tissues. In tumor epithelium, mitochondria predominantly exhibited low-aspect-ratio profiles (median ratio: 1.165; interquartile range [IQR]: 1.116-1.230), with 90.8% displaying an aspect ratio below 1.5, indicating significant mitochondrial shortening (Fig. 5a, b). In stark contrast, mitochondria in adjacent non-tumor epithelium were much more elongated (median ratio: 1.819; IQR: 1.356-2.664); 63.6% had an aspect ratio ≥ 1.5 , especially that 21.9% exhibited a ratio ≥ 3.0 (Fig. 5c, d). These results suggest that mitochondrial fission in human prostate cancer may be more active than in the normal control cells.

We further recapitulated this mitochondrial shortening phenotype in cell lines, observing that malignant prostate cancer 22Rv1 cells displayed substantially shorter mitochondrial branches (0.646 μm) compared to normal prostate epithelial RWPE-1 cells (1.350 μm) (Fig. 5e, f). Furthermore, we demonstrated that mitochondrial shortening in cancer cells results from hyper-fission mediated by increased IMM LPO. Flow cytometric analysis of MitoPeDPP fluorescence revealed that 22Rv1 cells exhibited higher basal IMM LPO levels than RWPE-1 control cells (Fig. 5g). Importantly, reducing IMM LPO in 22Rv1 cells via mitoGPX4 overexpression restored mitochondrial network integrity, increasing mean mitochondrial branch length from 0.498 μm to 1.279 μm (Fig. 5h, i). Supporting that mitochondrial shortening in 22Rv1 cells stems from excessive fission, treatment with the DRP1 inhibitor Mdivi-1 also induced mitochondrial elongation, yielding an increase in branch length comparable to that observed in mitoGPX4-overexpressing 22Rv1 cells (Extended Data Fig. 5a, b). Notably, both mitoGPX4 overexpression and DRP1 inhibition exerted minimal effects on mitochondrial length in control RWPE-1 cells (Fig. 5h, i, Extended Data Fig. 5a, b), suggesting that mitochondrial fission is inactive in these normal control cells, consistent with their low IMM LPO levels (Fig. 5g). These results indicate that prostate cancer cells exhibit active LPO-mediated mitochondrial fission, which can be suppressed by either decreasing IMM LPO or inhibiting DRP1. Therefore, IMM LPO-mediated

fission represents a general mechanism governing mitochondrial dynamics across diverse cell types in both homeostatic and disease contexts.

Discussion

This study identifies homeostatic focal surges in IMM LPO as an upstream signal that initiates mitochondrial fission, resolving long-standing questions about site selection and their coordination with fate determination (Fig. 5j). Crucially, our work re-contextualizes the role of LPO in cell biology. While best known as the catastrophic executioner of ferroptosis, driven by overwhelming oxidative damage³², we demonstrate a fundamentally different role: IMM LPO as a localized, and regulated physiological signal. We show that cells utilize spatially confined IMM LPO as a precise signaling moiety to direct mitochondrial dynamics. This discovery reframes IMM LPO from an unregulated destructive force to a core component of mitochondrial homeostasis.

The suitability of IMM LPO for initiating fission and encoding fate stems from its unique properties as a signal integrator. The IMM's enrichment in polyunsaturated fatty acids (PUFAs) renders it a highly poised platform for lipid peroxidation^{33, 34}. Nearly all fluctuations in mitochondrial state, from metabolic flux to Ca²⁺ transients³⁵, converge on altering electron flow through the ETC³⁶, which can trigger local ROS production and subsequent IMM LPO induction³⁷. Consequently, the local level of IMM LPO serves as a real-time, integrated readout of mitochondrial health, positioning it perfectly to translate this internal state into the physical act of fission and a corresponding quality control decision.

Our findings identify a homeostatic focal surge in IMM LPO as a molecular requirement for mitochondrial fission site selection. This event could precede ER recruitment, a previously-known early fission steps⁷, establishing IMM LPO as one of the earliest instructive signals in the fission cascade. A key mechanistic insight is the discovery of a positive feedback loop between IMM LPO and MERCS-mediated Ca²⁺ influx. We show that focal IMM LPO can precede and promote ER recruitment, while the resulting Ca²⁺ influx reciprocally amplifies IMM LPO. This self-amplifying loop functions as a commitment switch, ensuring that fission proceeds only at sites where the fission signal surpasses a critical threshold. This mechanism also establishes an integrative hub, allowing the fission machinery to respond dynamically to diverse cellular states that influence either IMM LPO or MERCS. Beyond fission site selection, our data reveal that the magnitude of the IMM LPO signal encodes daughter mitochondrion fate, explaining how fission is coupled to

mitochondrial quality control. This elucidates the documented yet poorly understood fission site bias of mitochondrial quality control, providing a rationale for how the biochemical state of a fission site, not merely its location, governs the fate of daughter mitochondria.

The IMM LPO-mediated pathway is a general mechanism for fission, underscored by its activation during homeostatic conditions and pathological hyperactivation in prostate cancer. Moreover, our findings open several exciting avenues for future research. A critical next step is to define the IMM LPO signature by identifying the specific peroxidized lipid species that act as the signal for fission. Second, the mechanism of trans-membrane communication, by which the IMM LPO signal affects the MERCS that involves the OMM and ER machinery, remains a key puzzle, possibly involving changes in membrane curvature³⁸. Finally, while mitoGPX4 is a key negative regulator, elucidating the full network of antioxidant systems that spatially and temporally confine the IMM LPO signal to prevent runaway fission will be crucial for a complete understanding. In conclusion, we identify IMM LPO as a primary, quantitative signal that governs where and with what consequence mitochondrial fission occurs, thereby integrating organelle dynamics with quality control in both health and disease.

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Author contributions Statement

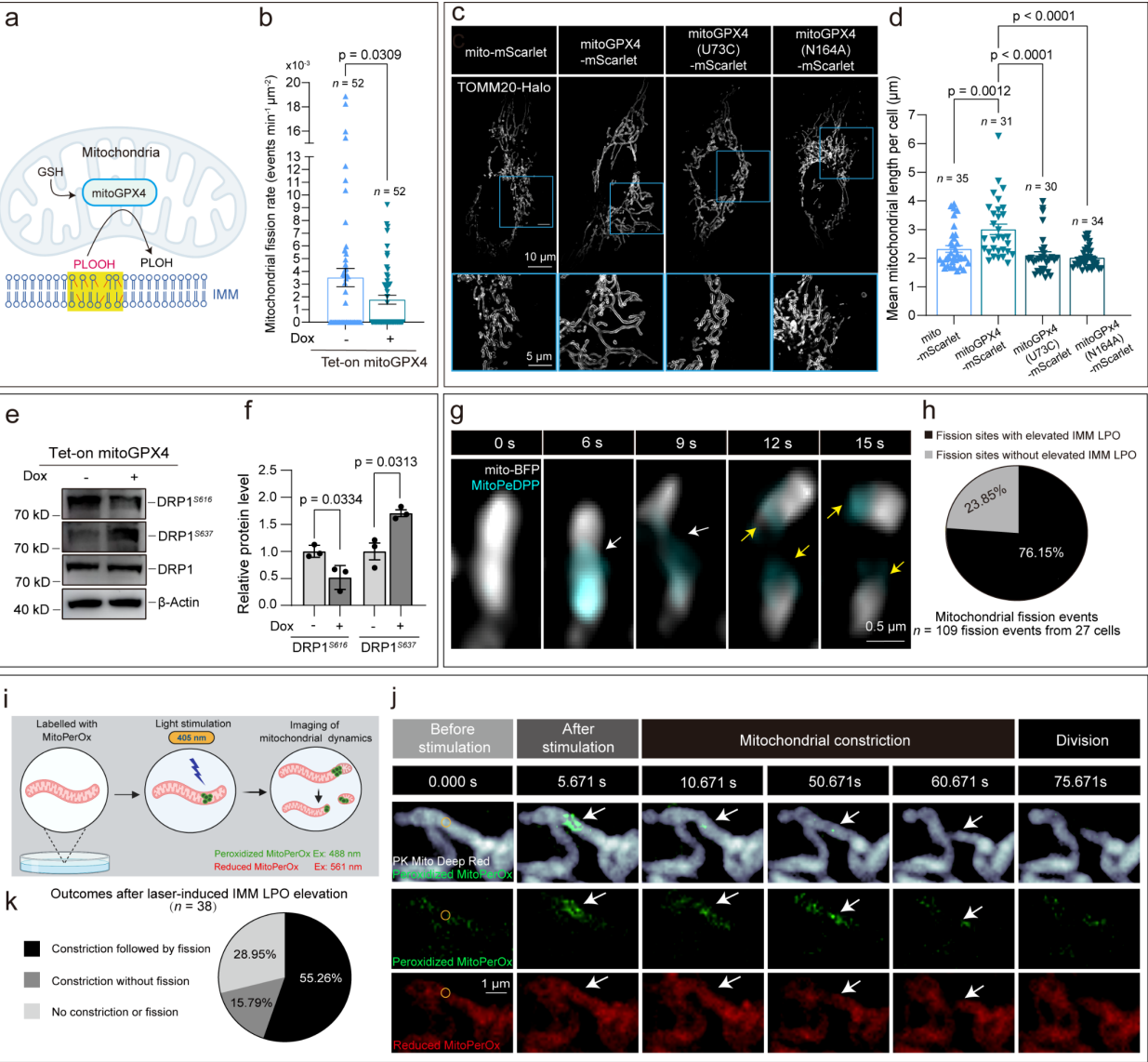
Conceptualization: H.T., B.X. and X.H.; Project administration: H.T. (overall) and B.X. (clinical sample component); Methodology, Software, Validation, Formal analysis, Imaging: X.H.; Investigation and Data curation: X.H. and Z.W.; Resources, Supervision, Funding acquisition: H.T. and B.X.; The manuscript was drafted by X.H. and Z.W., with critical review and editing by H.T. and B.X.

Declaration of interests

The authors declare no competing interests.

348 **Figure legends**

Fig. 1



349

350 **Fig. 1 | Homeostatic IMM LPO is necessary and sufficient to initiate mitochondrial fission.**

351 **(a)** Schematic illustrating the action of mitochondrial-targeted glutathione peroxidase 4

352 (mitoGPX4). MitoGPX4 reduces phospholipid hydroperoxides (PLOOH) to their corresponding

353 alcohols (PLOH), thereby suppressing inner mitochondrial membrane (IMM) lipid peroxidation

354 (LPO).

(b) Quantification of mitochondrial fission rates in Tet-on mitoGPX4 U-2 OS cells treated with or without doxycycline (Dox; 1 μ M) for 48 h. Mitochondrial fission events were quantified from live-cell time-lapse imaging data and normalized to imaging duration and mitochondrial area, yielding units of events $\text{min}^{-1} \mu\text{m}^{-2}$. Dox-induced mitoGPX4 expression significantly reduced the mitochondrial fission rate. Each point represents the mitochondrial fission rate from one individual cell.

(c and d) Representative SIM images (c) and quantification of mean mitochondrial length (d) in U-2 OS cells expressing mito-mScarlet, wild-type mitoGPX4-mScarlet or the catalytically inactive mitoGPX4 mutants U73C and N164A. Only wild-type mitoGPX4 induced mitochondrial elongation. The blue-boxed regions are shown at higher magnification below. Each dot in (d) represents the mean mitochondrial length from one individual cell.

(e and f) Immunoblot analysis (e) and corresponding quantification (f) of DRP1 phosphorylation in Tet-on mitoGPX4 U-2 OS cells treated with or without Dox for 48 h. MitoGPX4 induction decreased pro-fission phosphorylation at DRP1-Ser616 and increased anti-fission phosphorylation at DRP1-Ser637, indicating reduced DRP1 activity. Phospho-DRP1 levels were normalized to total DRP1, and β -actin served as a loading control. Each dot in (f) represents the phospho-DRP1-to-total DRP1 ratio from one independent biological experiment.

(g and h) Focal IMM LPO enrichment precedes mitochondrial fission. (g) Representative live-cell time-lapse SIM images of a U-2 OS cell expressing mito-BFP as a mitochondrial matrix marker and labeled with MitoPeDPP as an IMM LPO probe. Focal MitoPeDPP enrichment was detected at the prospective mitochondrial fission site before constriction and division. Arrows indicate a focal increase in IMM LPO (6 s) at the prospective fission site, followed by constriction (9 s) and division (12 s). The complete process is shown in Supplementary Video 1. White arrows indicate the prospective fission site, and yellow arrows indicate the daughter mitochondria after division. Pseudocolors are applied for optimal visualization. (h) Quantification shows that 76.15% of mitochondrial fission events were preceded by focal IMM LPO elevation.

(i) Schematic of the experimental setup used to induce a focal increase in IMM LPO through light stimulation. A defined mitochondrial region in HeLa cells labeled with the ratiometric IMM LPO probe MitoPerOx was subjected to focal 405-nm laser stimulation to induce local IMM LPO elevation, followed by live-cell imaging of mitochondrial dynamics. Peroxidized MitoPerOx was

385 detected using 488-nm excitation, whereas reduced MitoPerOx was detected using 561-nm
386 excitation.

387 **(j and k)** Focal induction of IMM LPO by light stimulation is sufficient to trigger mitochondrial
388 constriction and fission. (j) Representative time-lapse Olympus FV3000 confocal images of a
389 HeLa cell labeled with MitoPerOx (green, peroxidized; red, reduced) and PK Mito Deep Red
390 (IMM marker). Pseudocolors are applied for optimal visualization. The yellow circle marks the
391 region of light stimulation; white arrows indicate IMM LPO induction, subsequent constriction,
392 and fission. The complete process is shown in Supplementary Video 3. (k) Quantification of
393 outcomes from 38 stimulated sites exhibiting LPO induction shows that 55.26% of stimulated
394 mitochondrial sites underwent constriction followed by fission, 15.79% underwent constriction
395 without subsequent fission during the observation period, and 28.95% showed neither constriction
396 nor fission.

397 **Data information:** Data in (b), (d) and (f) are presented as mean $\pm$ S.E.M. from at least three
398 independent biological experiments. Statistical significance in (b) was assessed using two-tailed
399 unpaired Student's *t*-tests. Statistical significance in (d) was assessed using one-way ANOVA
400 followed by Tukey's multiple-comparisons test. Statistical significance in (f) was assessed using
401 paired two-tailed Student's *t*-tests. The schematics in (a) and (i) were created with BioRender.com.

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Fig.2

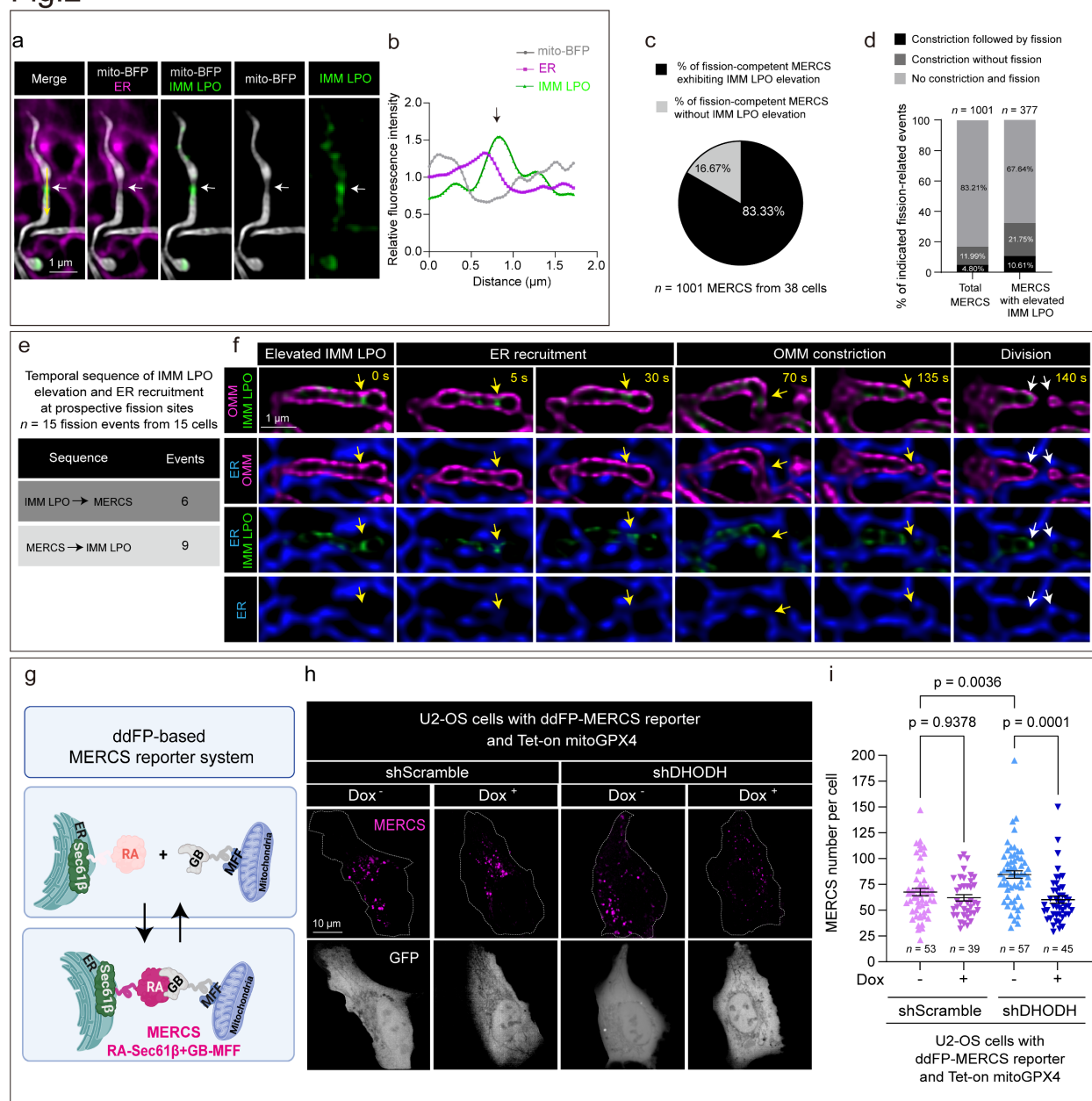


Fig. 2 | Fission-competent mitochondria and ER contact sites (MERCS) are predominantly marked by elevated IMM LPO, which promotes ER-mitochondrial contact to drive fission.

(a and b) Focal surges of IMM LPO at ER-mediated mitochondrial constriction sites. **(a)** Representative SIM image of a U-2 OS cell expressing Calnexin-mScarlet (ER) and mito-BFP (mitochondrial matrix), and labeled with MitoPeDPP (IMM LPO probe). The white arrow indicates a constriction site where focal IMM LPO coincides with ER contact. Pseudocolours are

applied for optimal visualization. (b) Line-scan analysis across the constriction site reveals increased IMM LPO at the MERCS. The black arrow indicates a mitochondrial constriction site where focal IMM LPO coincides with ER contact.

(c) Percentage of fission-competent MERCS exhibiting focal IMM LPO elevation. Quantification shows that 83.33% of fission-competent MERCS exhibited focal IMM LPO elevation, with the other 16.67% showing no detectable focal IMM LPO elevation.

(d) IMM LPO surges enhance constriction and fission propensity at MERCS. Stacked bars show the proportions of sites undergoing constriction followed by mitochondrial fission or constriction without subsequent fission during the observation period. Among total MERCS, 4.80% proceeded to complete division and 11.99% underwent constriction without division. Among MERCS exhibiting focal IMM LPO elevation, 10.61% proceeded to complete division and 21.75% underwent constriction without division.

(e) Quantification of the temporal ordering of focal IMM LPO elevation and ER recruitment at mitochondrial fission sites shows that either signal can precede the other, suggesting a reciprocal relationship between IMM LPO elevation and MERCS formation. A total of 15 events exhibiting a discernible temporal order between MERCS and IMM LPO were evaluated.

(f) Time-lapse spinning-disk confocal imaging (Nikon) of a U-2 OS cell expressing TOMM20-Halo (OMM) and Calnexin-mScarlet (ER), and labeled with MitoPeDPP (IMM LPO probe). At 0 s, elevated IMM LPO was observed at the prospective fission site (yellow arrow). Then, the ER is recruited to the IMM LPO-enriched site from 5-30 s. OMM constriction then occurs at this newly formed MERCS from 70-135 s, followed by mitochondrial division at 140 s. Yellow arrows track the prospective fission site; White arrows indicate the daughter mitochondria post-fission. Images were acquired every 5 s.

(g) Schematic of the dimerization-dependent fluorescent protein (ddFP)-based reporter system for quantifying MERCS. Reconstitution of the ER-targeted RA-Sec61 β fragment and the OMM-targeted GB-MFF fragment at MERCS generates a fluorescent signal.

(h and i) Elevated IMM LPO promotes MERCS formation. Representative three-dimensional images (h) and quantification (i) of MERCS number in U-2 OS cells expressing the ddFP reporter with indicated treatments. Increasing IMM LPO through shRNA-mediated DHODH knockdown

increased MERCS number, an effect reversed by scavenging IMM LPO through Dox-induced mitoGPX4 expression. MERCS were visualized using the ddFP reporter system, and cytosolic GFP indicates cell morphology. Each dot in (i) represents the number of ddFP-positive MERCS in one individual cell.

Data information: Data in (i) are presented as mean $\pm$ S.E.M. from at least three independent biological experiments. Statistical significance in (i) was assessed using two-way ANOVA followed by Tukey's multiple-comparisons test. The schematic in (g) was created with BioRender.com.

Fig. 3

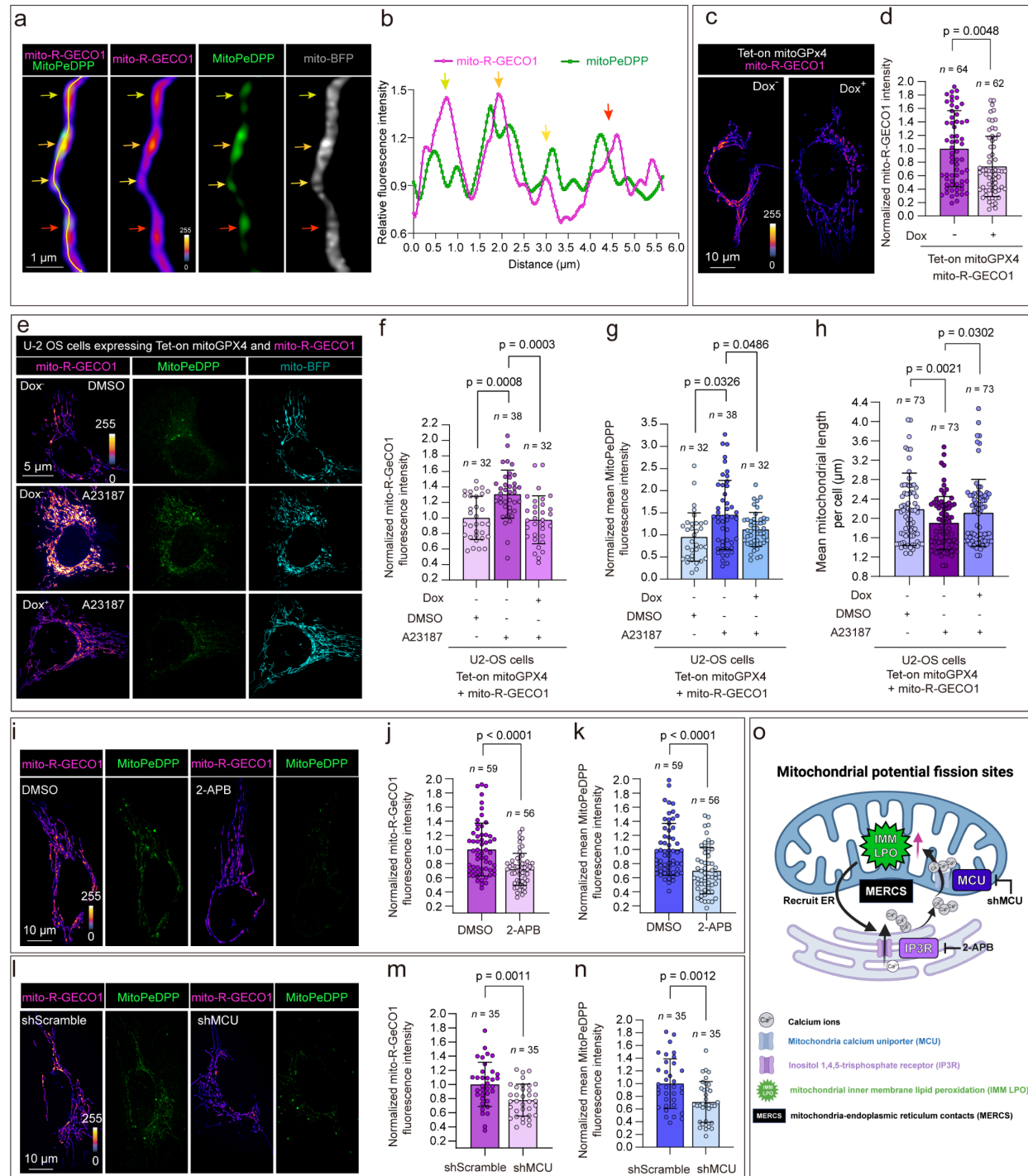


Fig. 3 | IMM LPO and ER-to-mitochondria Ca²⁺ transfer form a positive feedback loop.

(a and b) Local IMM LPO enrichment spatially coincides with mitochondrial matrix Ca²⁺ accumulation. (a) Representative SIM images of a U-2 OS cell expressing mito-R-GECO1 (Ca²⁺

sensor) and mito-BFP (mitochondrial matrix), and labeled with MitoPeDPP (IMM LPO probe). Arrows indicate sites of co-enrichment, likely at the constriction of mitochondrial inner compartment (CoMIC) region. Pseudocolors are applied for optimal visualization. (b) Line-scan analysis showing spatial alignment of mito-R-GECO1 and MitoPeDPP fluorescence peaks.

(c and d) Scavenging IMM LPO through mitoGPX4 overexpression reduces basal mitochondrial Ca^{2+} . Representative images (c) and quantification (d) show that Dox-induced mitoGPX4 expression reduces mito-R-GECO1 fluorescence in U-2 OS cells. Each dot in (d) represents the mean mito-R-GECO1 fluorescence intensity from one individual cell.

(e-h) Elevated Ca^{2+} induces IMM LPO and mitochondrial shortening, effects that are attenuated by mitoGPX4 overexpression. Representative images are shown in (e). Treatment of U-2 OS cells with the Ca^{2+} ionophore A23187 increased mitochondrial Ca^{2+} (e, f), elevated IMM LPO (e, g) and induced mitochondrial shortening (h). These effects were attenuated by scavenging IMM LPO through Dox-induced mitoGPX4 expression. Each dot in (f), (g) and (h) represents the mean mito-R-GECO1 fluorescence intensity, the mean MitoPeDPP fluorescence intensity and the mean mitochondrial length from one individual cell, respectively.

(i-k) Inhibition of IP3R-mediated ER Ca^{2+} release with 2-APB reduces both mitochondrial Ca^{2+} (j) and basal IMM LPO (k). Representative images are shown in (i). Each dot in (j) and (k) represents the mean mito-R-GECO1 intensity and mean MitoPeDPP intensity of an individual cell, respectively.

(l-n) Genetic suppression of mitochondrial Ca^{2+} uptake via MCU shRNA knockdown reduces both mitochondrial Ca^{2+} (m) and IMM LPO (n). Representative images are in (l). Each dot in (m) and (n) represents the mean mito-R-GECO1 intensity and mean MitoPeDPP intensity of an individual cell, respectively.

(o) Model of the positive feedback loop between IMM LPO and MERCS-mediated Ca^{2+} signaling. Local IMM LPO promotes MERCS-mediated Ca^{2+} influx via IP3R and MCU; this Ca^{2+} influx reciprocally amplifies IMM LPO, creating a commitment switch for fission.

Data information: Data in (d), (f-h), (j), (k), (m) and (n) are presented as mean $\pm$ S.E.M. from at least three independent biological experiments. Statistical significance in (d), (j), (k), (m) and (n) was assessed using two-tailed unpaired Student's *t*-tests. Statistical significance in (f-h) was

481 assessed using one-way ANOVA followed by Tukey's multiple-comparisons test. The schematic
482 in (o) was created with BioRender.com.

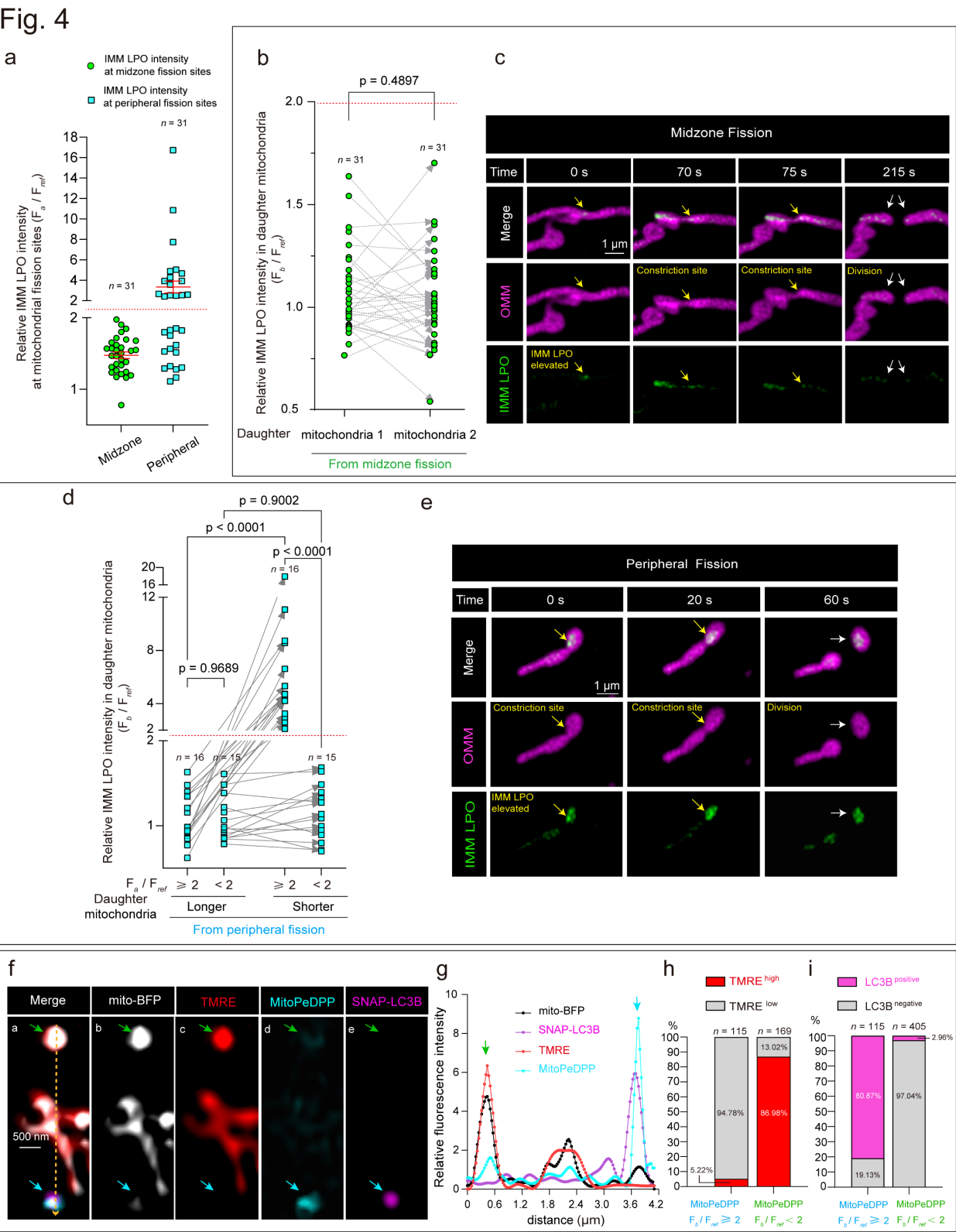


Fig. 4 | Focal IMM LPO magnitude predicts daughter mitochondrial fate.

485 **(a)** The distribution of IMM LPO intensity at fission sites of midzone fission and peripheral fission.
 486 F_a and F_{ref} denote the MitoPeDPP values at the prospective fission site and the background
 487 MitoPeDPP signal across the mother mitochondrion, respectively. The red dotted line indicates the
 488 twofold IMM LPO elevation threshold ($F_a/F_{ref} = 2$). Each dot represents the F_a/F_{ref} ratio from one
 489 individual fission event.

490 **(b and c)** Paired quantification of IMM LPO inherited by the two daughter mitochondria generated
 491 by midzone fission and the corresponding representative spinning-disk confocal time-lapse images
 492 of midzone fission in a U-2 OS cell labeled with TOMM20-Halo (OMM), and MitoPeDPP (IMM
 493 LPO probe) indicating that following midzone division, the resulting daughter mitochondria (white
 494 arrows) exhibit low, basal levels of LPO. (b) Both daughter mitochondria inherit comparable,
 495 modest IMM LPO levels. F_b and F_{ref} denote MitoPeDPP values at the division end of the daughter
 496 mitochondria and the background MitoPeDPP signal across the mother mitochondrion,
 497 respectively. Each dot represents the F_b/F_{ref} ratio for an individual midzone fission event and lines
 498 connect the two daughter mitochondria generated from the same fission event. The red dotted line
 499 indicates $F_b/F_{ref} = 2$. (c) at 0 s, a modest, focal elevation of IMM LPO is detected at a prospective
 500 midzone fission site. This signal precedes visible morphological changes and is followed by OMM
 501 constriction at 70 s, with final division occurring at 215 s. Images were acquired every 5 s.

502 **(d and e)** Paired quantification of IMM LPO inherited by the longer and shorter daughter
 503 mitochondria generated by peripheral fission and the corresponding representative spinning-disk
 504 confocal time-lapse images of peripheral fission in a U-2 OS cell labeled with TOMM20-Halo
 505 (OMM), and MitoPeDPP (IMM LPO probe), indicating that post-division, the severely elevated
 506 LPO signal is asymmetrically partitioned and retained by the smaller daughter mitochondrion
 507 (white arrows). (d) During peripheral division, when the initial F_a/F_{ref} ratio was ≥ 2 , the shorter
 508 daughter mitochondrion inherited substantially higher IMM LPO than its paired longer daughter;
 509 by contrast, when the initial F_a/F_{ref} ratio was < 2 , both daughter mitochondria retained modest
 510 IMM LPO levels. Each dot represents the F_b/F_{ref} ratio of one daughter mitochondrion, and lines
 511 connect the longer and shorter daughter mitochondria generated from the same fission event. The
 512 red dotted line indicates $F_b/F_{ref} = 2$. (e) From 0-20 s, a severe focal IMM LPO signal progressively
 513 intensifies at a prospective peripheral fission site. Mitochondrial division occurs at 60 s. Images
 514 were acquired every 5 s.

515 **(f-i)** Correlation between daughter mitochondrial IMM LPO levels, mitochondrial state, and fate.
 516 (f) Representative image of fragmented mitochondria in a U-2 OS cell expressing mito-BFP
 517 (mitochondrial matrix) and SNAP-LC3B (autophagosome), and labeled with TMRE
 518 (mitochondrial membrane-potential probe) and MitoPeDPP (IMM LPO probe). The low-LPO
 519 mitochondrial fragment indicated by the green arrow retains TMRE fluorescence and lacks LC3B
 520 association, whereas the high-LPO fragment indicated by the cyan arrow exhibits reduced TMRE
 521 fluorescence and positive LC3B association. (g) Line-scan analysis along the indicated region in
 522 (f) showing the relative fluorescence intensity profiles of mito-BFP, TMRE, MitoPeDPP and
 523 SNAP-LC3B. (h) Quantitative association between inherited IMM LPO and mitochondrial
 524 membrane potential. Daughter mitochondria were classified as severe-LPO ($F_b/F_{ref} \geq 2$) or
 525 modest-LPO ($F_b/F_{ref} < 2$). Among severe-LPO ones, 94.78% were TMRE-low, whereas 86.98%
 526 of modest-LPO ones were TMRE-high. (i) Quantitative association between inherited IMM LPO
 527 and LC3B recruitment. Among severe-LPO daughter mitochondria, 80.87% were LC3B-positive,
 528 whereas 97.04% of modest-LPO ones were LC3B-negative.

529 **Data information:** Data in (a), (b) and (d) are presented as mean $\pm$ S.E.M. from at least three
 530 independent biological experiments. Statistical significance in (b) and (d) was assessed using a
 531 paired two-tailed Student's *t*-test and two-way ANOVA followed by Tukey's multiple-comparisons
 532 test, respectively.

Fig. 5

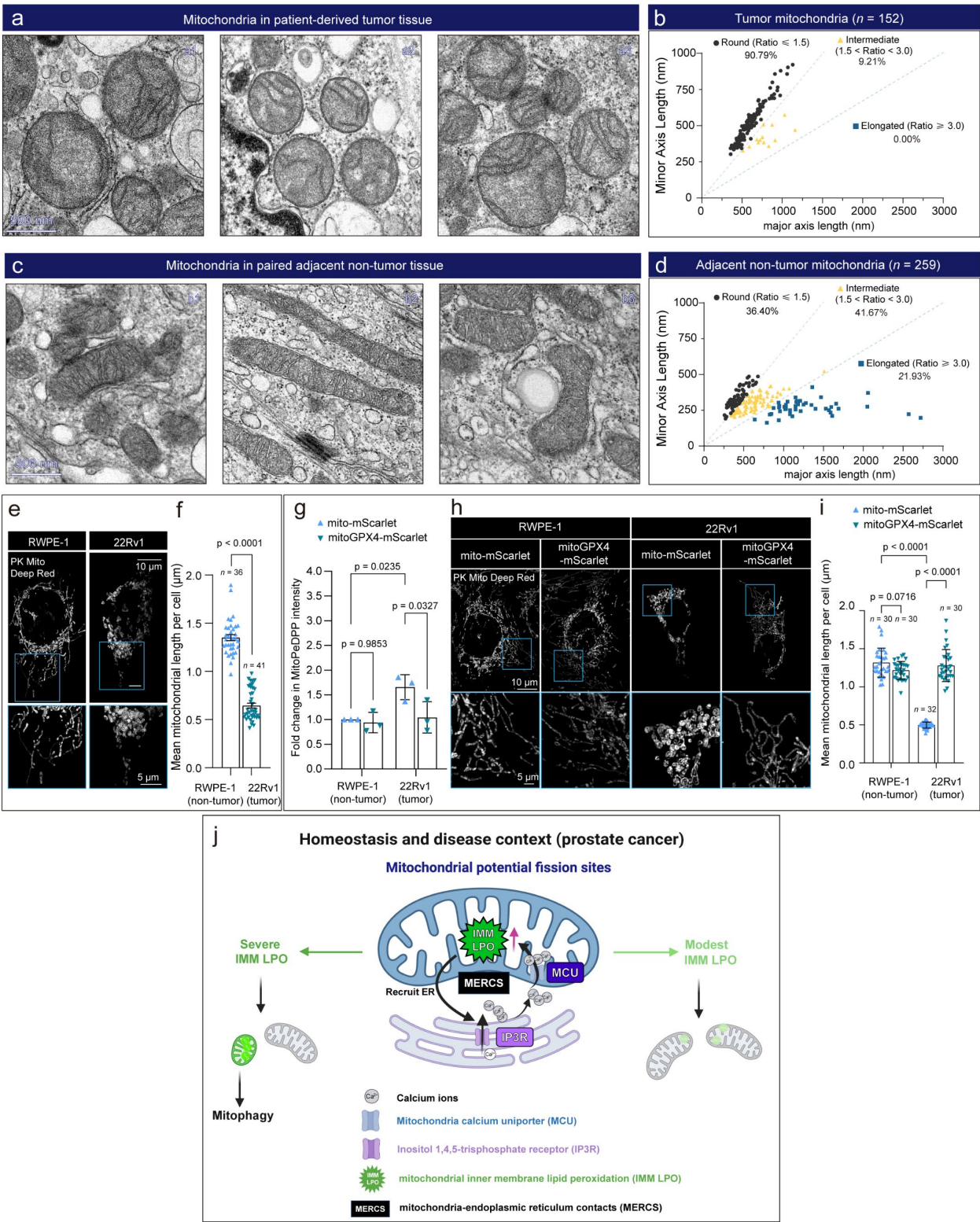


Fig. 5 | IMM LPO-mediated fission is pathologically hyperactivated in prostate cancer.

(a-d) Mitochondria are shortened in human prostate cancer tissue. Representative transmission electron microscopy (TEM) images of mitochondria in tumor tissue (a) and paired adjacent non-tumor tissue (c) from prostate cancer patients. Tumor and paired adjacent non-tumor tissues were histopathologically confirmed using H&E-stained sections. Epithelial regions selected for mitochondrial morphometry were further verified using large-scale electron microscopy imaging and reconstruction based on tissue architecture and cellular morphology. Scatter plots in (b) and (d) show individual mitochondria according to their major- and minor-axis lengths and classify mitochondrial morphology on the basis of aspect ratio, calculated as the major-axis length divided by the minor-axis length: round, $\text{ratio} \leq 1.5$; intermediate, $1.5 < \text{ratio} < 3.0$; and elongated, $\text{ratio} \geq 3.0$. Tumor tissue contained predominantly round mitochondria (90.79%), whereas adjacent non-tumor tissue showed a broader distribution of round (36.40%), intermediate (41.67%) and elongated (21.93%) mitochondria. Each dot in (b) and (d) represents the aspect ratio of one individual mitochondrion.

(e and f) Prostate cancer cells exhibit mitochondrial shortening. Representative SIM images (e) and quantification (f) show that malignant 22Rv1 cells have shorter mitochondrial branches (0.646 μm) compared to normal RWPE-1 epithelial cells (1.350 μm). Mitochondria were labeled with PK Mito Deep Red. The blue-boxed regions are shown at higher magnification below. Each dot in (f) represents the mean mitochondrial branch length from one individual cell.

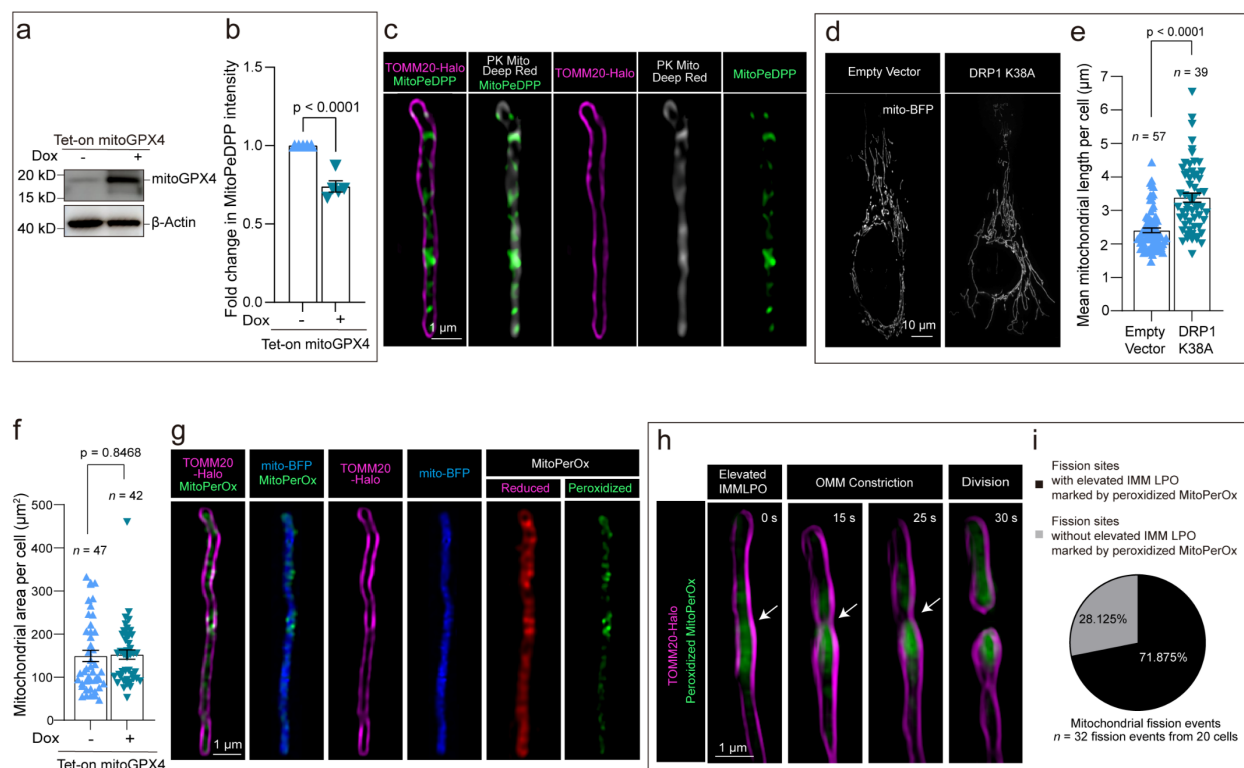
(g) Prostate cancer cells exhibit elevated basal IMM LPO. Flow cytometry analysis shows higher MitoPeDPP fluorescence in 22Rv1 cells compared to RWPE-1 cells. This elevated IMM LPO is suppressed by mitoGPX4 expression. Each dot represents the relative mean MitoPeDPP fluorescence intensity from one independent biological experiment; 10,000 cells were analyzed per sample.

(h and i) Scavenging IMM LPO reverse mitochondrial shortening in prostate cancer cells. Representative SIM images (h) and quantification (i) show that mitoGPX4 expression significantly increases mitochondrial branch length in 22Rv1 cells (from 0.498 μm to 1.279 μm) but has minimal effect in control RWPE-1 cells. Cells expressed mito-mScarlet or mitoGPX4-mScarlet, and mitochondria were labeled with PK Mito Deep Red. The blue-boxed regions are shown at higher magnification below. Each dot in (i) represents the mean mitochondrial branch length from one individual cell.

(j) Model summarizing IMM LPO-mediated fission and mitochondrial fate determination. Homeostatic focal surges of IMM LPO act as an upstream signal for mitochondrial fission by promoting ER recruitment and ER-to-mitochondria Ca^{2+} transfer. The magnitude of this IMM LPO signal dictates daughter mitochondrial fate. This program is pathologically enhanced in prostate cancer, leading to excessive fragmentation.

Data information: Data in (f), (g) and (i) are presented as mean $\pm$ S.E.M. from three independent biological experiments. Statistical significance in (f) was assessed using a two-tailed unpaired Student's *t*-test. Statistical significance in (g) and (i) was assessed using two-way ANOVA followed by Tukey's multiple-comparisons test. The schematic in (j) was created with BioRender.com.

Extended Data Fig.1



Extended Data Fig. 1 | Suppression of IMM LPO induces mitochondrial elongation that requires GPX4 catalytic activity and phenocopies DRP1 inhibition.

(a) Immunoblot analysis validating the Dox-inducible Tet-on system for mitoGPX4 expression in U-2 OS cells. Cells were treated with Dox (1 μM) for 48 h. β-actin served as a loading control.

(b) Flow cytometry analysis confirming that Dox-induced mitoGPX4 expression reduces basal IMM LPO in Tet-on mitoGPX4 U-2 OS cells labeled with MitoPeDPP. Each dot represents the relative mean MitoPeDPP fluorescence intensity from one independent biological experiment; 10,000 cells were analyzed per sample.

(c) Validation of the IMM localization of MitoPeDPP. Representative SIM images of a U-2 OS cell expressing TOMM20-Halo (mitochondrial outer membrane, OMM) and labeled with PK Mito Deep Red (IMM marker) and MitoPeDPP (IMM LPO probe).

(d and e) Expression of dominant-negative DRP1(K38A) phenocopies mitoGPX4-induced mitochondrial elongation. Representative SIM images (d) and quantification of mean

mitochondrial branch length (e) in U-2 OS cells expressing either an empty vector or DRP1(K38A). Mitochondria were visualized using mito-BFP. Each dot in (e) represents the mean mitochondrial length from one individual cell.

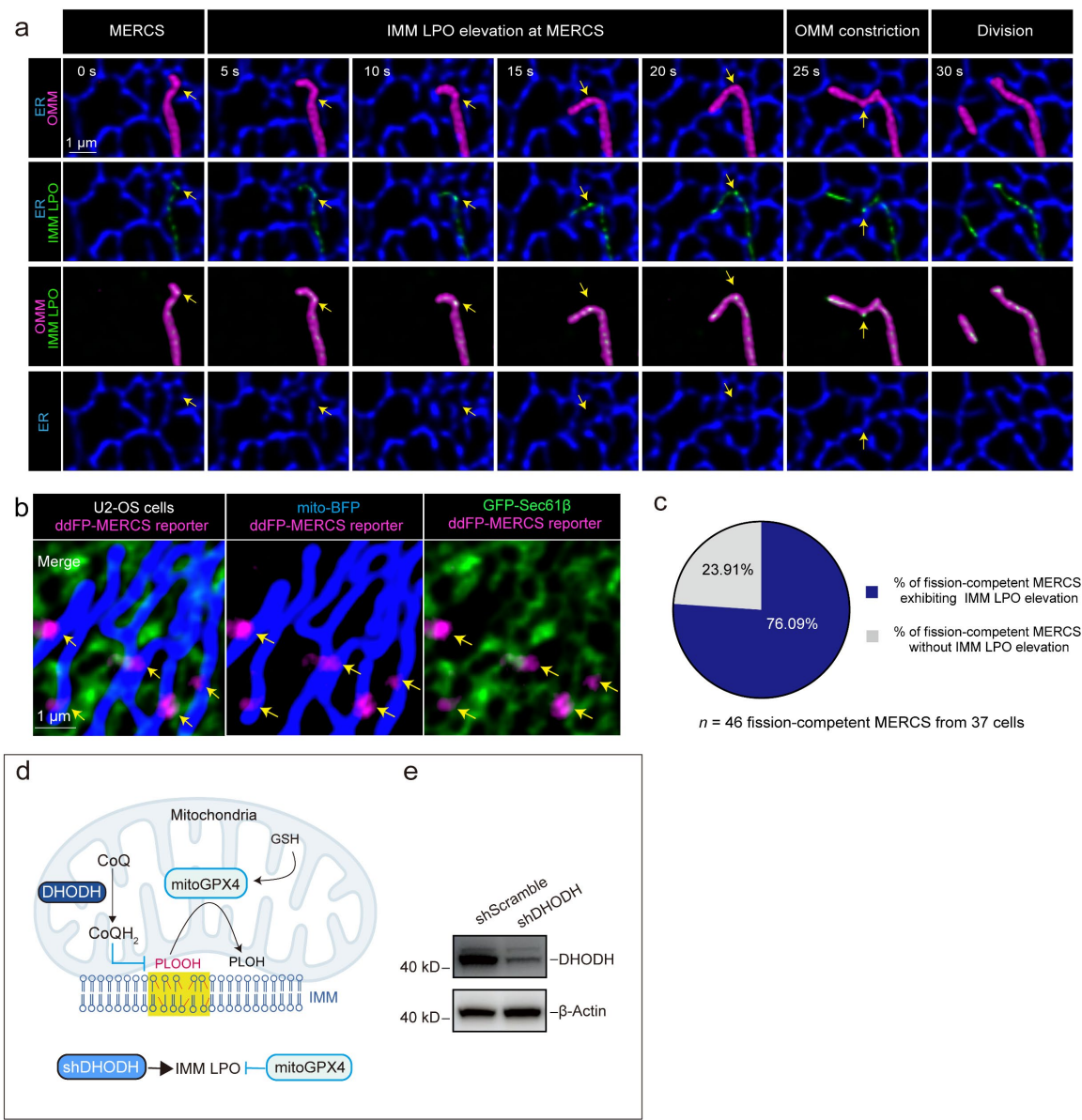
(f) Quantification of total mitochondrial area per cell in Tet-on mitoGPX4 U-2 OS cells treated with or without Dox. The absence of a significant difference indicates that mitoGPX4 expression does not alter total mitochondrial abundance. Each dot represents the total mitochondrial area from one individual cell.

(g) Validation of the mitochondrial localization of the ratiometric LPO probe MitoPerOx. Representative SIM images show MitoPerOx signals within mitochondria marked by TOMM20-Halo (OMM) and mito-BFP (mitochondrial matrix). Reduced MitoPerOx is shown in red and peroxidized MitoPerOx in green.

(h and i) MitoPerOx imaging confirms focal IMM LPO elevation preceding mitochondrial fission. (h) Representative time-lapse images showing focal accumulation of peroxidized MitoPerOx at a prospective mitochondrial fission site, followed by OMM constriction and division. White arrows indicate the prospective fission site. The complete process is shown in Supplementary Video 2. (i) Quantification of 32 fission events reveals that 23 fission events (71.88%) were preceded by local LPO enrichment.

Data information: Data in (b), (e) and (f) are presented as mean $\pm$ S.E.M. from at least three independent biological experiments. Statistical significance in (b) was assessed using two-tailed paired Student's *t*-tests. Statistical significance in (e) and (f) was assessed using two-tailed unpaired Student's *t*-tests.

Extended Data Fig.2



Extended Data Fig. 2 | MERCS formation and IMM LPO can precede one another, and the ddFP reporter confirms that IMM LPO occurs at MERCS.

(a) Time-lapse spinning-disk confocal imaging of a U-2 OS cell expressing TOMM20-Halo (OMM) and Calnexin-mScarlet (ER), and labeled with MitoPeDPP (IMM LPO probe). At 0 s, there is an MERCS at the prospective fission site (yellow arrow). From 5-20 s, a focal IMM LPO

signal progressively accumulates at this pre-existing MERCS. This is followed by OMM constriction at 25 s and mitochondrial division at 30 s. Images were acquired every 5 s.

(b) Validation of the localization of the ddFP-based MERCS reporter. Representative images of U-2 OS cells expressing the ddFP-MERCS reporter, mito-BFP as a mitochondrial matrix marker and GFP-Sec61 β as an ER marker show that reconstituted ddFP signals localize at contact sites between mitochondria and the ER. Yellow arrows indicate ddFP-positive MERCS.

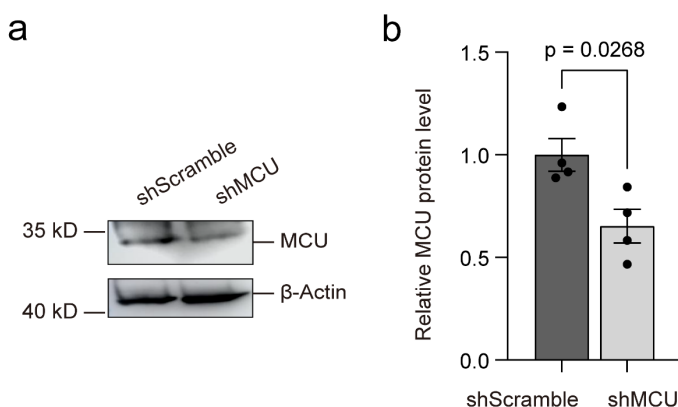
(c) Quantification showing that among 46 fission events occurring at ddFP-defined MERCS, 76.09% showed elevated IMM LPO prior to division.

(d) Schematic illustrating the bidirectional manipulation of IMM LPO. DHODH knockdown increases IMM LPO by impairing the DHODH-CoQH₂ mitochondrial lipid antioxidant pathway, whereas mitoGPX4 overexpression scavenges IMM phospholipid hydroperoxides through a glutathione-dependent mechanism.

(e) Immunoblot confirming efficient knockdown of DHODH protein by shRNA in U-2 OS cells. β -Actin served as a loading control.

Data information: Data are from at least three independent biological replicates. The schematic in (d) was created with BioRender.com.

Extended Data Fig.3

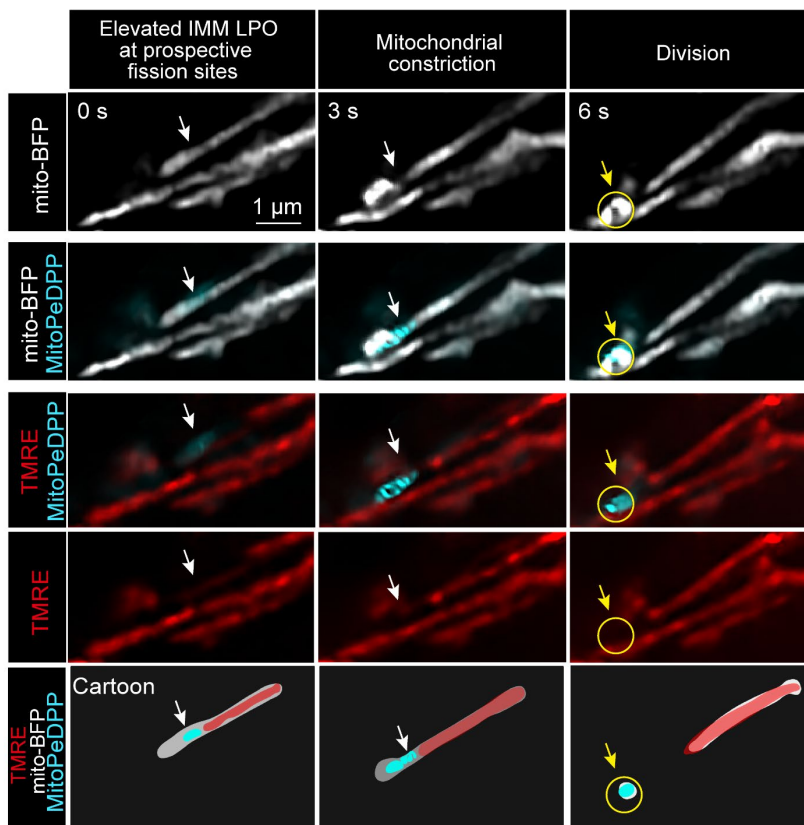


636

637 Extended Data Fig. 3 | Validation of MCU knockdown.

638 **(a and b)** Immunoblot analysis (a) and quantification (b) confirming reduction of MCU protein
639 levels in U-2 OS cells expressing shMCU compared to a scramble control (shScramble). β -Actin
640 served as a loading control. Each dot represents one biological replicate. Data are presented as
641 mean $\pm$ S.E.M from 4 independent biological replicates. Statistical significance was determined
642 using a paired two-tailed Student's t-test.

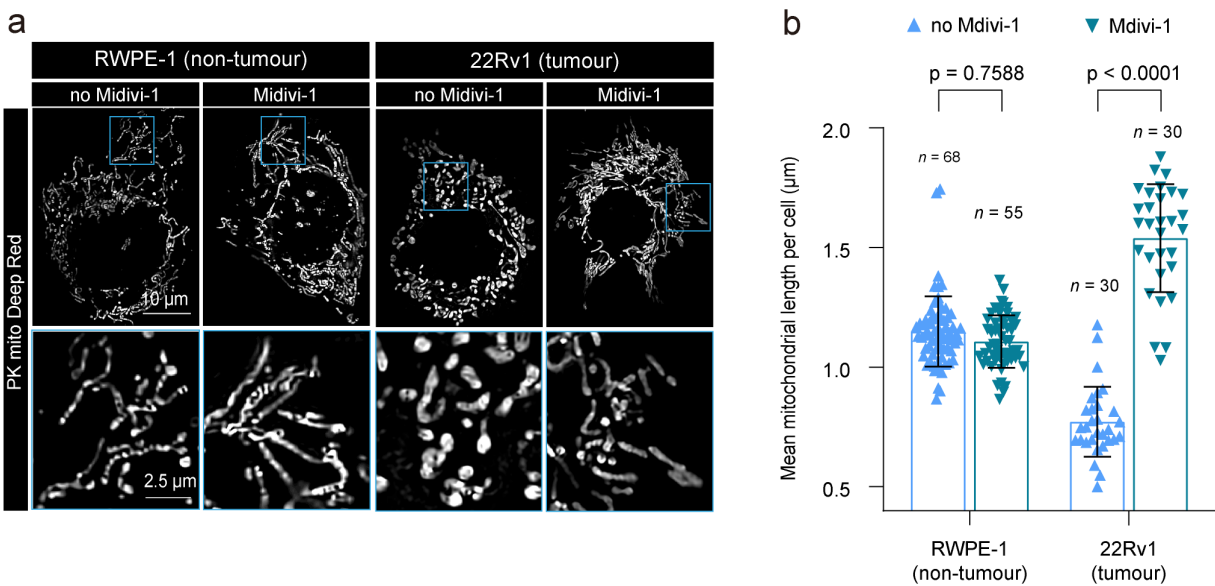
Extended Data Fig.4



Extended Data Fig. 4 | Visualization of the asymmetric inheritance of IMM LPO and membrane potential state following peripheral fission.

Representative time-lapse SIM imaging of a U-2 OS cell expressing mito-BFP (mitochondrial matrix) and labeled with TMRE (mitochondrial membrane-potential probe) and MitoPeDPP (IMM LPO probe). From 0-3s, the focal IMM LPO signal intensifies, followed by mitochondrial constriction at 3 s and division at 6 s. Following division, the shorter daughter mitochondrion (indicated by yellow arrow and circle) inherits the elevated MitoPeDPP signal and exhibits a concomitant loss of TMRE fluorescence, indicating depolarization. In contrast, the longer daughter maintains its TMRE signal. The white arrow indicates the area with increasing MitoPeDPP fluorescence. Images were acquired every 3 s. These images are representative of more than 10 similar events.

Extended Data Fig.5



Extended Data Fig. 5 | DRP1 inhibition phenocopies mitochondrial elongation induced by IMM LPO scavenging in prostate cancer cells.

(a and b) The DRP1 inhibitor Mdivi-1 elongates mitochondria in prostate cancer cells. Representative SIM images (a) and quantification (b) demonstrate that Mdivi-1 treatment significantly increases mitochondrial branch length in 22Rv1 prostate cancer cells, whereas it exerts no significant effect in control RWPE-1 cells. The extent of elongation resulting from DRP1 inhibition is comparable to that observed in cells overexpressing mitoGPX4 (Fig. 5i), indicating that the fragmented mitochondrial phenotype in 22Rv1 cells is driven by DRP1-dependent hyperfission. Mitochondria were labeled with PK Mito Deep Red. Each dot represents the mean mitochondrial length of an individual cell. Data in (b) are presented as mean $\pm$ S.E.M. from three independent biological experiments. Statistical significance was assessed using two-way ANOVA followed by Tukey's multiple-comparisons test.

Supplementary Video 1 | Focal IMM LPO elevation precedes mitochondrial constriction and division under homeostatic conditions (related to Fig. 1g).

Time-lapse SIM imaging of a U-2 OS cell labeled with mito-BFP (mitochondrial matrix) and the IMM LPO probe MitoPeDPP. The video shows the sequence of events leading to mitochondrial fission. A focal increase in MitoPeDPP fluorescence is observed at the prospective fission site (white arrow) from 3 s. This is followed by mitochondrial constriction from 6 s, culminating in mitochondrial division at 12 s. Images were acquired every 3 s.

Supplementary Video 2 | Focal surges in IMM LPO precedes mitochondrial division, visualized with MitoPerOx (related to Extended Data Fig. 1h).

Time-lapse spinning-disk confocal imaging of a U-2 OS cell expressing TOMM20-Halo (OMM) and labeled with the IMM LPO sensor MitoPerOx (green = peroxidized). Focal peroxidized MitoPerOx enrichment is detected at the prospective fission site at 0 s. This is followed by OMM constriction from 15-25 s and mitochondrial division at 30 s. White arrows indicate the prospective fission site. Images were acquired every 5 s.

Supplementary Video 3 | Focal photo-induction of IMM LPO is sufficient to trigger mitochondrial fission (related to Fig. 1j).

Time-lapse confocal imaging (Olympus FV3000) of a HeLa cell labeled with PK Mito Deep Red (IMM marker) and the ratiometric LPO sensor MitoPerOx (green = peroxidized). The yellow circle indicates the region targeted for focal 405-nm laser stimulation. Following baseline acquisition, stimulation occurs between 5.000 s and 5.671 s. A rapid, localized increase in peroxidized MitoPerOx fluorescence is detected immediately post-stimulation. This is followed by mitochondrial constriction from 10.671 s to 60.671 s, and complete fission at 75.671 s. White arrows track the stimulated site.

Supplementary Online Material

Supplementary Fig 1 | Gating strategy for MitoPeDPP-based flow cytometry analysis.

Representative flow cytometric plots illustrate the hierarchical gating and analytical logic used to measure MitoPeDPP fluorescence. Intact cell populations were first identified using FSC-A versus SSC-A to exclude debris, followed by single-cell isolation via FSC-A versus FSC-H gating. The MitoPeDPP signal was then quantified by measuring the mean fluorescence intensity in the FITC channel (FL1-A). Representative data are shown for U-2 OS Tet-on mitoGPX4 cells, either left unstained (Blank) or stained with MitoPeDPP, following culture in the absence or presence of Dox (1 μ M) for 48 h.

Supplementary Table 1: List of plasmids used in this study

Supplementary Table 2: List of shRNA plasmids used in this study

Supplementary Table 3: List of stable cell lines used in this study

Supplementary Table 4: List of antibodies used in this study

Methods

Cell Lines and Culture Conditions

HEK293T (ATCC CRL-3216), HeLa (ATCC CCL-2), U-2 OS (ATCC HTB-96), 22Rv1 (ATCC CRL-2505), and RWPE-1 (ATCC CRL-11609) cells were obtained from ATCC. HEK293T, HeLa, and U-2 OS cells were maintained in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, D6429) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, F0193) and 1% penicillin/streptomycin (Yeasen, 60162ES76). 22Rv1 cells were cultured in RPMI-1640 medium (Gibco, C11875500BT) with 10% FBS and 1% penicillin/streptomycin. RWPE-1 cells were cultured in Keratinocyte-SFM (1X) (Gibco, 17005042) supplemented with bovine pituitary extract (BPE, 25 mg), recombinant human epidermal growth factor (EGF, 2.5 µg), and 1% penicillin/streptomycin. All cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ and were routinely tested for mycoplasma contamination.

Chemicals and Treatments

The following reagents were utilized: Lipo8000 (Beyotime, C0533), HBSS (Solarbio, H1145), PBS (GENOM, GNM20012), A23187 (YEASEN, 50403ES03), DMSO (Sigma-Aldrich, D2650), 2-APB (MCE, HY-W009724), doxycycline (MCE, 564-25-0), and Mdivi-1 (Beyotime, SC8028). Unless otherwise stated, cells were treated with the following final concentrations and durations: A23187 (1 µM for 10 min), 2-APB (100 µM for 1 h prior to imaging), doxycycline (1 µM for 48 h), or Mdivi-1 (10 µM for 24 h).

Human Prostate Tissue Analysis

Paired prostate tumor and adjacent non-tumor tissues were obtained from needle-biopsy specimens of three patients with early-stage prostate cancer. The study was approved by the Institutional Review Board of Shanghai Ninth People's Hospital, and written informed consent was obtained from all participants. Tumor and adjacent non-tumor tissues were first confirmed by H&E staining and pathological evaluation and then corresponding samples were processed for TEM analysis. For TEM processing, tissues were immediately immersed in fixative (2% paraformaldehyde, 2.5% glutaraldehyde) at 4 °C. Specimens were then rinsed in buffer, post-fixed in 1% osmium tetroxide, and stained en bloc with 1% aqueous uranyl acetate. Tissues were dehydrated through a graded ethanol series (30-100%), transitioned through propylene oxide,

infiltrated with epoxy resin (Epon 812), and polymerized at 60 °C for 24-48 h. Ultrathin sections (70 nm) were then prepared for imaging.

Plasmid Construction

Plasmids pRSV-Rev (Addgene #12253), pMDLg (#12251), pVSVG (#12259), and the mito-R-GECO1 source plasmid (#46021) were obtained from Addgene. Templates for TOMM20-Halo, GB, RA, and Snap tags, and the Tet-on inducible expression system were generous gifts from Dr. Dong Li (Tsinghua University), Dr. Xin Zhang (Westlake University), and Dr. Peiguo Yang (Westlake University), respectively. Other cDNAs were obtained from the Westlake University Plasmid Library. Expression constructs were generated by PCR-amplifying respective cDNAs and subcloning into vectors (e.g., pBOBI) using the ClonExpress Ultra One Step Cloning Kit (Vazyme, C115). Standard restriction cloning with BamHI (NEB, R3104S) and EcoRI (NEB, R3142S) was also employed. Site-directed mutagenesis was performed using the QuikChange II Kit (Agilent, 200524). All newly generated plasmids were verified by Sanger sequencing. A complete plasmid list is provided in Supplementary Table 1.

For shRNA plasmid generation, target sequences were designed using the Thermo Fisher RNAi Designer tool. Annealed shRNA oligonucleotides were cloned into the BamHI and EcoRI sites of the pLKO.1-puro vector using T4 DNA ligase (NEB, M0202L). Positive clones were validated by Sanger sequencing. pLKO.1-shScramble-GFP (Tsingke Biotechnology) was used as a negative control. All shRNA sequences are listed in Supplementary Table 2.

Generation of stable transgenic and knockout cell lines

Stable cell lines were generated via lentiviral infection. Lentivirus was produced in HEK293T cells by co-transfecting a transfer plasmid with packaging plasmids (pMDLg, pRSV-Rev, pVSVG) using Lipo8000. Viral supernatants were collected at 24-48 h post-transfection, filtered (0.22 µm), and supplemented with polybrene (10 µg/mL; Sigma-Aldrich, TR-1003). Target cells were infected by spinfection and subsequently selected with puromycin (2 µg/mL for 5 days) and/or fluorescence-activated cell sorting (FACS). Details of all stable cell lines are provided in Supplementary Table 3.

Western Blot Analysis

Cells were washed with PBS and lysed in RIPA buffer (Beyotime, P0013K) containing protease inhibitors (Servicebio, G2006). Lysates were sonicated, cleared by centrifugation ($12,000 \times g$ for 12 min at 4 °C), and protein concentration was quantified using a BCA assay (Beyotime, P0010). Equal amounts of protein (20-40 µg) were resolved by SDS-PAGE and transferred to PVDF membranes (Merck Millipore, ISEQ00010). Membranes were blocked with 5% non-fat skimmed milk or BSA, incubated with primary antibodies (listed in Supplementary Table 4), and subsequently with HRP-conjugated Goat Anti-Rabbit (Genscript, A00098) or Goat Anti-Mouse (Genscript, A00160) secondary antibodies. Protein bands were visualized using a Li-Cor Odyssey system or a Bio-Rad chemiluminescence apparatus. Each blot was repeated at least three times.

Flow Cytometry and Cell Sorting

Cells were trypsinized, neutralized, centrifuged, and resuspended in FACS buffer (PBS containing 2% FBS). Single-cell suspensions were filtered through a 40-µm mesh prior to analysis. Data were acquired on a CytoFLEX LX flow cytometer (Beckman Coulter), with a minimum of 1×10^4 target events analyzed per sample. Data analysis was performed using FlowJo (v10.8). For sorting, cells were processed similarly and sorted on an MA900 Multi-Application Cell Sorter (Sony) into collection tubes containing complete culture medium. The gating strategy used for MitoPeDPP-based flow cytometric analysis is shown in Supplementary Fig. 1.

Live-Cell Staining and Labeling

All staining procedures were performed under light-protected conditions. (1) Mitochondrial State: For inner membrane labeling, cells were incubated with 250 nM PK Mito Deep Red (Genvivo; excitation/emission [ex/em] 644/670 nm) for 30 min. Membrane potential was assessed by incubating cells with 500 nM TMRE (Thermo Fisher, T669; ex/em 549/575 nm) for 30 min. (2) Lipid Peroxidation: To detect lipid peroxidation (LPO) in the inner mitochondrial membrane (IMM), cells were incubated with either 0.5 µM MitoPeDPP (Dojindo, M466; ex/em 452/470 nm) for 30 min or 200 nM MitoPerOx (Aladdin, M275561) for 1 h. MitoPeDPP is a triphenylphosphonium (TPP)-conjugated dye that exhibits intense fluorescence upon oxidation by lipid peroxides within the IMM. (3) HaloTag and SnapTag Labeling³⁹: HaloTag fusions were labeled with 0.2 µM HaloTagTM TMR or 0.5 µM HaloTagTM SiR ligands (kindly provided by Dr.

Zhixing Chen, Peking University). SNAP-tag fusions were labeled with 0.3 μ M SNAP-Cell® 647-SiR (NEB, S9102S). Following labeling, cells were rinsed three times and subjected to a 30-min washout period.

Microscopy and Image Acquisition

All live-cell imaging was performed in pre-warmed Leibovitz medium at 37°C with 5% CO₂.
(1) Spinning Disk Confocal: Live-cell imaging was primarily conducted on a Nikon Ti2-E microscope equipped with a Yokogawa CSU-W1 spinning disk system, a perfect focus system (PFS) for focus locking, and a high-sensitivity sCMOS camera. Time-lapse imaging was typically performed at intervals of 3-10 s. **(2) Multimodality Structured Illumination Microscopy (Multi-SIM):** Super-resolution imaging was performed on a NanoInsights SIM X system with a Zeiss Observer 7, Kinetix sCMOS camera, and 405/488/561/640 nm lasers. Multi-color time-lapse images were captured every 3-5 s. **(3) Olympus FV3000 confocal microscope:** Targeted live-cell laser-stimulation and subsequent imaging were conducted on an inverted Olympus FV3000 confocal microscope. Predefined regions of interest (ROIs) were irradiated using specific 405 nm laser lines, immediately followed by time-lapse scanning. **(4) Calcium Analysis:** Mitochondrial Ca²⁺ was measured as the mean fluorescence intensity of mito-R-GECO1 in ImageJ.

Image and Data Analysis

Mitochondrial fission rate and site: 3D mitochondrial mean branch length was quantified in Fiji using the MiNA plugin⁴⁰. Fission rates were quantified from time-lapse 3D datasets using Imaris 10.1.0 by counting the total number of fission events over a 5-min period, normalized to imaging time and mitochondrial network area, and expressed as events min⁻¹ μ m⁻². Peripheral fission was defined as yielding a daughter $\leq$ 20% the length of the parent and all other events were classified as midzone fission¹².

LPO Quantification: For LPO quantification at fission sites, the mean MitoPeDPP fluorescence intensity at the site immediately preceding fission (F_a) was normalized to the reference fluorescence of the corresponding mother mitochondrion (F_{ref}), yielding F_a/F_{ref} . Daughter mitochondrial IMM LPO intensity was quantified as the mean MitoPeDPP fluorescence in the resulting daughter mitochondrion (F_b) normalized to the same reference fluorescence (F_{ref}), yielding F_b/F_{ref} . Events with $F_a/F_{ref} \geq 2$ were classified as exhibiting severe local IMM LPO

enrichment, whereas events with F_a/F_{ref} between 1 and 2 were classified as exhibiting modest IMM LPO enrichment.

TEM Morphometry of Human Samples: In TEM micrographs of human prostate tissue, epithelial cells were identified based on ultrastructural criteria. For each mitochondrion in cancerous or adjacent normal cells, the major and minor axes were measured in Velox software. Mitochondrial morphology was categorized according to aspect ratio as round (ratio ≤ 1.5), intermediate ($1.5 < \text{ratio} < 3.0$), or elongated (ratio ≥ 3.0).

Statistical Analysis

All data derive from at least three biological replicates. Statistical analyses were performed using GraphPad Prism 10. Data are presented as mean $\pm$ S.E.M.. For comparisons between two groups, unpaired or paired two-tailed Student's t-tests were used as appropriate. Matched daughter mitochondria from the same fission event were analyzed using paired t-tests or Wilcoxon matched-pairs signed-rank tests. One-way or two-way ANOVA with appropriate multiplicity correction was used for multigroup experiments. 3D images were analyzed with Imaris 10.1.0, 2D images with Fiji, and flow data with FlowJo.

Data Availability

The data are available within the paper and its Supplementary Information files. Raw data and further materials are available from the corresponding authors upon request.

Ethics Statement

Human prostate biopsy specimens were obtained with approval from the Institutional Review Board of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. All procedures involving human participants were conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from all participants.

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