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ER-MITOCHONDRIA CONTACTS ARE PRIME HOTSPOTS OF PHOSPHOLIPID PEROXIDATION DRIVING FERROPTOSIS

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Abstract

Ferroptosis is a form of regulated cell death characterized by peroxidation of phospholipids (PLs). The following thesis critically reviews a recent article, which investigates how this process starts at endoplasmic reticulum-mitochondria contact sites (EMCSs). In this study, super-resolution live-cell imaging has been used to reconstruct the spatiotemporal dynamics of ferroptosis at the interorganelle level. The authors demonstrated that EMCSs expand rapidly upon initial PL peroxidation and, afterward, oxidative stress propagates to mitochondria, leading to mitochondrial ROS production and fission. Oxidative lipidomic analysis further revealed that EMCSs are enriched in proferroptotic polyunsaturated phospholipids. Functional experiments, performed in murine embryonic fibroblasts and human triple-negative breast cancer cells, showed that disrupting ER-mitochondria tethering reduced PL peroxidation and ferroptosis, whereas stabilizing these contact sites enhanced both processes, suggesting a therapeutic strategy for inducing ferroptosis in cancer cells. In this thesis, the experimental approach, the main findings and the wider implications of this research are evaluated.

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1 Introduction

1.1 Organelle biology: from isolation to interaction

For decades, biologists have studied the characteristics of the different organelles within eukaryotic cells. These compartments were traditionally analyzed as isolated units, each delimited by intracellular membranes and characterized by distinct biochemical functions. However, in recent years, this reductive view has shifted; increasing attention is being given to the physical and functional interactions between these compartments and their contribution to the maintenance of cellular homeostasis. This type of communication occurs in «areas of close apposition between the membranes of two organelles»¹, known as contact sites. Several proteins are localized at these sites and can be classified into one or more of the following four categories: structural, functional, regulator and sorter/recruiter proteins¹.

1.2 ER-mitochondria contact sites (EMCSs)

Among these contact sites, the ones between the endoplasmic reticulum and mitochondria have emerged as central hubs involved in a great plethora of key intracellular activities and signaling pathways.

EMCSs play an important role in calcium transfer between the two compartments. The core channels involved in this exchange are the inositol trisphosphate receptors (IP3Rs) on the ER, which interact with the mitochondrial voltage-dependent anion channel 1 (VDAC1) in the outer mitochondrial membrane, while the chaperone glucose-regulated protein 75 (Grp75) acts as a physical linker between them².

EMCSs are also crucial for lipid homeostasis. Lipid transport between organelles was long thought to occur mainly through vesicular trafficking. However, mitochondria are excluded from the classical vesicular pathway, making it unclear how they could receive lipids synthesized in other compartments. This enigma was solved when lipid synthesizing enzymes have been found in mitochondria-associated membranes (MAMs) in 1990. From this point forward, EMCSs were identified as the sites of phospholipid synthesis and non-vesicular lipid transfer between the ER and mitochondria. The PLs involved in this transfer are phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylcholine (PC). For instance, the ER synthesizes PS, which is transferred to the mitochondria, where it is rapidly converted into phosphatidylethanolamine (PE). Subsequently, part of the PE is transported back to the ER where it can turn into PC.

These regions are also implicated in other signaling processes, including apoptosis, mitochondrial dynamics and metabolism. Although they are essential in these physiological conditions, their activities may also lead to pathological processes under certain circumstances, particularly those involving altered lipid metabolism and oxidative stress.

1.3 Phospholipid peroxidation

PL peroxidation (PLOx) consists in an aberrant flow of ions across a cellular membrane from one PL to another. Specific PLs containing polyunsaturated fatty acids (PUFAs) are involved in this process, in fact these fatty acids contain C–H bonds with low dissociation energies. Therefore, they are subjected to oxidation by reactive oxygen species (ROS), initiating a chain reaction. A hydroxyl radical $\cdot\text{OH}$ abstracts a hydrogen atom from a PUFA-PL, generating a carbon-centered lipid radical $\text{L}\cdot$ that reacts with molecular oxygen to form a peroxy radical $\text{LOO}\cdot$. Further carbon- or oxygen-centered radicals are formed in subsequent steps, where each one stabilizes itself by abstracting H atoms from neighbored lipids. Each reaction generates a hydroperoxide LOOH and another lipid radical, allowing the process to continue. This constitutes the propagation phase of PL peroxidation, which represents a key mechanism underlying ferroptosis³.

1.4 Ferroptosis: mechanisms, inducers and inhibitors

Ferroptosis is a form of iron-dependent cell death characterized by excessive PL peroxidation. The peroxidation may be driven by free intracellular iron facilitating the Fenton reaction, in which Fe^{2+} is oxidized creating a hydroxyl radical $\text{OH}\cdot$ that starts the chain reaction³. In addition, lipid hydroperoxides may be synthesized by iron-dependent enzymes, such as arachidonate lipoxygenases⁴. Lipid hydroperoxides can further react with iron to generate highly reactive lipid radicals, which additionally fuel oxidation of PUFA-PLs. This continued oxidation will lead to thinning and enhanced curvature of the cell membrane, consequently causing micellization, pore formation and membrane disruption.

Ferroptosis is normally inhibited by the continuous activity of cellular lipid hydroperoxide detoxification systems that prevent their accumulation in membranes to toxic levels. One of these systems is glutathione peroxidase 4 (GPX4), a selenium-dependent enzyme capable of reducing potentially toxic PL-hydroperoxide (PL-OOH) to the respective stable alcohol (PL-OH) that is less dangerous. Cysteine and selenium are two metabolites essential for GPX4 to perform its function. Cysteine (Cys) is required to synthesize glutathione (GSH) and can be obtained from the hydrolysis of cystine, which is imported by the cell surface system x_c^- cystine-glutamate antiporter. Endocytosis of extracellular cystine-rich proteins constitutes an additional route by which cysteine becomes available for GSH synthesis.

Mitochondria and ER have both been linked to the regulation of ferroptosis individually. For example, the radical-trapping antioxidant ferrostatin-1 (Fer-1) targeted to mitochondria is a potent inhibitor of ferroptosis fostered by cysteine starvation or inhibitors of the system x_c^- ⁵, whilst ER-targeted Fer-1 protects from ferroptosis triggered by RAS-selective lethal RSL3, a small molecule inhibitor of GPX4⁶. RSL3 is classified as a class II ferroptosis inducer (FIN).

Similarly, mitochondrial DHODH and coenzyme Q₁₀ have been identified as intrinsic suppressors of ferroptosis, specifically under conditions of cysteine starvation and GSH depletion⁴. This framework can be achieved with class I FINs, such as erastin and its more potent derivative imidazole ketone erastin (IKE), which inhibit the x_c⁻ system. It was also demonstrated that inhibiting the mitochondrial electron transport chain blunts this type of ferroptosis⁴.

Ferroptosis is involved in different processes, both physiological and pathological. Physiological processes include tumor suppression, immunity, development and aging. In addition to the physiological functions described above, ferroptosis has been implicated in many pathological contexts: neurodegeneration, tumorigenesis, infectious and autoimmune diseases⁴.

1.5 Role of ER-mitochondria contacts in ferroptosis

Given the established role of EMCSs in lipid and calcium transport, their contribution to ferroptosis has been presumed. Zhang et al. have discovered a small molecule that inhibits ferroptosis by binding to the sigma-1 receptor (σ -1R). This receptor is a chaperone primarily localized in MAMs, where it modulates Ca²⁺ signaling. It interacts with IP3R3 to support an abundant Ca²⁺ flow crucial for ferroptosis. CGI1746, the small molecule, interrupts the communication between ER and mitochondria by preventing the σ -1R from linking with IP3R3. This lack of interaction results in a decreased calcium accumulation in the mitochondria; hence, there is reduced ROS production and PL peroxidation. Furthermore, it alters the normal lipid transport coordinated by EMCSs, prompting the cell to increase PUFA-containing triacylglycerol (TAG) in lipid droplets. In this manner, lipid droplets protect the cell from ferroptosis by storing PUFAs away from membrane PLs².

In this study, they additionally demonstrated that chemical and genetic inhibition of different EMCSs components blocks ferroptosis and suggested that the remodeling of these sites may be used as a clinical strategy to inhibit ferroptosis in pathological contexts².

While these studies implicate a role of EMCSs in the regulation of ferroptosis, the precise spatiotemporal dynamics of these contacts under conditions causing PLox remain unclear. Having a better understanding of where these events occur at the subcellular level is essential to deepen our knowledge in the initial stages of ferroptosis and to delineate sites of intervention to regulate its lethal progression.

2 Experimental approach

In this study, to verify the hypothesis that EMCSs are the primary hubs of early PLox, Sassano et al. combined three pivotal experimental procedures: high-resolution live cell imaging, oxidative phospholipidomics of subcellular fractions and EMCS genetic perturbation.

2.1 Cell models and transfection systems

2.1.1 Cell lines

The authors have made use of the following cell lines:

- *SV40-immortalized MEFs*: Mouse Embryonic Fibroblasts (MEFs) are primary fibroblasts purified from mouse embryos. Primary MEFs are limited cell lines, thus they cannot grow indefinitely. Nevertheless, different strategies are available to immortalize MEF so that culture maintenance time is reduced and near limitless amounts of protein lysate can be produced. Researchers have used MEFs already immortalized via Simian Virus-40 (SV40) transduction, which involves the introduction of the proto-oncogene SV40 large T-antigen (SV40 Ta) using a recombinant lentiviral vector. SV40 Ta transforms cell lines by inactivating the retinoblastoma and p53 tumor suppressor proteins. Thus, cells enter S phase and continuously replicate their DNA⁷.

In this study, SV40-immortalized MEFs proficient or deficient for PERK (PERK^{WT} and PERK^{KO}, respectively) were used, along with MEFs expressing either the wild-type eIF2 α (S/S) or its non-phosphorylatable S51A mutant (eIF2 α S/A).

- *Triple-negative breast cancer (TNBC) cell lines* (MDA-MB-157, HCC1937, HCC1143, Hs578T): TNBC cells are characterized by the absence of estrogen and progesterone receptors and low levels of human epidermal growth factor receptor 2. It should be noted that this article adopted the Fudan University Shanghai Cancer Center (FUSCC) subtype classification. Yang et al. identified 4 distinct subtypes: mesenchymal-like, basal-like and immune-suppressed, luminal androgen receptor (LAR) and immunomodulatory. MDA-MB-157 is classified as LAR subtype, the others are non-LAR subtypes.

All the cell lines were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose and 0.11 g/l sodium pyruvate, supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% fetal bovine serum (FBS) (all Sigma Aldrich).

2.1.2 Ferroptosis induction

Ferroptosis was induced by FINs: RSL3 (Selleckchem) and IKE (Selleckchem), whose mechanisms had been previously described (see *Section 1.4*). Cell death was prevented by the iron chelator deferoxamine mesylate salt (DFO₂ (Sigma Aldrich)), the radical-trapping antioxidant Fer-1 (Sigma Aldrich) and the reducing agent beta-mercaptoethanol (BME). BME was used only when IKE was involved, in fact it makes cysteine available to the cell without needing the system x_c^- . To confirm that the cell death observed was ferroptosis, Bodipy 581/591 C11, a probe for PL peroxidation, was used, while selective inhibitors for other types of regulated cell death served as negative controls. Inhibitor of caspases (ZVAD, Bachem) and necrostatin-1s (Sigma Aldrich) (blocker of necroptosis) failed to rescue cells from death.

2.1.3 Cell transfection and CRISPR-Cas9 gene knockout

Two different transfection methods were used:

- *TransIT-X2* (Mirus Bio): it is a low toxicity and dynamic transfection system engineered to enable high efficiency transfection of many cell types, including primary cells. This chemical reagent creates complexes with multiple types of macromolecules including plasmid DNA and siRNA to allow the transfection. The system utilizes an advanced, non-liposomal polymeric technology that avoids the toxicity often associated with liposomal reagents. It does not require media replacement and can be carried out in serum-containing medium.
- *Electroporation with 4D-nucleofector* (Lonza Bioscience): it delivers DNA, RNA, ribonucleoprotein complexes (RNPs) and proteins directly into the cell's nucleus thanks to optimized electrical parameters and cell type-specific solutions. Electric impulse creates transient pore in the membranes to allow the transfection. New units are optimized for CRISPR-based genome engineering. Genes were deleted by nucleofecting RNPs into MDA-MB-157 cells. RNPs consisted of sgRNA pools conjugated with Cas-9 nuclease (Synthego).

The cells were either replated after 24h for imaging or collected for lysate after 48h.

2.2 Microscopy and imaging techniques

2.2.1 Live cell imaging

To study the dynamics of the EMCSs, the researchers acquired time-lapse 3D live cell images, that are images stacks along the z axis (z-stacks) to recreate the cells volume at different time stamps. They utilized the high-resolution confocal Zeiss LSM 880–Airyscan, objective 63×, numerical aperture 1.4 oil, equipped with temperature 37 °C, CO₂ and a humidity-controlled incubator. Confocal microscopes

use a focused laser beam to scan samples point by point and a pinhole to reduce out-of-focus light.

To visualize mitochondria, the cells were incubated with MitoTracker green or Far Red (Thermo Fisher Scientific). They are fluorescent dyes that passively diffuse across the plasma membrane and accumulate in the mitochondria of live cells, since they are positively charged.

To visualize ER, the cells were transfected with BFP-KDEL (Addgene plasmid #49150), that is a plasmid encoding for the blue fluorescent protein fused to the ER retention signal. At times, the cells were transfected with Sec61 β -mCherry (Addgene plasmid #121160) to visualize the ER membrane in red.

To manipulate the EMCSs, the cells were transfected with the following synthetic constructs capable of artificially spacing the two membranes apart (~20 nm) or bringing them closer together (up to 5 nm).

- 9xL spacer (AKAP1 (34-63)-9x-mRFP-yUBC6).
- OMM-ER linker (AKAP1 (34-63)-mRFP-yUBC6).
- AKAP1-mRFP was used as control.

AKAP1 (A-Kinase Anchoring Protein 1) is a scaffold protein of the outer mitochondrial membrane (OMM). Here, the plasmids encode for a sequence of this protein that acts as an OMM targeting sequence.

yUBC6 is an ER membrane targeting sequence derived from the yeast ubiquitin conjugating enzyme E2 protein.

Monomeric red fluorescent protein (mRFP) is a tag that allows to visualize the location of the protein to which it is fused.

The authors first took images of the dish in untreated condition, later RSL3 or IKE were added directly to induce ferroptosis and MitoSOX (Thermo Fisher Scientific) staining, Bodipy 581/591 C11 (Thermo Fisher Scientific) or Liperfluo were added to visualize mitochondrial ROS or PL peroxidation respectively.

2.2.2 Electron microscopy

The Transmission Electron Microscope (TEM) provides nanometer-scale resolution (a few nm), which is far superior to that of confocal microscopy (around 200 nm). This enables the direct visualization of cellular ultrastructure, including individual membranes and the inter-organelle membrane contact sites, details that are impossible to resolve optically. In this study MEFs, PERK_{WT} and PERK_{KO}, expressing HRP-KDEL-myc were fixed on coverslips with glutaraldehyde and analyzed at different experimental conditions: untreated, pretreated with RSL3 for 15 min or RSL3 cotreated with Fer-1. HRP-KDEL-myc is an ER Lumen marker. The construct components are:

- Horseradish Peroxidase (HRP): it is a metalloenzyme that, in the presence of H₂O₂ and diaminobenzidine (DAB), catalyzes the oxidation of DAB, forming an electron-dense precipitate visible under TEM.
- ER retention signal KDEL.

- Myc: a small tag used to identify the protein with antibodies.

Thin sections were imaged on a Tecnai-20 electron microscope (Philips-FEI). ImageJ/Fiji Software made it possible to directly measure the reduction in the physical distance between the ER and mitochondria membranes and to calculate the ER-mitochondria contact coefficient. ERMICC was calculated as follows:

$$\frac{\text{interface length}}{\text{mitochondrial perimeter} \times \text{distance ER-mitochondria}}$$

2.2.3 Ca^{2+} imaging

MEFs were transiently transfected with mito-R-GECO1 and, subsequently, incubated with Cal520 at room temperature in modified Krebs solution. The recombinant genetically encoded calcium indicator (GECO) is expressed in the mitochondrial matrix and increases its red fluorescence when it binds Ca^{2+} . Instead, Cal520 is a fluorescent dye that measures cytosolic Ca^{2+} . A de-esterification step is crucial for removing the ester groups that allowed the dye to cross the membrane. Extracellular Ca^{2+} was eliminated with EGTA. The cytosolic Ca^{2+} release (Cal520, excitation 493, emission 515) and mitochondrial Ca^{2+} uptake (mito-R-GECO1, excitation 560, emission 584) were monitored via live cell imaging using a Zeiss Axio Observer Z1 inverted microscope. Baseline values were recorded for 60 s prior to the addition of RSL3 (for 15 min) and ATP, that promote the release of calcium stored in the ER.

2.2.4 FEMP imaging and high-throughput EMCS measurements

The MEFs, PERK_{WT} and PERK_{KO}, were infected with an adenovirus carrying the FEMP probe complementary DNA. The FRET-based ER–Mitochondria Probe (mAKAP1-YFP-Tav2-CFP-Sac1) is designed to measure the basal and maximal proximity between the two organelles. In fact, FEMP contains a FKBP-FRB dimerization domain that, following rapamycin treatment, allows maximal juxtaposition of the two fluorescence proteins: CFP, donor, and YFP, acceptor. A self-cleaving Tav2A peptide was inserted downstream of the YFP sequence. After translation, the peptide undergoes self-cleavage and releases YFP and CFP, which are targeted to the mitochondria and the ER, respectively, via the Akap1 and Sac1 targeting sequences⁸.

After 24 h, the cells were imaged using an Operetta CLS High Content imaging system (Revvity) with the following filter settings: CFP (excitation 410–430, emission 460–500), YFP (excitation 490–510, emission 520–560) and YFP FRET (CFP excitation, YFP emission). During FRET, energy is transferred directly from the donor to the nearby acceptor without the emission of light. FRET intensity is inversely proportional to the sixth power of the distance between the two fluorophores and occurs when they are at less than 15 nm⁸.

After measuring the baseline FRET intensity of the untreated samples, RSL3, RSL3 + Fer-1, RSL3 + DFO were added to the wells. The images were analyzed using

Harmony 3.5 image analysis software (Revvity). The YFP channel was chosen to mark the region of interest (ROI) and around each ROI, a second boundary was drawn to measure the background intensity. FRET Ratio was calculated as: FRET/CFP.

The TNBC cells were plated and transfected with the Sec16 β –EGFP plasmid using the cationic jetOPTIMUS transfection reagent (Polyplus). Sec16 defines ER exit sites, specialized regions of the ER from which COPII vesicles originate. Thus, this plasmid was used to mark ER in green. After 24 hours, the cells were incubated with MitoTracker Far Red, imaged using Operetta CLS and analyzed.

2.3 Immunochemical techniques

2.3.1 Western Blotting

Western blotting allows proteins to be separated based on molecular weight (via SDS-PAGE), transferred onto a membrane and detected using specific antibodies, thereby providing a semi-quantitative measure of protein abundance. In this study, the lysates were separated using a 4%–12% Bis–Tris gradient gel on the Criterion system (Bio-Rad Laboratories). It was transferred to Protran 2- μ m-pored nitrocellulose paper (PerkinElmer) and blocked for 1 h at room temperature in TBS-T buffer containing 5% non-fat dry milk. The blots were incubated with appropriate antibodies for chemical (HRP-based) detection (Cell Signaling Technologies) or Infrared detection (Thermo Fisher Scientific).

2.3.2 Proximity Ligation Assay

A Proximity Ligation Assay (PLA) was performed using Duolink in Situ Red Started Kit Mouse/Rabbit (Sigma Aldrich) to detect the proximity between two contact-site components on opposing membranes. Specifically mitochondrial protein VDAC1 and ER protein IP3R3 after treatment with RSL3 at the different timepoints.

Two primary antibodies raised in different species are used to bind the two protein targets. A pair of oligonucleotide-labeled secondary antibodies (the PLA probes) then binds to the primary antibodies. Connector oligonucleotides hybridize to the PLA probes only if they are in proximity; a ligase then forms a closed circular DNA template that undergoes repeated amplification cycles (rolling circle amplification). Finally, fluorescence oligonucleotides hybridize to complementary sequences within the amplicon and are visualized as distinct dots (PLA signals) with an Olympus IX73 microscope. In the images, the nuclei are blue (DAPI) and the PLA signals are red. Each dot coincides with an interaction between the two proteins⁹. The images were quantified manually with ImageJ/Fiji Software.

2.3.3 Immunostaining

The cells were permeabilized utilizing saponin in normal goat serum and the images were taken with the Zeiss LSM 880–Airyscan.

2.4 Lipidomic analysis by LC-MS/MS

2.4.1 Subcellular fractionation

The researchers intended to obtain two isolated samples: EMCS fraction and pure mitochondria in order to compare their lipid compositions. To achieve this goal, the MEFs were collected, pelleted and homogenized after treatment with RSL3 and Fer-1 at different timepoints. The cell homogenate was subjected to differential centrifugation at increasing speeds to isolate the crude mitochondria fraction which was then subjected to Percoll gradient ultracentrifugation (30% and 15%) to separate the EMCS and pure mitochondria fractions.

2.4.2 Lipidomics and redox lipidomics

Thereafter, the different lipid species, in each sample, were separated through liquid chromatography (LC). Lipids move at different speeds depending on their chemical composition and exit the column in succession. Afterwards, they enter the mass spectrometry, which determines their mass-to-charge ratio (m/z). In this way, it was possible to identify the PL species, its abundance and whether it is oxidized. A multivariate statistical analysis using OPLS-DA (orthogonal partial least squares-discriminant analysis) and VIP scores allowed for the identification of which lipid species most clearly distinguish the different experimental conditions.

Following transient transfection of MEFs with the AKAP1–mRFP control or the 9xL spacer, cell pellets were analyzed by mass spectrometry-based lipidomics using the Lipometrix platform.

2.5 Functional assays

2.5.1 Flow cytometry

After ferroptosis induction, the cells were collected at different timestamps and stained using MitoPerox or Bodipy 581/591 C11, probes for lipid peroxidation assay. Their oxidation causes a shift in the fluorescence emission peak from red to green. Bodipy C11 localizes to all membranes in live cells, while MitoPerox is mitochondria targeted. The samples were processed using BD FACS Canto IIBD Canto II or Attune flow cytometer. The cells flow one at a time in front of a laser beam and the instrument measures the fluorescence intensity. FlowJo software was utilized for the analysis.

2.5.2 Cell death detection

The cell death was evaluated by the fluorescent viability dye SYTOX Green, a dye that enters only in cells with damaged membranes, binds the DNA and radiates green fluorescence. The latter was quantified either by using the Flexstation plate reader at 488/523 nm or by counting the number of SYTOX-Green-positive cells normalized on the total amount of cells using an Olympus IX73 or Operetta CLS.

2.6 Gene set expression analysis

RNAseq and lipidomics data of patients from FUSCC TNBC cohort and The Cancer Genome Atlas were analyzed. Each tumor was classified into one of the subtypes described by Yang et al. and an EMCS score was assigned to each based on a single-sample Gene Set Enrichment Analysis (ssGSEA). Starting from a list of EMCS associated genes (full or manually curated), they computed the enrichment scores. A higher score reflects a higher expression level of the EMCS genes. The EMCS scores were compared across the different subtypes and determined whether the observed differences were statistically significant using Wilcoxon rank-sum test.

2.7 Image processing, analysis and statistics

2.7.1 Image processing

After acquiring 2D and 3D images with the confocal microscopes, the EMCS area or volume was quantified with the ImageJ/Fiji software. First of all, the software must understand which pixels/voxels belong to the ER and which to the mitochondria. To create the ER and mitochondria mask (binary image), the intensity threshold was determined using the IsoData algorithm. Once the mask is obtained, the software can count the pixels or voxels and calculate area or volume. The EMCS mask was created keeping only the overlapping pixels/voxels from the two images. Regarding the lipid peroxidation, the operations performed were:

$$\text{Bodipy C11} = \frac{\text{Intensity of oxidized Bodipy C11 (green)}}{\text{Total Bodipy C11 signal (red)}}$$

$$\text{Liperfluo} = \frac{\text{Integrated Density}}{\text{Total cell Volume}} = \frac{\text{Area} \times \text{Average intensity}}{\text{Total cell Volume}}$$

2.7.2 Mander's coefficient and fragmentation index

Colocalization analysis was performed using Mander's overlap coefficient. Two channels were compared: the Liperfluo (or Bodipy) mask and the EMCS one. The overlapping of these two signals is quantified by Mander's coefficient (*Fig. 1*). To reduce the background, Otsu's automatic thresholding method was utilized.

Imaris 10.1 software was used for 3D reconstructions and to measure mitochondrial morphology. A healthy mitochondrial network consists of a few large elements. The fragmentation index ranges from 0 to 1 and was calculated as the sum of small fragments volumes normalized on the total mitochondrial volume.

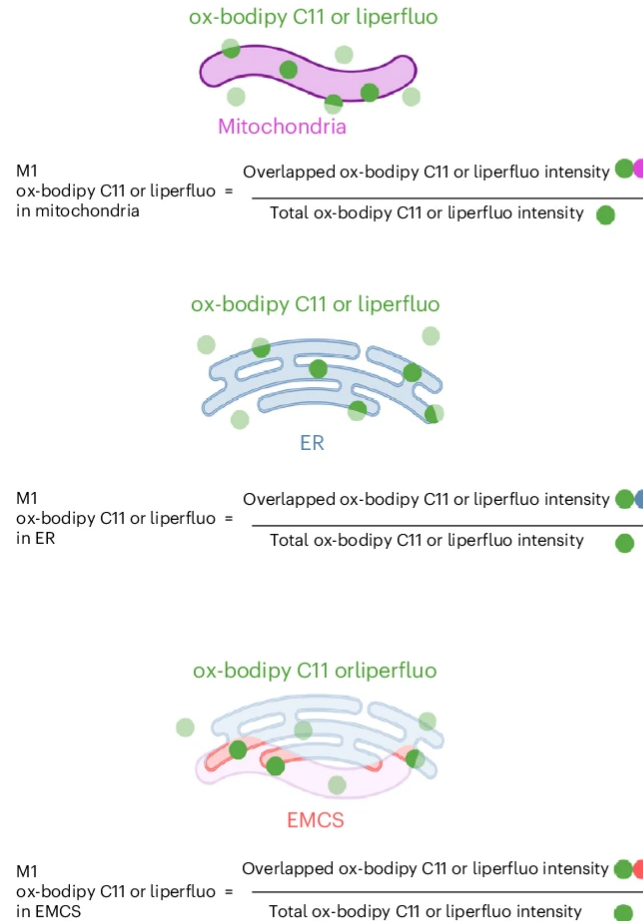


Fig. 1: A schematic representation of Mander's coefficient (M1). The M1 quantifies the fraction of the signal intensity of the lipid peroxidation probe that colocalizes in each subcellular compartment/ organelle relative to the total signal intensity of the probe

2.7.3 Statistics

All data are represented as the mean $\pm$ standard error of the mean (s.e.m.).

The statistical analysis was performed with Prism v9.0f, GraphPad. For two group comparisons, an unpaired two-tailed t-test with F-testing or a one-sample t-test was applied. The statistical significance between multiple groups was determined by a one-way analysis of variance (ANOVA) followed by Tukey's or Šídák's post hoc correction. Multiple pairwise t-tests would inflate the type I error rate (false +).

The data distribution was assumed to be normal, but this was not formally tested. The statistical significance was set at $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

3 Results

3.1 ER-mitochondria contacts rapidly expand during ferroptosis

The researchers conducted time-lapse live cell imaging and a 3D rendering of acquired z-stacks to monitor early EMCS remodeling in MEFs during ferroptosis (see *Sections 2.2.1 and 2.7.1*). These techniques revealed that the contact surface between mitochondria and the ER increases by 15% already after 15 minutes of RSL3 treatment (*Fig. 2*). A PLA analysis confirmed the rapid expansion of EMCSs and their persistence up to 1 hour following RSL3 exposure. However, as revealed by TEM observations, not only does the contact surface area increase, but also the distance separating the two organelles decreases, going from an average of 20 nm to 9 nm. The ERMICC coefficient (see *Section 2.2.2*) was calculated and compared with the untreated condition. It increased by more than four times in 15 minutes.

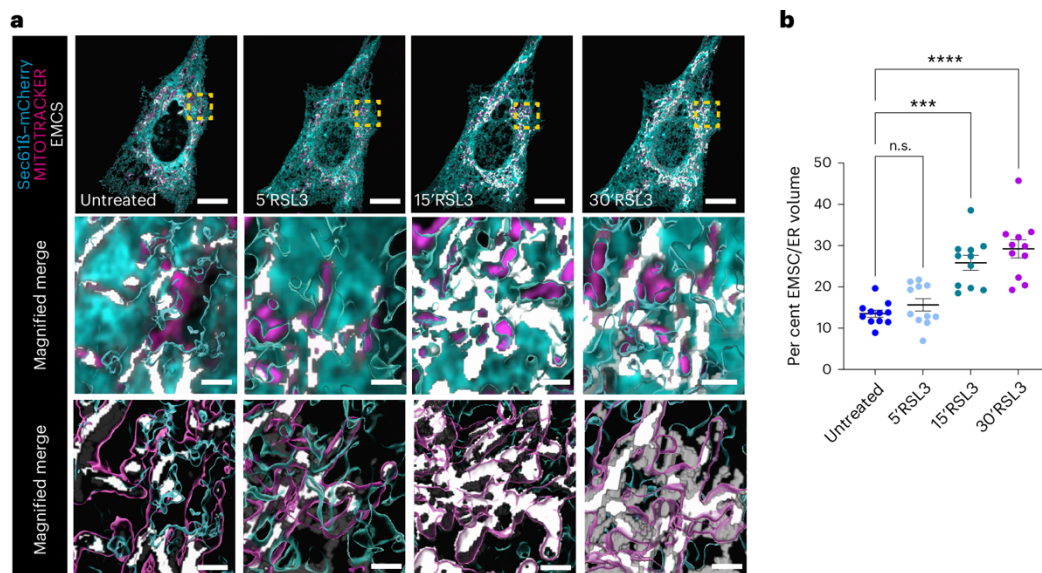


Fig. 2: a, Volume-rendered 3D reconstructions of confocal z-stacks from time-lapse imaging of MEFs expressing the ER marker Sec61B-mCherry (cyan) and stained with MitoTracker Far Red (magenta), at 0, 5, 15 and 30 min after RSL3 (0.5 μ M). The EMCS masked volume, representing colocalization of ER and mitochondria, is indicated in white. Scale bar, 10 μ m; zoom, 1 μ m. **b**, The per cent of EMCSs normalized on ER volume at the same timepoints ($n = 3$ biological replicates; same 11 cells analyzed per timepoint).

Mitochondrial and cytosolic Ca^{2+} levels have been monitored (see *Section 2.2.3*). RSL3 bolstered mitochondrial Ca^{2+} uptake, without altering the overall cytosolic Ca^{2+} levels. They, therefore, wondered whether the abundant Ca^{2+} flow, during ferroptosis, is the cause of EMCS structural reorganization or purely a consequence. If Ca^{2+} was the signal inducing EMCS expansion, then preventing Ca^{2+} increases using BAPTA-AM should preclude it. Instead, EMCSs have expanded nonetheless; hence, elevation of Ca^{2+} is not the main trigger of EMCS remodeling in early ferroptosis. Fer-1, the small molecule that blocks PLox propagation, hinders EMCS expansion (*Fig. 3*). As a result, ER-mitochondria tethering is fostered by PLox.

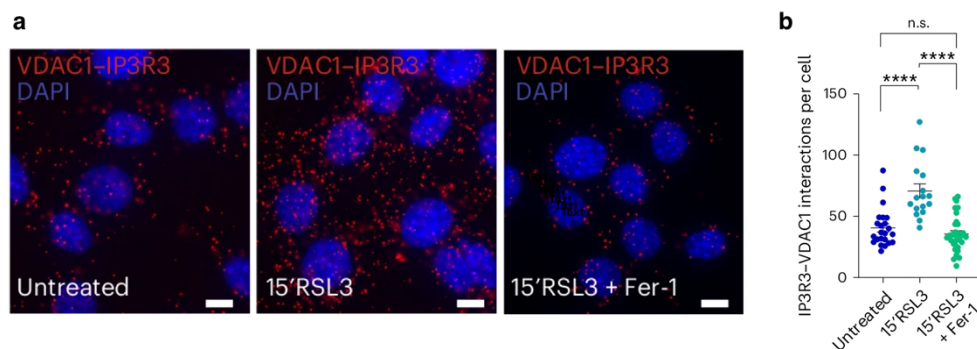


Fig. 3: Representative *in situ* PLA images (a) and quantification (b) of IP3R3–VDAC1 interaction dots per nucleus in MEFs, untreated or treated for 15 min with RSL3 (0.125 μ M) $\pm$ Fer-1 (1 μ M) ($n = 3$ biological replicates; images: $n = 23$ untreated, $n = 17$ RSL3, $n = 32$ RSL3+Fer-1). Scale bar, 10 μ m.

3.2 PL peroxidation starts at EMCSs and propagates to the mitochondria

To investigate the interorganelle transfer of lipid-ROS the researchers followed the spatial pattern of oxidized Bodipy C11 through live cell imaging and calculated its distribution in the ER, mitochondria and EMCSs (see *Section 2.7.2*). During RSL3 treatment, the ox-Bodipy C11 signal did not change in the ER, instead a significantly larger fraction of it overlapped with EMCSs starting from 5 minutes after RSL3 treatment. By 15-30 min, PLox increased in the mitochondria too, while it showed a decline elsewhere in the cell. They performed 3D rendering of live imaging also employing the LiperFluo probe (*Fig. 4*), which emits green fluorescence upon LOOH reduction, and IKE treatment, obtaining the same results.

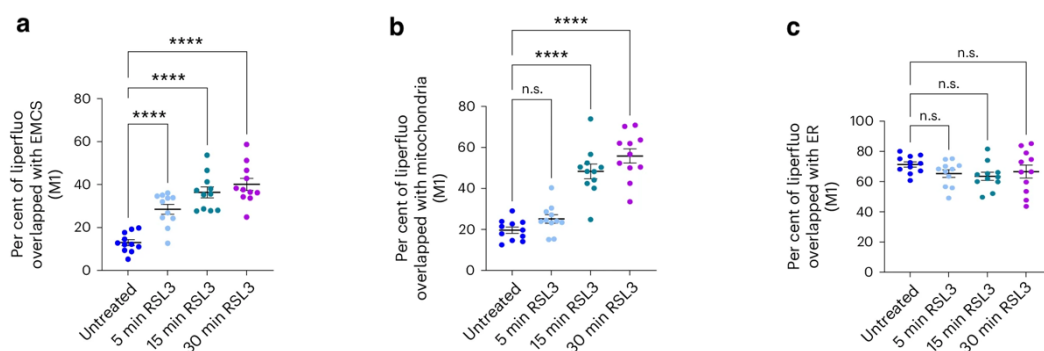


Fig. 4: The colocalization (M1%) of Liperfluo in the EMCSs (a), mitochondria (b) and ER (c) masks at time 0, 5, 15 and 30 min after RSL3 (0.5 μ M) ($n = 3$ biological replicate, the same 11 cells were acquired and analyzed at each timepoint per condition).

After conducting LC-MS/MS-based lipidomics and redox lipidomics (see *Section 2.4*), the fractional distribution of total PE, PC and mitochondrial cardiolipin (CL), along with all their oxidized forms, was computed in the input, EMCS and mitochondrial fraction. In non-oxidizing conditions (Fer-1), EMCSs were enriched in PE and PC species compared with the input and the mitochondrial fraction. Consistent with the imaging data, after 30 min of RSL3, both PEOx and PCox increased in the mitochondrial fraction and in EMCSs, suggesting a relocation of oxidized PLs between these two compartments. In contrast, CL oxidation remained

confined to mitochondria. Mitochondrial PLox accumulation at 30 min was accompanied by network fragmentation (see *Section 2.7.2*) and superoxide production (visualized using MitoSOX), at a rate that depends on cell type. Overall, these results show that, during ferroptosis, PLox originates at EMCSs and subsequently spreads to mitochondria, driving their morphological and redox changes.

3.3 Spacing EMCSs protects mitochondria from lipid-ROS attack

To test whether preventing ER–mitochondria tethering affects ferroptosis, Sassano et al. used the FEMP probe (see *Section 2.2.4*) to measure ER–mitochondria proximity in PERK^{WT} and PERK^{KO} cells. PERK is an unfolded protein response (UPR) sensor, with a secondary scaffold function at the EMCSs independent of its kinase activity. PERK loss impairs EMCS formation. PERK^{KO} cells showed both lower basal and maximal FEMP signal, indicating their inability to expand EMCSs after RSL3. In PERK^{WT} cells, Fer-1 reduced EMCS expansion to a level comparable to that of PERK^{KO} cells (*Fig. 5a*), supporting the previous findings stating that PLox prompts ER-mitochondria tethering.

Live imaging was conducted in MEFs expressing the 9xL mitochondria-ER spacer (see *Section 2.2.1*) and exposed to RSL3 for 30 min. The spacer blunted the overall LiperFluo signal, the primary PLox accumulation at EMCSs (*Fig. 5b*), EMCS expansion and the secondary PLox accumulation in mitochondria, phenocopying PERK^{KO}.

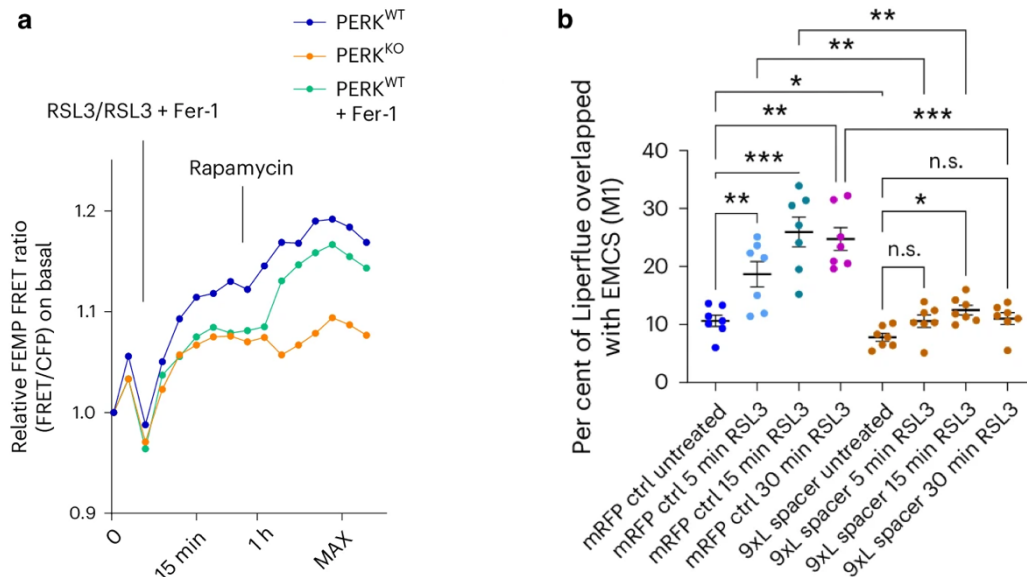


Fig. 5: **a**, Time-lapse traces of FEMP ratio in PERK^{WT} and PERK^{KO} MEFs following RSL3 treatment, with or without Fer-1. Where indicated, rapamycin was added to induce maximal FRET. **b**, Colocalization (Mander's coefficient M1 %) of LiperFluo in the EMCS masks at 0, 5, 15 and 30 min after RSL3 (0.5 μ M) in MEFs expressing AKAP1-mRFP (control) or the 9xL spacer ($n = 3$ biological replicates; same 7 cells per condition analyzed at each timepoint).

Neither PERK deficiency nor 9xL expression altered the global lipidome or mitochondrial network in untreated cells, but both blunted mitochondrial fragmentation during ferroptosis. PERK deficiency also abolished downstream mitochondrial lipid-ROS (3 h) and overall cellular PLox (6 h) production. Together, these results indicate that restricting EMCS dynamics, genetically or synthetically, prevents the initial formation of PLox at these sites, its spread to mitochondria, mitochondrial network remodeling and further lipid-ROS production.

3.4 Impaired EMCS expansion blunts oxidation of proferroptotic PUFA-PLs

Oxidized PUFA-containing PE and PC species (mono- and di-PUFA) are known to promote ferroptosis. Following the early increase, EMCSs remained stable during the first 6 hours of RSL3 treatment, coinciding with peak PLox formation. Therefore, the researchers wondered whether preventing EMCS expansion affects PLox abundance and composition at these sites. LC-MS/MS-based lipidomics, redox lipidomics and subsequent OPLS-DA (see *Section 2.4*) were performed on whole cells (input) and EMCS fractions from PERK^{WT} and PERK^{KO} cells treated with RSL3 ± Fer-1. In PERK^{WT} cells, EMCSs were enriched in oxidation-prone PUFA-containing PE and PC species compared with the input, while CL enrichment exclusively in the input confirmed the purity of the EMCS fraction. In contrast, PERK^{KO} cells showed a drastically reduced PL diversity in EMCSs, with far fewer unsaturated species and no significant PUFA-PL enrichment (*Fig. 6*).

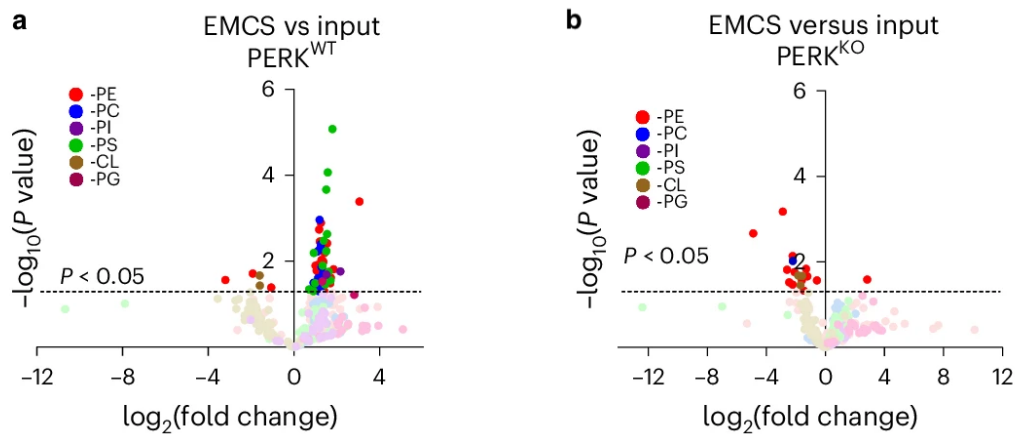


Fig. 6: Volcano plots depicting changes in PL content between input and EMCS fractions in PERK^{WT} (a) and PERK^{KO} (b) MEFs. Each dot represents an individual PL species: its horizontal position indicates whether that species is enriched in EMCSs (right) or in the input (left), while its vertical position reflects the statistical confidence of this difference; *n* = 3 biological replicates.

After RSL3 treatment, Fer-1 protected nearly all PL classes (except CL) from oxidation. PERK^{WT} EMCSs displayed a diverse PLox landscape spanning multiple PL classes, including PEx and PSx species not found in PERK^{KO} cells; the highly oxidizable di-PUFA PE (40:8) was strongly enriched in PERK^{WT} EMCSs but not in PERK^{KO} ones, where PLox was limited to only three species. PERK deficiency did not significantly affect antioxidant machinery expression or the MUFA/PUFA ratio.

Overall, these results reveal that EMCSs possess a lipidome particularly vulnerable to oxidative attack.

3.5 Enforcing ER–mitochondria tethering promotes ferroptosis

PERK-deficient cells were significantly resistant to RSL3- and IKE-induced ferroptosis compared with wild-type cells. To determine whether this protection stemmed from PERK's kinase-mediated UPR signaling or its tethering function, they re-expressed a kinase-dead PERK mutant (PERK^{KD}), which restores only the tethering function, in PERK^{KO} cells; this fully rescued ferroptosis sensitivity. Consistently, chemical inhibition of PERK kinase activity, or expression of a phosphorylation-dead eIF2 α mutant (the UPR effector downstream of PERK), did not protect cells from ferroptosis. Thus, PERK's tethering function, not its UPR signaling, is critical for ferroptosis.

These findings were validated using MFN2, another ER–mitochondria tether: MFN2 loss protected against ferroptosis, while re-expressing MFN2 or its EMCS-specific splice variant ERMIT2 restored vulnerability. The synthetic 9xL spacer similarly protected cells, phenocopying PERK and MFN2 loss. Conversely, forcing EMCSs closer together, via a synthetic OMM–ER linker (see *Section 2.2.1*) or overexpression of the σ -1 receptor, increased EMCS formation and restored or enhanced ferroptosis sensitivity, even in PERK^{KO} cells, indicating that artificially tightening ER–mitochondria contacts can override the loss of a specific tether. Hence, shortening ER–mitochondria contacts potentiates ferroptosis (*Fig. 7*), while distancing EMCSs blunts it.



Fig. 7: Representative images of live PERK^{KO} MEFs transiently transfected with the OMM–ER linker at time 0 and 24 h after RSL3 (0.5 μ M). The images show SYTOX Green staining, BF and RFP. Scale bar, 100 μ m.

3.6 EMCSs shape ferroptosis vulnerability of TNBC subtypes

Since ER–mitochondria contact dysregulation is a hallmark of cancer and the LAR subtype of TNBC is known to be hypersensitive to FINs, the researchers asked whether this vulnerability reflects differences in EMCS integrity. Using ssGSEA (see *Section 2.6*), they found that the LAR subtype showed a distinct enrichment in EMCS-associated genes (EMCS^{high} signature) compared with the other subtypes (*Fig. 8*). Consistently, non-LAR TNBC cell lines showed reduced protein levels of these EMCS tethers and fewer ER–mitochondria contacts compared with the ferroptosis-vulnerable LAR cell line MDA-MB-157. The EMCS^{high} LAR subtype

was also associated with elevated levels of oxidized PE species, linking EMCS gene expression to lipid peroxidation susceptibility.

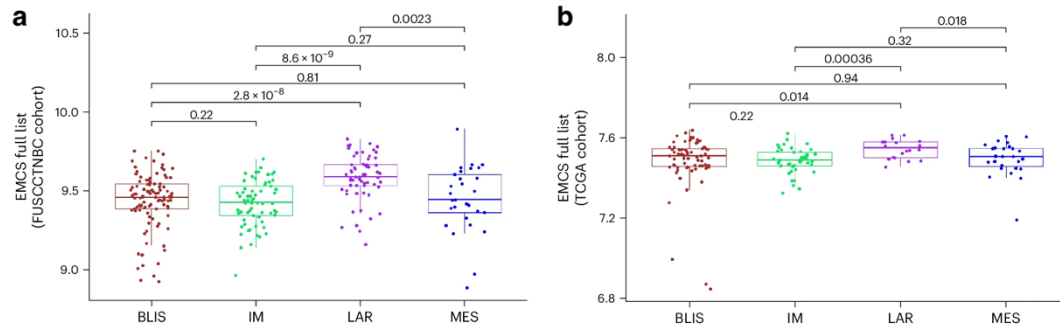


Fig. 8: Box plots (median, 1.5× interquartile range whiskers) of the EMCS-enrichment score (full gene list), calculated by ssGSEA, across TNBC subtypes (BLIS, IM, LAR, MES) from the FUSCCTNBC (**a**; n = 258 patients) and TCGA (**b**; n = 158 patients) cohorts. Statistical significance was determined by two-sided Wilcoxon's test.

To test this link functionally, the researchers disrupted EMCSs in MDA-MB-157 cells (via the 9xL spacer or PERK knockout), which conferred resistance to RSL3 comparable to that of non-LAR lines; restoring EMCS integrity with the OMM–ER linker rescued their ferroptosis sensitivity. Conversely, forcing EMCS formation via the OMM–ER linker in non-LAR cell lines (Hs578T, HCC1143) partially sensitized them to ferroptosis, though not to the same extent as MDA-MB-157.

Together, these results indicate that EMCS integrity underlies the heterogeneous ferroptosis vulnerability of TNBC subtypes and suggest that an EMCS^{high} gene signature could serve as a predictive biomarker for ferroptosis sensitivity in TNBC.

4 Discussion

This study provides strong evidence that EMCSs are not purely passive platforms for interorganelle communication, but active hotspots where PLox originates during the early phases of ferroptosis. By combining super-resolution live imaging with subcellular oxidative lipidomics, the authors show that EMCSs expand within minutes of GPX4 inhibition or system x_c^- blockade, that this expansion is causally driven by lipid-ROS rather than by calcium signaling and that the distinct PUFA-enriched phospholipid landscape of EMCSs renders them particularly susceptible to oxidation. Genetic or synthetic manipulations that restrict EMCS dynamics, whether through PERK, MFN2 or an artificial spacer, consistently blunted PLox propagation and protected cells from ferroptosis, while manipulations that tightened ER-mitochondria proximity had the opposite effect.

Although the study establishes lipid peroxidation as the trigger of EMCS expansion, the precise molecular mechanisms by which PLox is translated into structural remodeling of ER-mitochondria contacts remain to be elucidated, as does the potential propagation of PLox to membranes of other organelles in close contact. Relatedly, while the authors show that depleting individual EMCS tethering curbs ferroptosis, the downstream molecular consequences of losing these specific tethers are not fully resolved. PERK acts as a scaffold recruiting the ER-associated lipid transfer protein extended-synaptotagmin 1 (E-Syt1) to EMCSs, favoring PL transfer to mitochondria¹⁰. Disrupting endogenous tethers may destabilize such molecular complexes and impair lipid transfer even when other EMCS components remain present. This anti-ferroptotic effect may also involve the repair of peroxidized PLs before they reach the mitochondria¹¹. Whether disturbance of these lipid transfer or repair enzymes modulates ferroptosis requires future studies. It would also be interesting to explore how PL transfer via EMCSs interacts with the mitochondrial DHODH/CoQ antioxidant axis⁴ and under which metabolic contexts it becomes relevant.

Regarding the spatial origin of PLox, it is worth noting that an alternative model has been proposed, whereby di-PUFA-PC integrates directly into mitochondrial membranes and interacts with complex I of the electron transport chain, driving mitochondrial ROS generation, with PLox at the ER occurring only secondarily¹². This contrasts with the EMCS-first, mitochondria-second sequence established in the present study. Future work, reconciling these two models, would help clarify if these represent distinct pathways or complementary aspects of the same process.

A related consideration concerns the role of calcium signaling in this causal chain. The authors show that the calcium chelator BAPTA-AM does not prevent RSL3-induced EMCS expansion. This finding stands in apparent contrast with an article from Zhang et al., who observed that BAPTA-AM protected cells from both chemically and genetically induced ferroptosis². This discrepancy might reflect differences in the specific readout used (EMCS expansion at 15 minutes versus overall ferroptotic cell death at later time points), in cell type or in BAPTA-AM

concentration; alternatively, it may indicate that calcium plays a role downstream of the initial EMCS-driven PLox wave, as stated by the authors themselves. Nonetheless, I believe this aspect warrants further examination.

From a methodological standpoint, some considerations deserve mention. The study relies extensively on SV40 large T antigen-immortalized MEFs. Despite the robust immortalized cell lines that SV40 Ta transformation produces, there may be phenotypic and/or molecular differences when compared to wild-type lines⁷. While the authors do not explicitly address this point, it is plausible that such considerations were accounted for during experimental design and interpretation. Possibly validation in additional cellular contexts, such as primary or non-transformed cell lines, would further strengthen confidence in the generalizability of these findings. Additionally, the colocalization techniques (Airyscan imaging combined with Mander's coefficient) are under the resolution limit for EMCSs detection. Airyscan has a xy resolution of 90-140 nm, while EMCSs typically span only 5–70 nm. Consequently, the increase in ER–mitochondria colocalization may partly reflect simple organelle proximity rather than true tethering. Similarly, PLA used to confirm EMCS expansion is a technique with well-recognized limitations. PLA is a valuable tool for confirming protein-protein interactions in situ, but should not be relied upon to quantify the extent of membrane contact¹. It is worth noting, however, that in this study PLA was employed only as one of several lines of evidence, which substantially mitigates this limitation and strengthens confidence in the overall conclusion. It is also worth noting that, as explicitly acknowledged by the authors, sample sizes were not predetermined through statistical power analysis, normality of the data distribution was assumed, but not formally tested, and investigators were not blinded during data collection and analysis. Moreover, some of the presented data show a level of dispersion that may deserve caution, for instance in Fig. 2i and 5h from the original paper (likewise, the statistical pattern observed in Fig. 6c).

Beyond these considerations, the findings of this study in TNBC cells open several paths for future research and possible clinical translation. Therapeutic strategies aimed at enforcing ER–mitochondria tethering could complement existing approaches to sensitize cancer cells that are either tolerant or less sensitive to FINs. Recent discoveries include pharmacological inhibition of DHODH, which weakens the mitochondrial antioxidant defense¹³ as well as resveratrol, which promotes NEDD4L-mediated ubiquitination and degradation of GPX4¹⁴. Conversely, therapeutic strategies aimed at distancing EMCSs could protect healthy tissues from excessive PLox in degenerative conditions.

Overall, this study establishes EMCSs as key regulators of ferroptosis and promising targets for future therapeutic intervention.

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Appendix

Sassano ML, Tyurina YY, Diokmetzidou A, et al. Endoplasmic reticulum–mitochondria contacts are prime hotspots of phospholipid peroxidation driving ferroptosis. *Nat Cell Biol.* 2025;27(6):902-917. doi:10.1038/s41556-025-01668-z

Endoplasmic reticulum–mitochondria contacts are prime hotspots of phospholipid peroxidation driving ferroptosis

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The peroxidation of membrane phospholipids (PLs) is a hallmark of ferroptosis. The endoplasmic reticulum and mitochondria have been implicated in ferroptosis, but whether intracellular PL peroxidation ensues at their contact sites (endoplasmic reticulum–mitochondria contact sites, EMCSs) is unknown. Using super-resolution live imaging, we charted the spatiotemporal events triggered by ferroptosis at the interorganelle level. Here we show that EMCSs expand minutes after localized PL peroxides are formed and secondarily spread to mitochondria, promoting mitochondrial reactive oxygen species and fission. Oxidative lipidomics unravels that EMCSs host distinct proferroptotic polyunsaturated-PLs, including doubly proferroptotic polyunsaturated-acylated PLs, demonstrating their high propensity to undergo PL peroxidation. Endoplasmic reticulum–mitochondria untethering blunts PL peroxidation and ferroptosis, while EMCS stabilization enhances them. Consistently, distancing EMCSs protects the ferroptosis-susceptible triple-negative breast cancer subtype, harbouring high EMCS-related gene expression and basal PL peroxide levels. Conversely, in insensitive triple-negative breast cancer subtypes, bolstering EMCSs sensitizes them to ferroptosis. Our data unveil endoplasmic reticulum–mitochondria appositions as initial hubs of PL peroxide formation and posit that empowering EMCSs endorses ferroptosis in cancer cells.

In eukaryotic cells, the endoplasmic reticulum (ER) establishes a dynamic connection with mitochondria through membrane appositions called ER–mitochondria contact sites (EMCSs). EMCSs are stabilized by protein tethers that operate as bridges between these organelles and provide a molecular platform for signal communication^{1,2}. The dynamic regulation and remodelling of EMCSs ensure rapid coordination and integration of physiological and metabolic cues, vital for maintaining organelle and cellular function. A wealth of genetic and biochemical

studies validated the functional role of EMCSs in key signalling processes at the cellular and systemic/organ level, including Ca^{2+} fluxes, apoptosis, mitochondrial dynamics, metabolism and lipid homeostasis³.

EMCSs play an essential role in transporting phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS), from the ER where they are synthesized to the mitochondria^{4–6}, where PS is rapidly converted to phosphatidylethanolamine (PE) and transferred back to the ER^{4–6}. However, while EMCSs are crucial for the homeostatic

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transport of phospholipids (PLs) to mitochondria, our understanding of the spatiotemporal dynamics and remodelling of EMCSs under conditions causing PL peroxidation (P_{Lox}) is scant. Oxidized polyunsaturated fatty acids (PUFAs)-containing PEs are the main drivers of ferroptosis, an iron-dependent necrotic cell death caused by the blockade of cellular lipid hydroperoxide detoxification systems and capable of generating myriads of secondary oxidatively-truncated electrophilic species forming adducts with proteins^{7–10}. Glutathione peroxidase 4 (GPX4) can reduce hydroperoxyl-PLs to the respective stable alcohols, hence preventing ferroptosis^{7–10}. During ferroptosis, P_{Lox} produced in membranes rapidly spread⁹, ultimately causing plasma membrane damage and cell death. Ferroptosis has been implicated in several pathologies including neurodegeneration, cancer and cardiovascular diseases¹¹. However, while substantial progress has been made in understanding the molecular effector mechanisms of ferroptosis, the events that govern the initial P_{Lox} damage and its precise subcellular localization remain elusive.

Different organelles have been linked to the regulation of ferroptosis¹² in different cellular settings^{12–17}. For example, the radical-trapping agent ferrostatin-1 (Fer-1) targeted to mitochondria protects cells against ferroptosis induced by cysteine starvation or inhibitors of the system x_c^- , whereas ER-localized Fer-1 protects from ferroptosis induced by a small molecule inhibitor of GPX4 (refs. 13,14). In the cells exposed to diacyl-PUFA-PLs, mitochondria-targeted antioxidants blunt lipid reactive oxygen species (ROS) formation in the ER and reduce ferroptosis¹⁵. While these studies implicate primarily the ER and mitochondria in the regulation of ferroptosis, it remains unclear how these organelles precisely control the onset of P_{Lox} production and the propagation of these lethal signals during ferroptosis. Portraying the precise spatiotemporal dynamics of EMCSs under conditions eliciting P_{Lox} is paramount to gaining molecular insights into the initial stages of ferroptosis and identifying sites of interventions to regulate its lethal progression.

In this study, by combining high-resolution live cell imaging with oxidative phospholipidomics of subcellular fractions and EMCS genetic perturbation, we identify EMCSs as primary sites of early P_{Lox} in cells undergoing ferroptosis.

Results

ER–mitochondria contacts rapidly expand during ferroptosis

To assess the dynamics of EMCS remodelling induced by lipid-ROS in real time, we first validated the induction of ferroptosis in our cellular model. We used the RAS-selective lethal (1S,3R)RSL3 (ref. 18) GPX4 inhibitor and the cystine–glutamate antiporter system x_c^- blocker imidazole ketone erastin (IKE), which by impeding the import of cystine causes glutathione (GSH) depletion and inactivation of GPX4 among other GSH-depending enzymes¹⁹, as established class II and I

ferroptosis inducers (FINs), respectively. In murine embryonic fibroblasts (MEFs) (1S,3R)RSL3 (or just RSL3) but not the inactive diastereomer (1R,3R)RSL3 (ref. 20), induced cell death that was prevented by the iron chelator deferoxamine mesylate salt (DFO) and the radical scavenger and suppressor of arachidonoyl-PE oxidation²¹ Fer-1 (Extended Data Fig. 1a,b). Likewise, cell death induced by IKE was prevented by DFO, Fer-1 and by the reducing agent beta-mercaptoethanol (BME), which can promote cystine uptake through mechanisms bypassing system x_c^- (ref. 9) (Extended Data Fig. 1c,d). By contrast, inhibitors of caspases (Z-Val-Ala-DL-Asp(OMe)-fluoromethylketone, ZVAD) or RIPK1 (necrostatin-1s, Nec1s) (Extended Data Fig. 1a,c) failed to do so. The cell death was preceded by the Fer-1-inhibitable activation of P_{Lox}, as measured by the ratiometric fluorescent indicator of membrane vulnerability to P_{Lox} Bodipy 581/591 C11 (Bodipy C11), a commonly used biomarker of ferroptosis (Extended Data Fig. 1e). Together, these observations demonstrate the induction of bona fide ferroptosis by class II and I FINs in our settings.

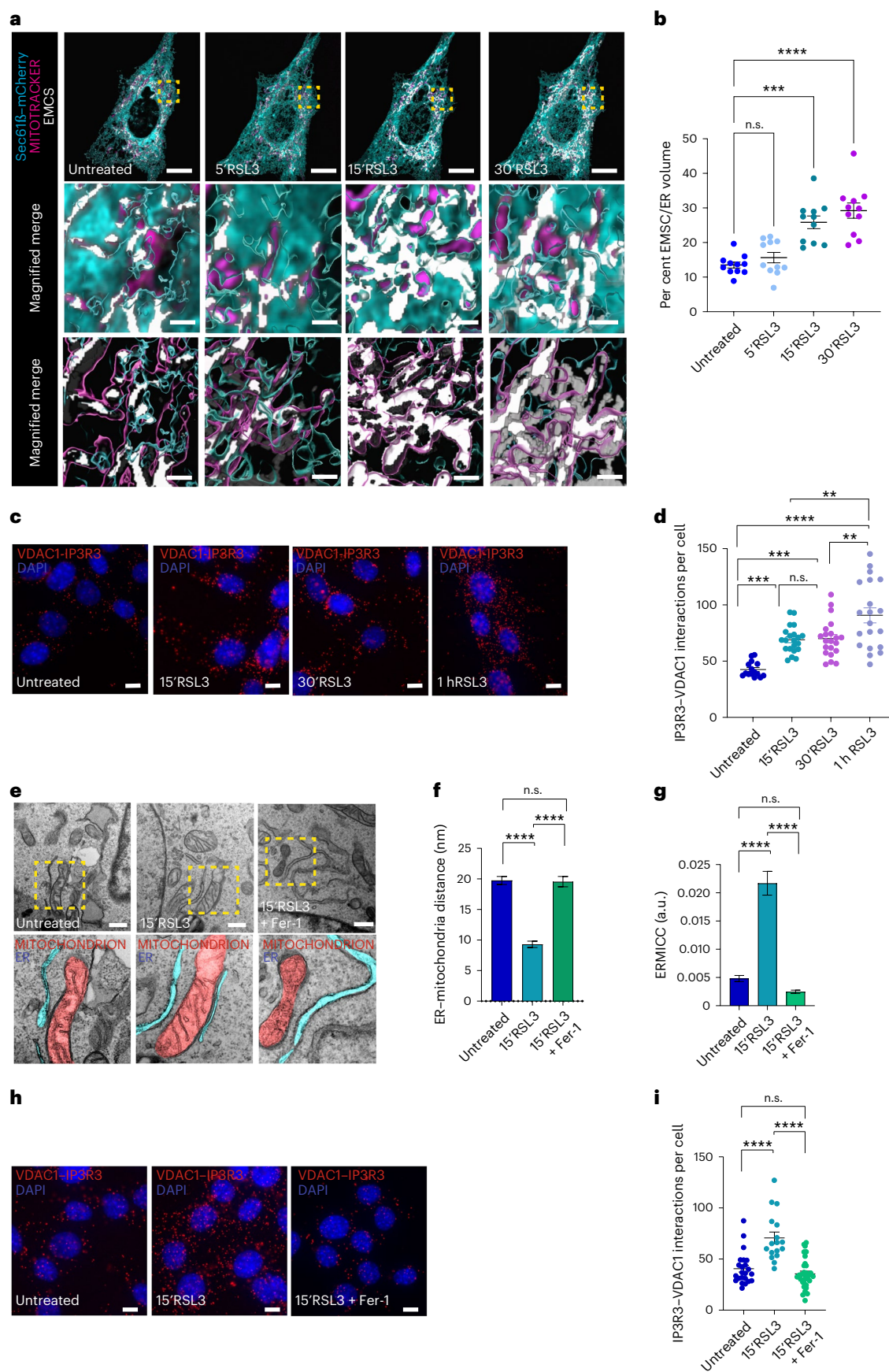
We then conducted time-lapse live cell imaging using super-resolution confocal Airyscan microscopy and a three-dimensional (3D) rendering of acquired z-stacks to visualize the early remodelling of EMCSs during ferroptosis in cells expressing Sec61β–mCherry (as an ER marker) and incubated with MitoTracker Far Red (to stain the mitochondrial network). As early as 15 min following RSL3 treatment, the ER surface engaged in contact with the mitochondria expanded from a steady-state level of ~15% in untreated cells to ~30%, plateauing at this level (Fig. 1a,b). A proximity ligation assay (PLA) between mitochondrial VDAC1 and ER-associated IP3R3, two known EMCS markers^{22,23}, confirmed the fast kinetics of EMCS expansion and their perdurance up to 1 h after RSL3 treatment (Fig. 1c,d). This rapid EMCS remodelling was not observed in cells treated with the DNA-damaging agent cisplatin (CDDP) or the pan-kinase inhibitor staurosporine, undergoing Fer-1-insensitive and ZVAD-inhibitable apoptosis (Extended Data Fig. 1f–i).

To characterize the impact of ferroptosis induction on EMCS architecture at the ultrastructural level, we then conducted transmission electron microscopy (TEM) and calculated the ER–mitochondria contact coefficient (ERMICC), which computes various parameters involved in contact sites²⁴. The distance separating the ER and mitochondria shortened from an average of 20 nm of the resting cells to 9 nm in response to RSL3 treatment (15 min) (Fig. 1e,f), and the ERMICC coefficient increased by more than fourfold as compared with untreated cells (Fig. 1e,g).

EMCSs play a crucial role in allowing efficient mitochondrial Ca^{2+} uptake upon ER Ca^{2+} depletion^{1,25}. Elevations in cytosolic²⁶ or mitochondrial^{27,28} Ca^{2+} have been implicated in the regulation of ferroptosis. Cells expressing the genetically encoded mitochondria-targeted Ca^{2+} indicator mito-R-GECO1 (ref. 29) and loaded with cytosolic Ca^{2+}

Fig. 1 | EMCSs rapidly expand at the onset of lipid peroxidation. a, Representative volume-rendered 3D reconstructions of confocal z-stacks from time-lapse live images of MEFs transiently transfected with ER marker Sec61β–mCherry (cyan) and stained with MitoTracker Far Red (magenta) at time 0 and 5, 15 and 30 min after RSL3 (0.5 μM) (5'RSL3, 15'RSL3 and 30'RSL3, respectively). The EMCS masked volume, representing colocalization of ER and mitochondria, is indicated in white. The magnified images represent a merge of ER, mitochondrial surfaces and EMCS mask. Scale bar, 10 μm. Zoom, 1 μm. **b**, The per cent of EMCSs normalized on ER volume (Sec61β–mCherry) at time 0 and 5'RSL3, 15'RSL3 and 30'RSL3 (0.5 μM) ($n = 3$ biological replicates, the same cells ($n = 11$) were acquired and analysed at each timepoint per condition). Similar experiments have been reproduced independently in Fig. 4, Supplementary Fig. 2 and Supplementary Fig. 3. **c,d**, Representative images (**c**) of in situ PLA in MEFs and quantification (**d**) of N of dots corresponding to IP3R3–VDAC1 interaction per nucleus in untreated cells and in cells treated for 15 and 30 min and 1 h with RSL3 (0.125 μM) ($n = 3$ biological replicates, images $n = 15$ for untreated, $n = 21$ for 15'RSL3, $n = 21$ for 30'RSL3, $n = 20$ for 1 h RSL3). Scale bar, 10 μm. **e–g**, Representative TEM images

of EMCSs at time 0 and 15 min after RSL3 (0.125 μM) and cotreatment with Fer-1 (1 μM) (**e**), quantification of EMCSs distance (**f**) and ER–mitochondria contact coefficient (ERMICC; interface length/(mitochondrial perimeter × distance ER–mitochondria)) (**g**), with a morphometric analysis of the ER located at less than 30 nm from the mitochondria ($n = 70$ images per condition, five cells per condition from two biological replicates). **h,i**, Representative images (**h**) of in situ PLA in MEFs and quantification (**i**) of N of dots corresponding to the IP3R3–VDAC1 interaction per nucleus in untreated cells and in cells treated for 15 min with RSL3 (0.125 μM) or cotreated with RSL3 (0.125 μM) and Fer-1 (1 μM) ($n = 3$ biological replicates, images $n = 23$ for untreated, $n = 17$ for 15'RSL3, $n = 32$ for 15'RSL3 + Fer-1). Scale bar, 10 μm. In **d**, and **i**, at least two wells per condition were imaged and quantified per biological replicate. Each image contained multiple cells. All quantitative data are the mean ± s.e.m. In **b**, the statistical significance was determined by a repeated-measures one-way ANOVA, Dunnett post hoc test. In **d**, **f**, **g** and **i**, the statistical significance was determined by a one-way ANOVA, Tukey post hoc test. n.s., not significant ($P > 0.05$), * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.



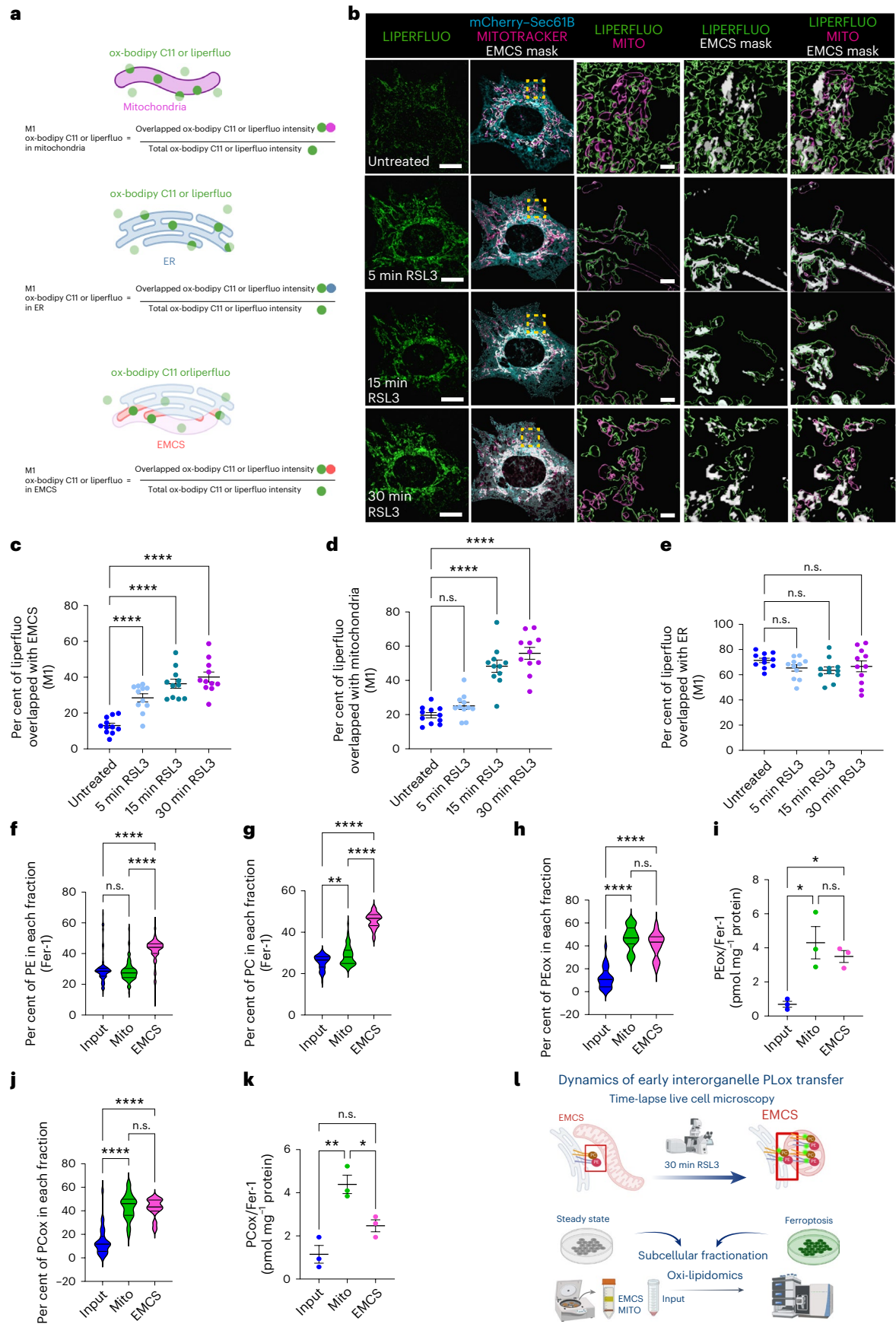


Fig. 2 | PLox initiates at EMCs and propagates to the mitochondria.

a, A schematic representation of Mander's coefficient (M1). The M1 quantifies the fraction of the signal intensity of the lipid peroxidation probe that colocalizes in each subcellular compartment/organelle relative to the total signal intensity of the probe. **b**, Representative volume-rendered 3D reconstructions of confocal z-stacks from time-lapse live images of MEFs transiently transfected with ER marker Sec61 β -mCherry (cyan) and stained with MitoTracker Far Red (magenta) and Liperfluo (green) at time 0 and 5, 15 and 30 min after RSL3 (0.5 μ M). The EMCs masked volume, representing colocalization of ER and mitochondria, is indicated in white. The magnified images represent, respectively, (i) Liperfluo and mitochondrial surfaces, (ii) Liperfluo surface and EMCs mask and (iii) Liperfluo and mitochondrial surfaces together with the EMCs mask. Scale bar, 10 μ m. Zoom, 1 μ m. **c–e**, The colocalization (M1%) of Liperfluo in the EMCs (**c**), mitochondria (**d**) and ER (**e**) masks at time 0 and 5, 15 and 30 min after RSL3 (0.5 μ M) ($n = 3$ biological replicates, the same cells ($n = 11$) were acquired and analysed at each timepoint per condition). Similar experiments and settings

have been reproduced independently in Fig. 4, Supplementary Fig. 2 and Supplementary Fig. 3. **f,g**, The per cent of total PE (**f**) and PC (**g**) in the input, EMCs and mitochondrial fraction ($n = 3$ biological replicates, 105 PE subspecies, 58 PC subspecies). **h–k**, The per cent of PEOx (**h**) and PCox (**j**) in the input, EMCs and mitochondrial fraction and PEOx (**i**) and PCox (**k**) fold changes compared with corresponding conditions cotreated with Fer-1 (1 μ M) ($n = 3$ biological replicates, 25 PEOx subspecies, 29 PCox subspecies). **i**, A schematic representation of the complementary approaches and methodology used to assess the dynamics of early interorganelle PLox transfer. All quantitative data are the mean $\pm$ s.e.m. In **c–e**, the statistical significance was determined by a repeated-measures one-way ANOVA, Dunnett post hoc test. In **f–h** and **j**, the statistical significance was determined by a repeated-measures one-way ANOVA, Tukey post hoc test. In **i** and **k**, the statistical significance was determined by a one-way ANOVA, Tukey post hoc test. n.s., not significant ($P > 0.05$), * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$. Panel **a** created with [BioRender.com](https://www.biorender.com).

indicator Cal520 to concomitantly measure mitochondrial and cytosolic Ca $^{2+}$ (cytCa $^{2+}$) levels were treated with RSL3 in a Ca $^{2+}$ -free medium for 15 min, followed by the addition of ATP to evoke Ca $^{2+}$ release from ER Ca $^{2+}$ stores (Extended Data Fig. 1j–m). RSL3 did not alter ATP-induced cytCa $^{2+}$ elevation (Extended Data Fig. 1j,k) but potentiated mitochondrial Ca $^{2+}$ uptake (Extended Data Fig. 1l,m). This suggests that early remodelling of EMCs facilitates the transfer of Ca $^{2+}$ to mitochondria, without affecting the overall cytCa $^{2+}$ levels. The cell-permeable high-affinity/fast-kinetic Ca $^{2+}$ chelator BAPTA-AM did not affect the EMCs expansion caused by RSL3 (Extended Data Fig. 1n,o). While these results do not confute the participation of Ca $^{2+}$ signals in ferroptosis at later stages, they suggest that elevation of Ca $^{2+}$ is not the main trigger of EMCs remodelling in the early phases of ferroptosis.

By contrast, in cells treated with RSL3, blockade of PLox by Fer-1 (Extended Data Fig. 1e) inhibited EMCs expansion as shown by TEM analysis (Fig. 1e–g) and PLA (Fig. 1h,i).

We conclude that PLox prompts ER–mitochondria tethering.

PLox starts at EMCs and propagates to the mitochondria

To capture early PLox and their interorganelle transfer we monitored by live imaging the subcellular distribution of oxidized Bodipy C11 (ox-Bodipy) signal by creating masks of the mitochondria, the ER, and the overlapping area between the ER and mitochondria, representing the EMCs (Extended Data Fig. 2a–d), in untreated cells and following 5, 15 and 30 min after RSL3 exposure. We then evaluate at each timepoint the fraction of oxidized Bodipy C11 signal that spatially overlapped with the ER, mitochondria and EMCs, using Mander's coefficient (M1) as a quantitative measure of the cooccurrence of ox-Bodipy C11 fluorescence with a subcellular organelle^{30,31} (Fig. 2a).

The steady-state levels of ox-Bodipy C11 in the ER, which houses important cellular redox reactions, were significantly higher compared with those of the EMCs and mitochondria (Extended Data

Fig. 2b–d). However, the ox-Bodipy C11 signal did not change throughout the time of RSL3 treatment in the ER; if anything, it showed a decreasing trend 30 min after RSL3 addition (Extended Data Fig. 2b). Of note, as soon as 5 min after RSL3 exposure, a significantly larger fraction of ox-Bodipy C11 signal overlapped with EMCs and persisted up to 30 min (Extended Data Fig. 2c), at the time when a marked ox-Bodipy C11 signal colocalized with mitochondria (Extended Data Fig. 2d). At variance, the residual cellular fraction of ox-Bodipy C11, obtained upon the removal of the ER and mitochondria and EMCs masks, showed a decline (Extended Data Fig. 2e). Since both Bodipy C11 and its oxidation product do not spontaneously leave lipid monolayers for at least over 2 h (ref. 32), diffusion events are unlikely to contribute to the rapid increase in the ox-Bodipy C11 signal in EMCs observed in our settings.

To chase the spatiotemporal dynamics of PLox within the same cell, we performed 3D rendering of time-lapse super-resolution live imaging of cells expressing Sec61 β -mCherry (as ER marker) and costained with MitoTracker Far Red and the Liperfluo probe. The Liperfluo green-fluorescent signal results from the reduction of lipid hydroperoxides, thus providing a direct assessment of PLox formation³³. We then analysed (Fig. 2a) the kinetics of PLox organelle redistribution in cells treated with RSL3 (Fig. 2b–e) or IKE (Extended Data Fig. 3a–e).

Consistent with the findings using Bodipy C11 (Extended Data Fig. 2c,d), after 5 min of RSL3 exposure, we observed a rapid increase in the fraction of Liperfluo signal spatially overlapping with the EMCs, which further redistributed to the mitochondria within 15 min (Fig. 2c,d), along with the steady expansion of the EMCs (Fig. 1a,b). No significant changes in the ER-associated Liperfluo signal were observed over the time of RSL3 treatment (Fig. 2b,e), mirroring the results with Bodipy C11 (Extended Data Fig. 2a,b). A similar spatial redistribution of Fer-1-inhibitable PLox was observed, albeit with slower kinetics, after

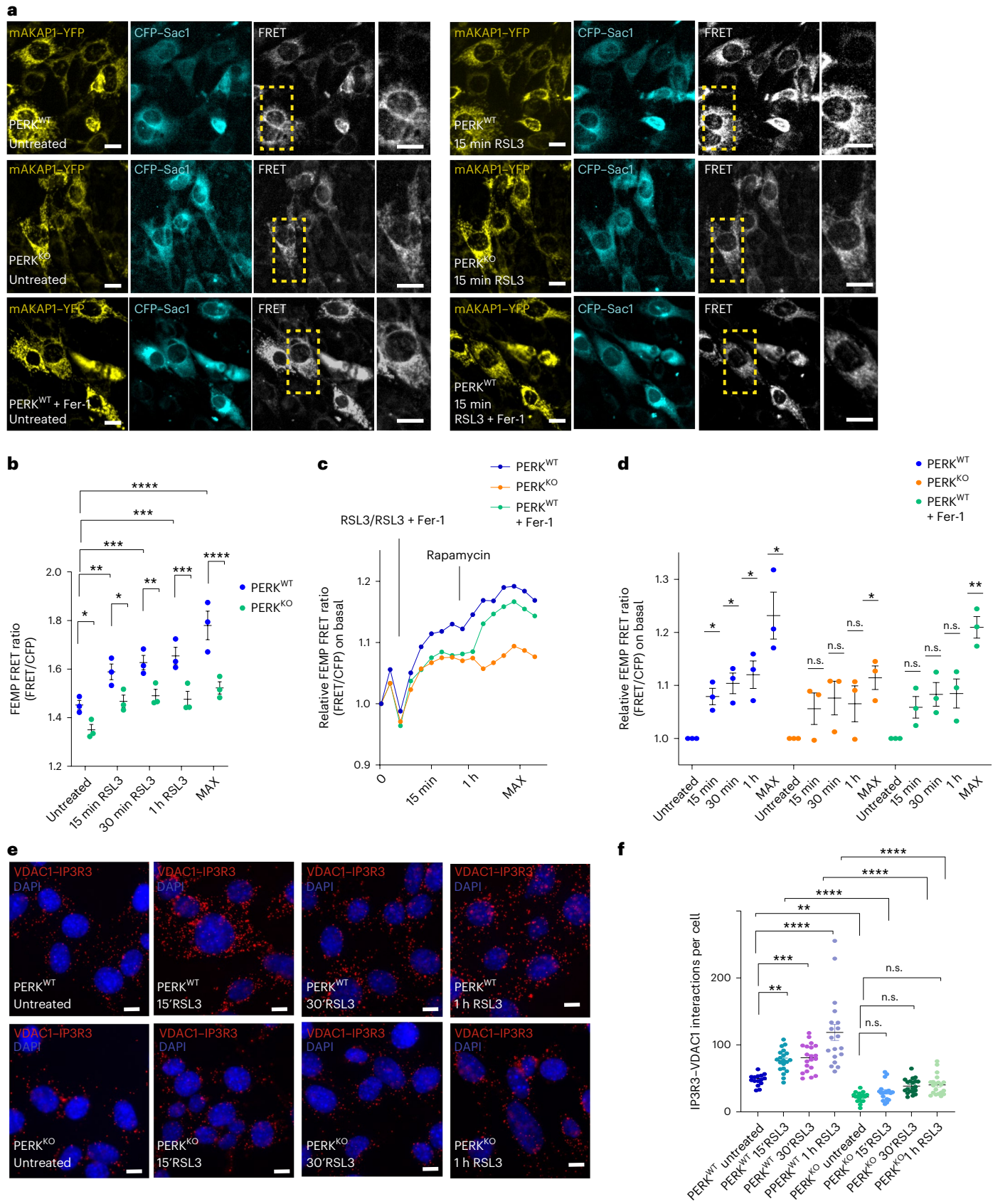
Fig. 3 | PERK deficiency protects from EMCs expansion during the early phases of ferroptosis.

a, Representative FRET live microscopy images of PERK WT and PERK KO MEFs transiently infected with FEMP probe (mAKAP1-YFP-Tav2-CFP-Sac1) and treated with RSL3 (0.5 μ M) and PERK WT cotreated with RSL3 (0.5 μ M) + Fer-1 (1 μ M) at time 0 and 15 min after RSL3 treatment. Scale bar, 10 μ m. Zoom, 10 μ m. **b**, The FEMP ratio at 0, 15 and 30 min and 1 h after RSL3 (0.5 μ M) and rapamycin treatment, in PERK WT MEFs transiently infected with a FEMP probe ($n = 3$ biological replicates, multiple areas in the same well were acquired and analysed per each timepoint per condition per biological replicate). MAX, maximum. **c**, Time-lapse imaging of the FEMP ratio traces, fold changes (on untreated) of PERK WT and PERK KO MEFs treated with RSL3 (0.5 μ M) and PERK WT cotreated with RSL3 (0.5 μ M) + Fer-1 (1 μ M) infected with the FEMP probe. Where indicated, the cells were treated with 100 nM rapamycin. **d**, The FEMP ratio at 0, 15 and 30 min and 1 h after RSL3 (0.5 μ M) treatment and rapamycin treatment, in PERK WT and PERK KO MEFs and PERK WT cotreated with RSL3 (0.5 μ M) + Fer-1

(1 μ M) infected with the FEMP probe ($n = 3$ biological replicates, multiple areas in the same well were acquired and analysed per each timepoint per condition and biological replicate). **e,f**, Representative images (**e**) of in situ PLA in PERK WT and PERK KO MEFs and quantification (**f**) of N of dots corresponding to IP3R3–VDAC1 interaction per nucleus in untreated cells and in cells treated for 15 and 30 min and 1 h with RSL3 (0.125 μ M) ($n = 3$ biological replicates, images $n = 15$ for PERK WT untreated, $n = 20$ for PERK WT 15 min of RSL3 (15'RSL3), $n = 20$ for PERK WT 30'RSL3, $n = 19$ for PERK WT for 1 h RSL3, $n = 17$ for PERK KO untreated, $n = 18$ for PERK KO 15'RSL3, $n = 20$ for PERK KO 30'RSL3, $n = 20$ for PERK KO for 1 h RSL3 and quantified as described in Fig. 1d). Scale bar, 10 μ m. All quantitative data are mean $\pm$ s.e.m. In **b**, the statistical significance was determined by a repeated-measures two-way ANOVA, Tukey post hoc test. In **d**, the statistical significance was determined by a one-sample t -test. In **f**, the statistical significance was determined by a two-way ANOVA, Tukey post hoc test. n.s., not significant ($P > 0.05$), * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

IKE (Extended Data Fig. 3a–e). Overall cellular PLox levels following RSL3 or IKE (that is, mean fluorescence intensity of ox-Bodipy C11 or LiperFluo) showed similar kinetics, with an early buildup of PLox steadily increasing over time (Extended Data Fig. 2f, g; Extended Data Fig. 3f).

Ferroptosis is associated with the generation of oxidation products from PUFA-PLs, particularly containing PC and PE^{3–5}, and EMCSSs are the spatially restricted sites of PC and PS transfer between the ER and mitochondria^{8–10}. We posited that following their expansion,



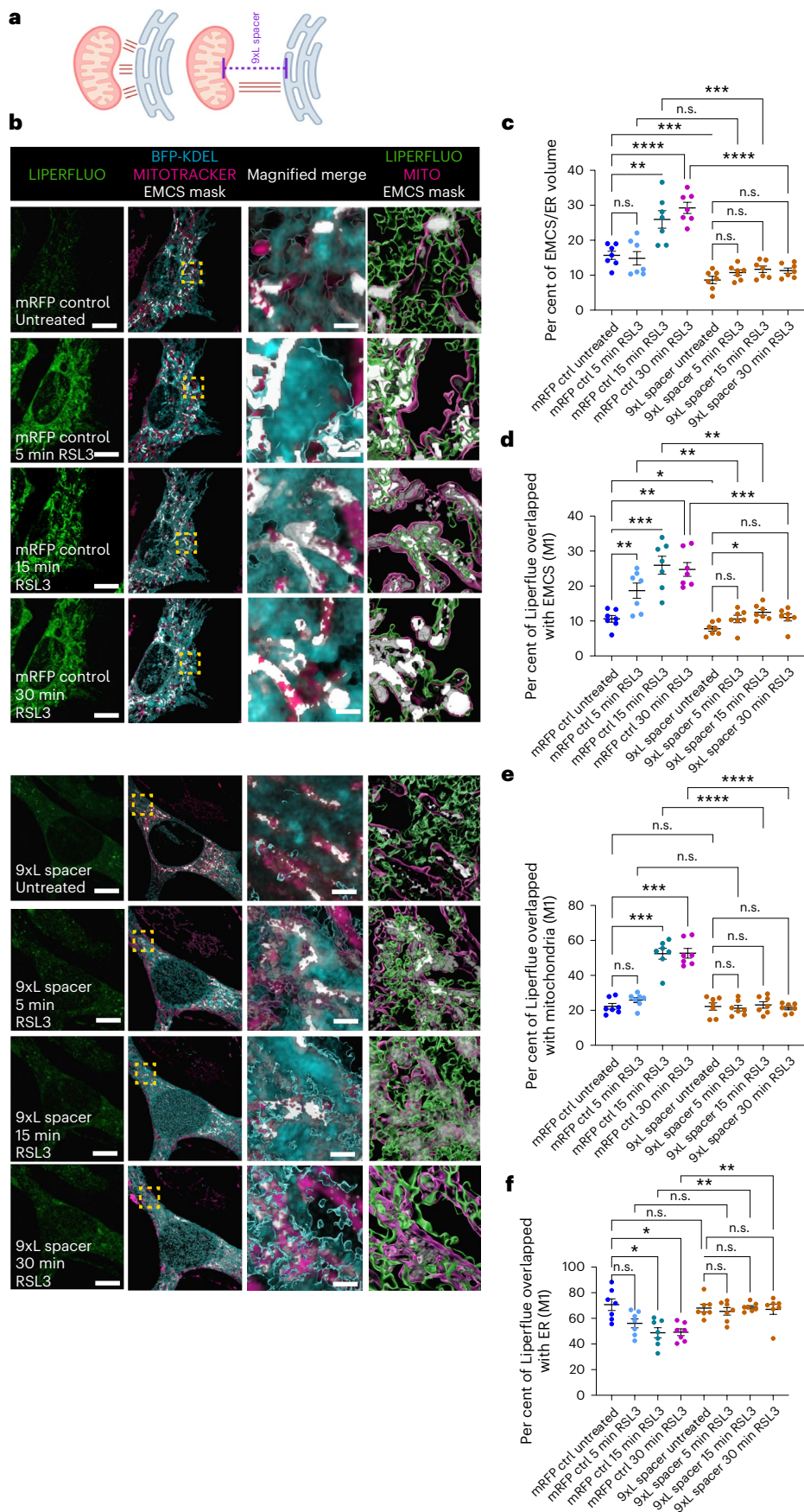


Fig. 4 | Spacing EMCs protects cells from EMCS-mediated mitochondrial PLox. **a**, A schematic representation of 9xL spacer. **b**, Representative volume-rendered 3D reconstructions of confocal z-stacks from time-lapse live images of MEFs transiently transfected with AKAP1-mRFP control or 9xL spacer and the ER marker BFP-KDEL (cyan) and stained with MitoTracker Far Red (magenta) and Liperfluo (green) at time 0, 5, 15 and 30 min after RSL3 (0.5 μ M). The EMCS masked volume, representing colocalization of the ER and mitochondria, is indicated in white. The magnified images represent, respectively, (i) the merge of ER, mitochondrial surfaces and EMCS mask and (ii) Liperfluo and mitochondrial surfaces together with EMCS mask. Scale bar, 10 μ m. Zoom, 1 μ m. **c**, A quantification of the per cent of EMCs normalized on the ER volume (BFP-KDEL) at time 0, 5, 15 and 30 min after RSL3 (0.5 μ M) in MEFs transiently

transfected with AKAP1-mRFP control or 9xL spacer ($n = 3$ biological replicates). ctrl, control. **d–f**, A colocalization (Mander's coefficient (M1), per cent) of Liperfluo in the EMCS (**d**), mitochondria (**e**) and ER (**f**) masks at time 0, 5, 15 and 30 min after RSL3 (0.5 μ M) treatment in MEFs transiently transfected with AKAP1-mRFP control or 9xL spacer ($n = 3$ biological replicates, the same cells ($n = 7$ mRFP control, $n = 7$ 9xL spacer) were acquired and analysed at each timepoint per condition). Similar experiments and settings have been reproduced independently on Fig. 2, Supplementary Fig. 2 and Supplementary Fig. 3. All quantitative data are the mean $\pm$ s.e.m. In **c**, **d**, **e** and **f**, the statistical significance was determined by a two-way repeated-measures ANOVA, Tukey post hoc test. n.s., not significant ($P > 0.05$), * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$. Panel **a** created with BioRender.com.

particularly oxidized PE (PEox) and oxidized PC (PCox) could be channelled to the mitochondria via ER-mitochondria juxtapositions. We exposed cells for 30 min to RSL3, either alone or in combination with Fer-1, to specifically block RSL3-induced PLox and isolated the cell's input, their respective EMCs and the enriched mitochondria fraction (that is, containing the mitochondria and associated EMCs) (Extended Data Fig. 4a) by ultracentrifugation. We conducted liquid chromatography tandem mass spectrometry (LC-MS/MS)-based lipidomics and redox lipidomics analysis and calculated the fractional distribution of total PE, PC and mitochondrial cardiolipin (CL), as well as all their oxidized forms in the input, EMCs and mitochondrial fraction (Fig. 2f–h,j). These data are also presented as fold changes compared with those of the samples cotreated with Fer-1 (Fig. 2i,k). In non-oxidizing conditions (Fer-1), EMCs were enriched in the overall PE and PC species compared with the input and the mitochondrial fraction (Fig. 2f,g). After 30 min of RSL3, the total content of PEox (Fig. 2h,i) and PCox (Fig. 2j,k) was elevated both in the mitochondrial fraction and EMCs, suggesting the relocation of these PLox between EMCs and the mitochondria, in agreement with the spatial data of the ox-Bodipy C11 (Extended Data Fig. 2a,c,d) and Liperfluo signal (Fig. 2b–d). By contrast, CL oxidation did not change and remained restricted to the mitochondrial fraction (Extended Data Fig. 4b,c). Thus the integration of real-time imaging with liquid chromatography with tandem mass spectrometry (LC-MS/MS)-based lipidomics and redox lipidomics of organellar fractions (Fig. 2l) reveals that EMCs are key interfaces for the initiation and subsequent mitochondrial propagation of PLox during ferroptosis.

Accompanying the accumulation of mitochondrial PLox at 30 min, we observed the fragmentation of the mitochondrial network (Extended Data Fig. 4d–k) the production of mitochondrial superoxide (Extended Data Fig. 4l,m) followed by a steady increase in mitochondrial PLox (Extended Data Fig. 4n). The rapid dynamics of Fer-1 inhibitable PLox redistribution (Extended Data Fig. 3a–f) and

subsequent mitochondrial fragmentation (Extended Data Fig. 4d–k) following IKE probably reflect cell context-dependent mechanisms that control the abundance of the glutathione pool and ferroptosis sensitivity, as described in previous studies^{34,35}.

Hence, during ferroptosis, early PLox ensues at the EMCs and spreads to the mitochondria, triggering their morphological and redox changes.

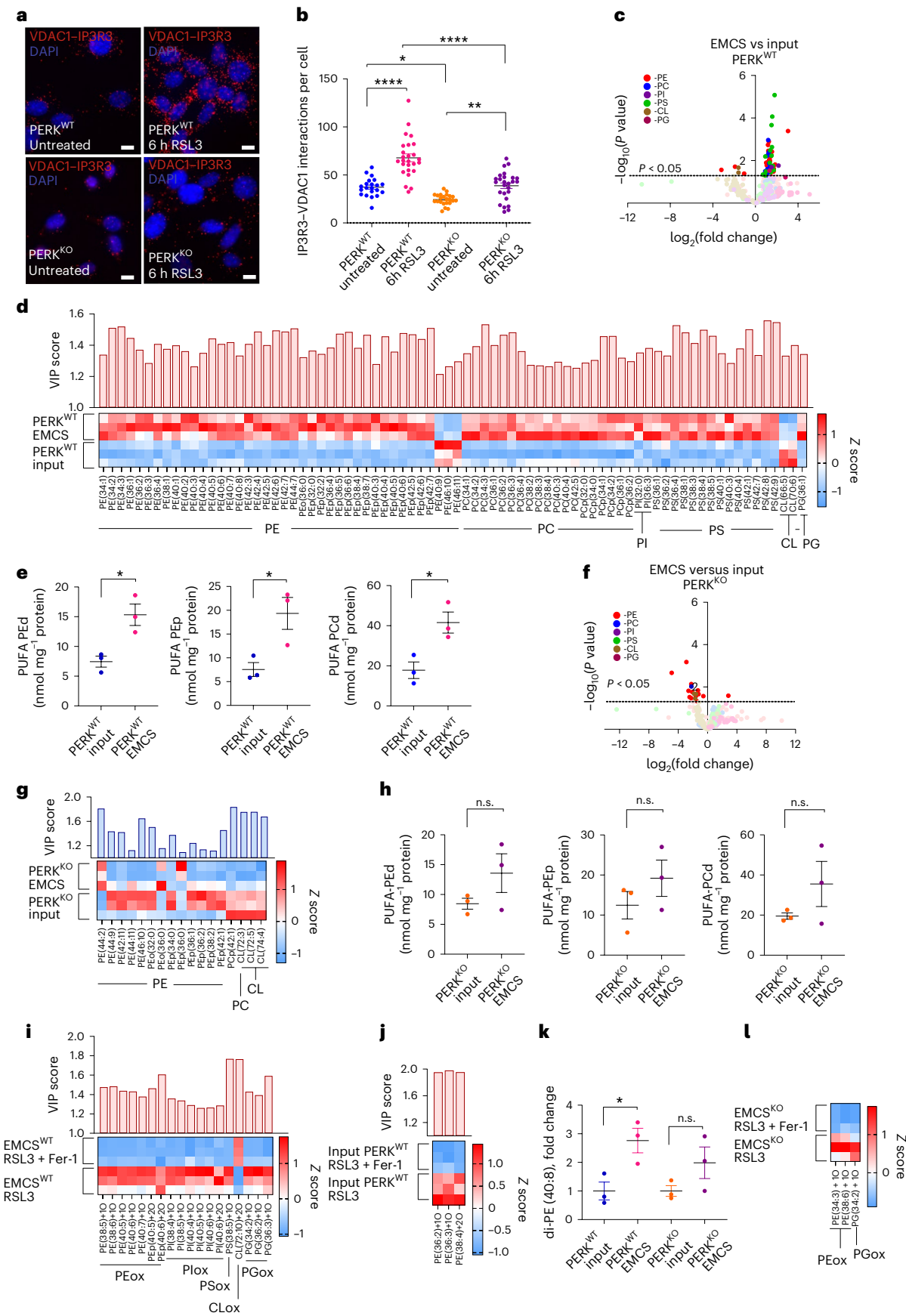
Spacing EMCs protects mitochondria from lipid-ROS attack

We then tested whether preventing ER-mitochondria tethering in response to lipid-ROS affected key steps of the ferroptosis cascade.

We first investigated the effects of the knockout of PERK, the unfolded protein response (UPR) sensor with moonlighting function at the ER-mitochondria contacts independent of its kinase activity^{36–38}. Consistent with our previous studies^{37,38}, the loss of PERK impaired EMCS formation in steady-state conditions (Extended Data Fig. 5a–c). We then transiently transduced wild-type PERK (PERK^{WT}) and PERK-knockout (PERK^{KO}) cells with the fluorescence resonance energy transfer (FRET)-based ER-mitochondria proximity probe (FEMP)²⁴. FEMP contains a FKBP-FRB dimerization domain that, following rapamycin treatment, allows maximal juxtaposition between the two spectral GFP variants targeted to the mitochondria and ER. A brief rapamycin pulse therefore allows measuring of not only the basal but also the extent of the maximal juxtaposition possible between the two organelles³⁹. In PERK^{WT} cells, EMCs rapidly increased after RSL3 treatment with kinetics such as those observed in our previous analysis (from 15 min to 1 h) (Fig. 3a,b). In line with the expected defect in EMCS formation, the basal and maximal FEMP signal was lower PERK^{KO} cells (Fig. 3a,b). Following RSL3, the FEMP signal rapidly and steadily increased in PERK^{WT} cells, whereas it remained consistently reduced in PERK-deficient cells, indicating their inability to form proficient EMCs in response to PLox (Fig. 3a–c). Fer-1 reduced RSL3-mediated EMCS expansion in PERK^{WT} cells to a similar extent to what was observed in

Fig. 5 | EMCs house ferroptosis-prone PUFA-containing PLs. **a**, Representative images (**a**) and quantification (**b**) of in situ PLA in PERK^{WT} and PERK^{KO} MEFs untreated and after RSL3 (0.125 μ M, 6 h) ($n = 3$ biological replicates, images $n = 21$ for PERK^{WT} untreated, $n = 26$ for PERK^{WT} 6h RSL3, $n = 23$ for PERK^{KO} untreated, $n = 26$ for PERK^{KO} 6h RSL3, quantified as in Fig. 1d). Scale bar, 10 μ m. **c**, A volcano plot depicting changes in PL content between input and EMCs in PERK^{WT} MEFs. **d**, A heat map visualizing the individual 78 PL species, based on the VIP scores and a two-sided unpaired *t*-test ($P < 0.05$) in PERK^{WT} MEFs quantified as pmol mg⁻¹ protein and auto-scaled to z-scores and coded blue (low values) to red (high values). The VIP scores for respective PL species are shown on the top ($n = 3$ biological replicates). **e**, The total contents of PUFAs in PUFA-Ped, PUFA-Pep and PUFA-PCd in EMCs versus input in PERK^{WT} MEFs ($n = 3$ biological replicates). **f**, A volcano plot depicting the differences in PL content between input and EMCs samples in PERK^{KO} MEFs ($n = 3$ biological replicates). **g**, A heat map visualizing the changes in the individual PL species based on the VIP scores and a two-sided unpaired *t*-test ($P < 0.05$) in EMCs versus input in PERK^{KO} MEFs. The data are quantified as in **d**. **h**, The total contents of PUFAs in PUFA-Ped, PUFA-PUFA-Pep and PUFA-PCd in EMCs versus input in PERK^{KO} MEFs ($n = 3$ biological

replicates). **i**, A heat map showing the content of the individual 18 PLox species based on the VIP scores and a two-sided unpaired *t*-test ($P < 0.05$) in EMCs from PERK^{WT} MEFs treated with RSL3 (0.125 μ M) in the absence or presence of Fer-1. The data are quantified as in **d** ($n = 3$ biological replicates). **j**, A heat map visualizing the changes in the content of three PEox species based on the VIP scores and a two-sided unpaired *t*-test ($P < 0.05$) in input PERK^{WT} MEFs. The data are quantified as in **d** ($n = 3$ biological replicates). **k**, The content of di-PUFA-PE (40:8) in PERK^{WT} and PERK^{KO} EMCs versus input ($N = 3$ biological replicates). **l**, A heat map depicting individual PEox and PGox species in EMCs from PERK^{KO} MEFs treated with RSL3 (0.125 μ M) with or without Fer-1 (1 μ M). The data are quantified as in **d**. OPLS-DA modelling for oxidized PLs from PERK^{KO} MEFs and EMCs from PERK^{KO} MEFs yielded zero components; therefore, the VIP scores were not available ($n = 3$ biological replicates). In **b**, **e**, **h** and **k**, the quantitative data are the mean $\pm$ s.e.m. In **b** and **k**, the statistical significance was determined by a two-way ANOVA, Tukey (for **b**) or Sidak (for **k**) post hoc test. In **c**, **e**, **f** and **h**, the statistical significance was determined by a two-sided unpaired *t*-test. n.s., not significant ($P > 0.05$), * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.



PERK^{KO} cells, supporting the causal link between lipid-ROS and the early expansion of ER-mitochondria juxtapositions (Fig. 3d). These findings were confirmed by PLA analysis of EMCSs in PERK^{WT} and PERK^{KO} cells (Fig. 3e,f).

Since the loss of PERK could have additional or indirect effects on the composition of the EMCSs^{36,40,41}, we tested whether preventing EMCS expansion and remodelling by an artificial tether could phenocopy the effects observed in PERK^{KO} cells. We introduced in MEFs the engineered tether 9xL containing the mitochondria-targeting sequence AKAP1 (34–63)–RFP linked to the ER marker yUBC6 by a sequence with nine tandem repeats, which we called ‘spacer’ since it separates contact sites at a membrane gap distance of approximately 20 nm (ref. 42) (Fig. 4a). In agreement, the expression of the 9xL spacer reduced EMCSs as measured by FEMP analysis (Extended Data Fig. 5d,e).

We conducted time-lapse imaging in live cells exposed to RSL3 for 30 min (Fig. 4b–g). Notably, transient expression of the 9xL spacer, blunted the overall cellular LiperFluo signal elicited by RSL3 (Extended Data Fig. 5f), the primary PLox accumulation in the EMCSs, the consequent expansion of EMCSs and the secondary accumulation of PLox in the mitochondria (Fig. 4b–f).

Together, these results suggest that distancing EMCSs and/or limiting their dynamics⁴³, either genetically or synthetically, thwarts the initial formation of PLox at these subcellular sites and their subsequent spreading to the mitochondria.

Conforming to our previous studies³⁷, PERK deficiency did not alter the global lipidome (Extended Data Fig. 5g) or the mitochondrial network (Extended Data Fig. 6a–d) in untreated cells. Similar results were obtained in cells transiently expressing the 9xL spacer (Extended Data Fig. 5h and Extended Data Fig. 6e,f). However, consistent with their effects on preventing EMCS expansion, both PERK deficiency and expression of the 9xL spacer blunted mitochondrial fragmentation (Extended Data Fig. 6a–f) in cells undergoing ferroptosis^{9,44}.

PERK deficiency also abolished the subsequent production of mitochondrial lipid-ROS (3 h) and overall cellular PLox (6 h) (Extended Data Fig. 6g,h).

Hence, the remodelling of the mitochondrial network, mitochondrial PL peroxidation and overall lipid-ROS are secondary to the expansion of EMCSs driven by FINs.

Thwarting EMCSs blunts oxidation of proferroptosis PUFA-PLs

Oxidation products from PUFA-PLs, particularly from PE and PC species with one PUFA located in sn-2 position (mono-PUFA) or two PUFAs localized in sn-1 and sn-2 positions of the PE (di-PUFA-PE) and PC molecule (di-PUFA PC) promote ferroptosis^{15,17}.

Following the early increase in ER-mitochondria tethering, the EMCSs remained stable through the first 6 h of RSL3 treatment corresponding to the apex of PLox formation (Extended Data Fig. 1c).

We examined whether preventing EMCS expansion during ferroptosis affects the abundance and composition of PLox at these subcellular sites. We chose PERK^{WT} and PERK^{KO} cells as models for several reasons: loss of PERK precluded (1) EMCS expansion (Figs. 3a–f and 5a,b), (2) the ensuing mitochondrial dysfunctions and (3) overall PLox (Extended Data Fig. 6a–d,g–h). Furthermore, PERK deficiency, by perturbing EMCS integrity, affects the homeostatic transfer of PLs from the ER to mitochondria³⁷, which may modify the proferroptosis lipid landscape of the EMCSs (Fig. 2f–k).

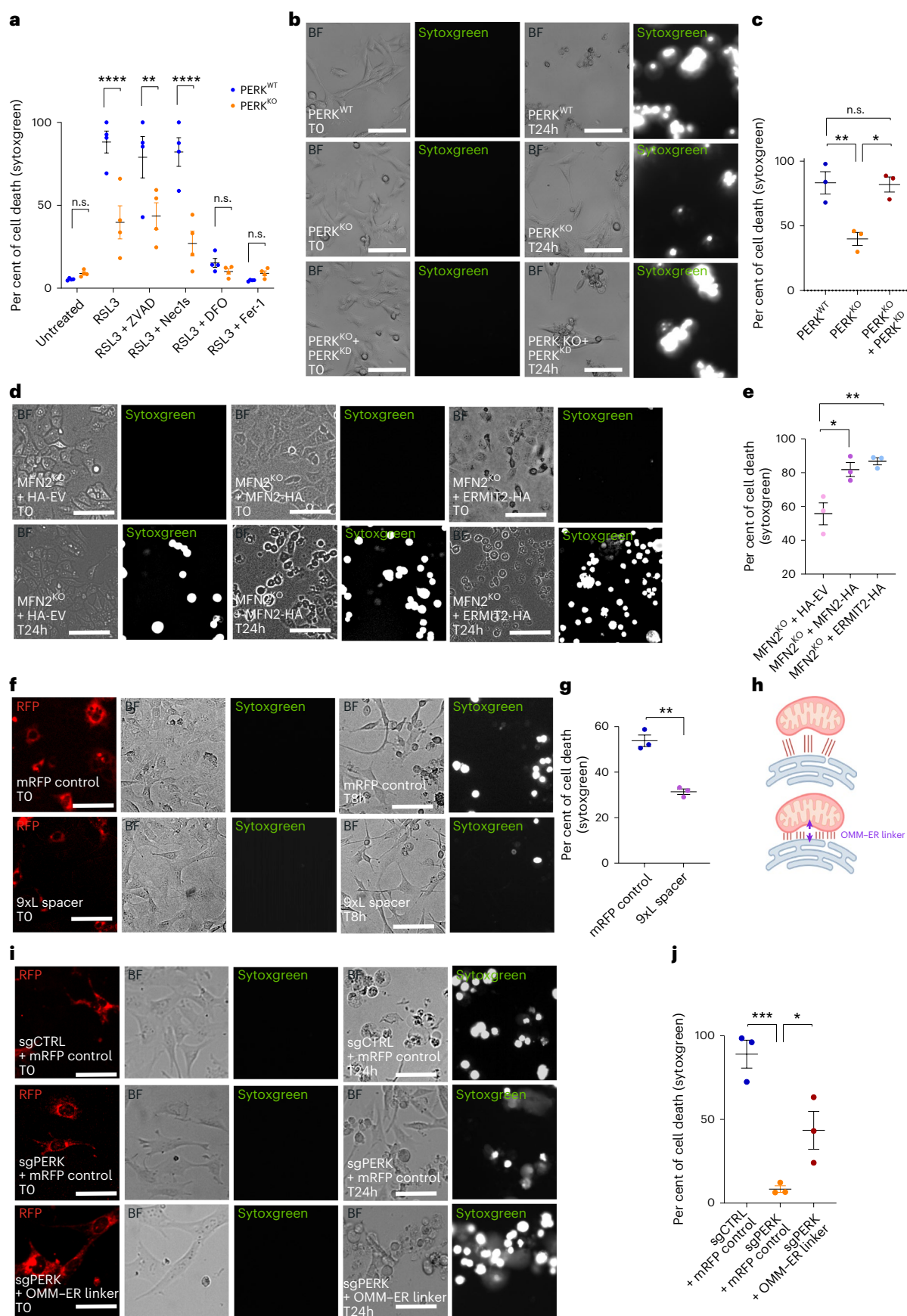
We performed LC-MS/MS-based lipidomics and redox lipidomics in PERK^{WT} and PERK^{KO} cells treated with RSL3 for 6 h, either alone or in combination with Fer-1, and isolated the cell's input and respective EMCSs by ultracentrifugation (Extended Data Fig. 7a).

Following LC-MS/MC phospholipidomics, we conducted orthogonal partial least squares discriminant analyses (OPLS-DA) using all identified non-oxidized PLs. This analysis revealed that input and EMCSs of PERK^{WT} cells clustered into separate groups (Extended Data Fig. 7b). Consistent with the phospholipidomics analysis of early timepoints (Fig. 2f,g) compared with the phospholipidome of the input, the EMCSs of the PERK^{WT} cells were enriched in several PEs and PCs. This fraction also contained several PI, PS, and phosphatidylglycerol (PG) species (Fig. 5c,d). A variable importance in projection (VIP) predictive score plot (with a threshold of 1) generated from OPLS-DA identified 78 PLs species distinguishing input from EMCSs (Fig. 5c). Particularly, 40 PEs, 19 PCs, 2 PIs, 14 PSs, 2 CLs and 1 PG species were identified as significant components that drove the differences between these two groups (Fig. 5d). In the EMCSs of these cells, the contents of 34 PE molecular species out of the total 40 were significantly increased and contained PUFAs with two or more double bonds, thus highly susceptible to oxidation^{9,45} (Fig. 5d). The total levels of PUFA-PE diacyls (PUFA-PEd), PUFA-PE plasmalogens (PUFA-PEp) and PUFA-PC diacyls (PUFA-PCd) were significantly upregulated in the EMCSs of the wild-type cells compared with their respective inputs (Fig. 5e). The same trend, although not significant, was observed for PUFA-PC-plasmalogen, PUFA-PS, PUFA-PI and PUFA-PG (Extended Data Fig. 7c). The inner mitochondrial membrane CL was enriched in the input of the PERK^{WT} cells (Fig. 5d and Extended Data Fig. 7d), thus ruling out cross-contamination of the isolated EMCS fractions by mitochondria membranes.

We analysed the effect of loss of EMCS integrity on their PL composition. OPLS-DA analysis separated input from the EMCSs of PERK^{KO} cells into two distinct clusters (Extended Data Fig. 7e) and identified 17 PLs with high VIP scores (Fig. 5f). Notably, PERK deficiency suppressed the rich PL variety and the highly unsaturated PL species of the EMCSs of the wild-type cells (Fig. 5c,d,f,g), restricting it to only three PE species, of which only one with double bonds (Fig. 5f,g). In these EMCS fractions, several oxidation-prone PUFAs, such as PUFA-PEd, PUFA-PEp and PUFA-PCd (Fig. 5h), were not significantly increased.

Fig. 6 | ER-mitochondria tethering fosters ferroptosis. **a**, The per cent of cell death of PERK^{WT} and PERK^{KO} MEFs at time 0 and after RSL3 (0.125 μ M, 24 h) in the absence or presence of ZVAD (30 μ M), Nec1s (30 μ M), DFO (50 μ M) or Fer-1 (1 μ M) ($n = 4$ biological replicates, three wells per condition per biological experiment). **b**, Representative images of live cells from PERK^{WT}, PERK^{KO} and PERK^{KO} MEFs after transient re-expression of PERK^{KO} at time 0 (T0) and 24 h (T24h) after RSL3 (0.5 μ M). The images show SYTOX Green staining and bright field (BF). Scale bar, 100 μ m. **c**, The per cent of cell death in PERK^{WT}, PERK^{KO} and PERK^{KO} MEFs after transient re-expression of PERK^{KO} after RSL3 (0.5 μ M, 24 h) ($n = 3$ biological replicates). **d**, Representative images of live cells from MFN2^{KO} MEFs transiently transfected with HA-tag-EV, MFN2-HA and ERMIT2-HA at T0 and after RSL3 (0.5 μ M, 24 h). The images show SYTOX Green staining and BF. Scale bar, 100 μ m. **e**, The per cent of cell death in MFN2^{KO} MEFs transiently transfected with HA-tag-EV, MFN2-HA and ERMIT2-HA after RSL3 (0.5 μ M, 24 h). Scale bar, 100 μ m ($n = 3$ biological replicates). **f**, Representative images of live cells from PERK^{WT} MEFs transiently transfected with AKAP1-mRFP control or 9xL spacer at time

0 and 24 h after RSL3 (0.5 μ M). The images show SYTOX Green staining, BF and RFP. Scale bar, 100 μ m. **g**, The per cent of cell death in PERK^{WT} MEFs transiently transfected with AKAP1-mRFP control or 9xL spacer after RSL3 (0.5 μ M, 8 h) ($n = 3$ biological replicates). **h**, A schema of the OMM-ER linker. **i**, Representative images of live cells from PERK^{WT} and PERK^{KO} MEFs transiently transfected with AKAP1-mRFP control and PERK^{KO} transiently transfected with the OMM-ER linker at time 0 and 24 h after RSL3 (0.5 μ M). The images show SYTOX Green staining, BF and RFP. Scale bar, 100 μ m. **j**, The per cent of cell death of PERK^{WT} and PERK^{KO} MEFs transiently transfected with AKAP1-mRFP control and PERK^{KO} transiently transfected with the OMM-ER linker after RSL3 (0.5 μ M, 24 h). Scale bar, 100 μ m ($n = 3$ biological replicates). All the quantitative data are the mean $\pm$ s.e.m. In **a**, the statistical significance was determined by a two-way ANOVA, Sidak post hoc test. In **c**, **e** and **j**, the statistical significance was determined by a one-way ANOVA, Tukey post hoc test. In **g**, the statistical significance was determined by a two-sided unpaired *t*-test. n.s., not significant ($P > 0.05$), * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.



We conducted redox lipidomics of the input and EMCS fractions of PERK^{WT} and PERK^{KO} cells after RSL3 treatment. OPLS-DA analysis based on the PLox species separated RSL3 and RSL3 + Fer-1 samples in different clusters for both input and EMCSs of the PERK^{WT} samples (Extended Data Fig. 7f,g). The presence of Fer-1 protected all major PL species from peroxidation (Fig. 5i,j and Extended Data Fig. 7h,i) except for CL. When compared with their inputs, which showed significant enrichment in three major PLox species (Fig. 5j and Extended Data Fig. 7i), the PLox landscape of the EMCSs of PERK^{WT} cells was highly diversified and contained oxidatively modified species in all major PL classes (Fig. 5i and Extended Data Fig. 7h). Among those, we detected five PLox and one PSox, whose precursors were elevated in the EMCSs of the PERK^{WT} cells but not in those of the PERK^{KO} cells (Extended Data Fig. 8a and Fig. 5k). In wild-type cells, the content of the highly oxidizable di-PUFA PE, PE(40:8), was almost 2.8 times higher in the EMCSs than in the input. By contrast, in the EMCSs of the PERK^{KO} cells, this di-PUFA was not enriched (Fig. 5k). Furthermore, in cells with defective EMCSs (that is PERK^{KO} cells) the presence of PLox was restricted to two PLox and one PGox species (Fig. 5l and Extended Data Fig. 8b).

PERK deficiency did not change the expression of the main cellular antioxidant machinery (Extended Data Fig. 8c–f) or the MUFA to PUFA ratio in any PL species within the input and EMCS fraction significantly (Extended Data Fig. 8g). However, we cannot exclude the possibility that the small changes observed contribute to the overall PERK phenotype.

Together, these results disclose the high vulnerability of the EMCS lipidome to ROS attack.

Enforcing ER–mitochondria tethering endorses ferroptosis

Given the role of ER–mitochondria appositions in controlling the onset and propagation of lipid-ROS signals, we then tested their causal role in ferroptosis. Consistent with their protective effects against EMCS-mediated formation of lipid-ROS, PERK-deficient cells were significantly resistant to ferroptosis induced by different RSL3 and IKE doses when compared with their wild-type counterparts (Fig. 6a and Extended Data Fig. 9a–e), indicating a general protective response against these FINs. ER stress and, in particular, the PERK–ATF4 axis of the UPR has been involved in ferroptosis^{46,47}. To discriminate the possible contribution of PERK kinase-mediated signalling function in ferroptosis, we expressed in PERK^{KO} MEFs the UPR-defective PERK kinase-dead mutant (PERK^{KD}) (Extended Data Fig. 9f), which restores only the PERK tethering function at the EMCSs^{37,38}. Re-expression of PERK^{KD} rescued the vulnerability of the PERK^{KO} cells to ferroptosis to a similar extent found in PERK-proficient cells (Fig. 6b,c), demonstrating that PERK-dependent tethering function is critical for RSL3 cytotoxicity. Consistent with this, chemical inhibition of PERK kinase activity failed to protect cells from ferroptosis (Extended Data Fig. 9g).

Likewise, ferroptosis progressed unaltered in cells expressing the S51A phosphorylation mutant of eIF2 α , the downstream effector of the PERK arm of the UPR (Extended Data Fig. 9h). Hence, loss of PERK impairs ferroptosis irrespective of its UPR function.

We further validated these findings by deleting mitofusin 2 (MFN2), a well-established ER–mitochondria tether^{24,48}. The recently identified MFN2 splice variant ERMIT2 localizes at the EMCS interface and tethers the ER with mitochondria⁴⁹. MFN2^{KO} cells in which MFN2-HA was re-expressed (Extended Data Fig. 9i) succumbed to RSL3-induced ferroptosis while MFN2 deletion (MFN2^{KO}) mitigated it (Fig. 6d,e). Consistent with its tethering function, rescuing ERMIT2-HA expression in MFN2^{KO} cells (Extended Data Fig. 9i) significantly corrected their ferroptosis vulnerability (Fig. 6d,e). Cells expressing the EMCS 9xL spacer were also protected from RSL3-mediated cell death (Fig. 6f,g), thus phenocopying the effects of the genetic loss of the PERK and MFN2 tethers.

We then sought orthogonal support for these findings. We first expressed in PERK^{KO} cells a synthetic outer mitochondrial membrane–ER (OMM–ER) linker, AKAP1 (34–63)–mRFP–yUbc6 (Fig. 6h), which shortens the EMCS proximity up to 5 nm while maintaining membrane identity²⁵. Expression of the OMM–ER linker increased EMCSs, as judged by FEMP imaging (Extended Data Fig. 9j,k) and restored ferroptosis susceptibility of PERK^{KO} cells (Fig. 6i,j). This suggests that forcing very narrow and/or less dynamic EMCSs⁴³ overrules the functional effects caused by the loss of a specific tethering molecule. Likewise, overexpression of the ER-associated Sigma1Receptor (S1R) stimulating ER–mitochondria proximity⁵⁰ augmented the sensitivity of both PERK^{WT} (Extended Data Fig. 9l,m) and PERK^{KO} cells (Extended Data Fig. 9n,o) to RSL3, consistent with a recent report linking the effect of a small molecule CGI1746 inhibitor of the S1R to ferroptosis sensitivity⁵¹.

Hence, all manoeuvres shortening ER–mitochondria contacts enforce ferroptosis, while distancing EMCSs blunts it.

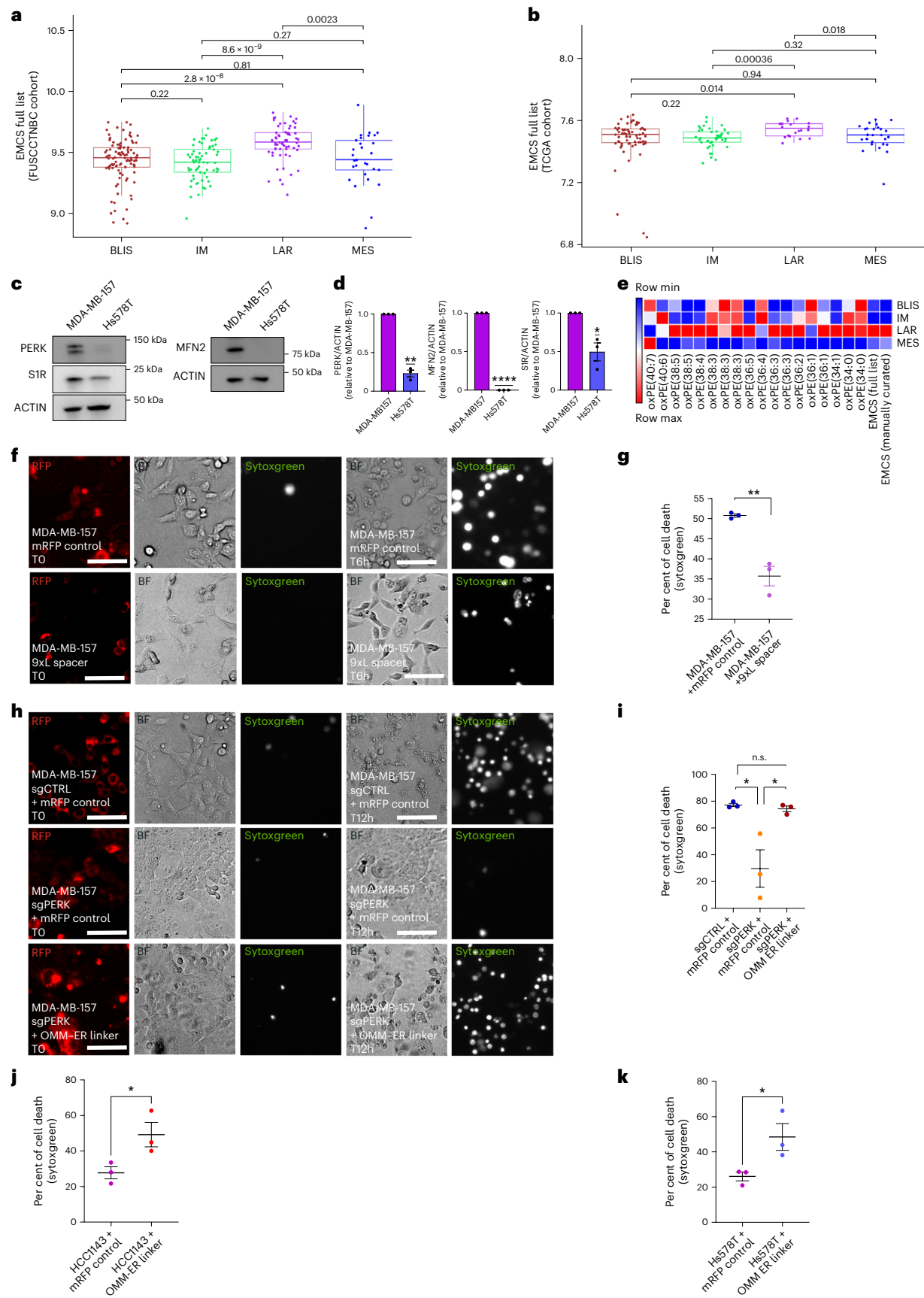
EMCSs shape ferroptosis vulnerability of TNBC subtypes

Dysregulation of ER–mitochondria contacts is a hallmark of several diseased conditions, including cancer^{52,53}. A recent study exploring the ferroptosis landscape of triple-negative breast cancer (TNBC) identified the luminal androgen receptor (LAR) subtype⁴⁵ as the subtype hypersensitive to FINs, compared with the mesenchymal-like (MES), immunomodulatory (IM) or basal-like and immune-suppressed (BLIS) subtypes⁵⁴. Congruently, the MDA-MB-157 cell line, a TNBC LAR subtype, succumbed to ferroptosis, while non-LAR TNBC cell lines were either tolerant (HCC1143, Hs578T) or less sensitive to RSL3 (HCC1937) (Extended Data Fig. 10a,b).

We wondered if the heterogeneity in ferroptosis vulnerability exhibited by the four TNBC subtypes from the extensive cohort of patients (FUSCCTNBC cohort)⁵⁴ could reflect a discrepancy in EMCS

Fig. 7 | EMCSs shape the responses of the triple-negative breast cancer subtypes to ferroptosis. a,b, Box plots of the EMCS-enrichment score, indicating the median line, (1.5 $\times$ interquartile range (whiskers)) (full list) of each sample calculated by ssGSEA across different TNBC subtypes from **a**: FUSCCTNBC cohort (two-sided Wilcoxon's test), $n = 258$ patient sample ($n = 102$ BLIS, $n = 65$ IM, $n = 62$ LAR, $n = 29$ MES) and from **b**: TCGA cohort (two-sided Wilcoxon's test) $n = 158$ patient samples ($n = 67$ BLIS, $n = 43$ IM, $n = 20$ LAR, $n = 28$ MES). **c,d**, Representative immunoblot (**c**) for PERK, S1R and MFN2 in MDA-MB-157 and Hs578T cells and relative quantification (**d**) to MDA-MB-157 cells ($n = 3$ biological replicates). **e**, A heat map visualizing the mean of the EMCS-enrichment score (manually curated and full list) and normalized abundances of PLox across different TNBC subtypes (FUSCCTNBC cohort). min, minimum; max, maximum. **f**, Representative images of cells from sgCTRL MDA-MB-157 cells transiently transfected with AKAP1–mRFP control or 9xL spacer at time 0 (T0) and after RSL3 (0.5 μ M, 6 h (T6h)). The images show SYTOX Green staining, bright field (BF) and RFP. Scale bar, 100 μ m. **g**, The per cent of cell death after RSL3 (0.5 μ M, 6 h) of sgCTRL MDA-MB-157 cells transiently transfected with AKAP1–mRFP control

or 9xL spacer ($n = 3$ biological replicates). **h**, Representative images of sgCTRL and sgPERK MDA-MB-157 cells transiently transfected with AKAP1–mRFP control and sgPERK transfected with OMM–ER linker at time 0 and after 12 h (T12h) of RSL3 (0.5 μ M). The images show SYTOX Green staining, BF and RFP. Scale bar, 100 μ m. **i**, The per cent of cell death (RSL3 (0.5 μ M, 6 h)) in sgCTRL MDA-MB-157 and sgPERK MDA-MB-157 cells transiently transfected with AKAP1–mRFP control and sgPERK with the OMM–ER linker ($n = 3$ biological replicates). **j**, The per cent of cell death after RSL3 (3 μ M, 24 h) of HCC1143 cells transiently transfected with AKAP1–mRFP control or OMM–ER linker ($n = 3$ biological replicates). **k**, The per cent of cell death after RSL3 (1 μ M, 24 h) of Hs578T cells transiently transfected with AKAP1–mRFP control or OMM–ER linker ($n = 3$ biological replicates). In **d**, **g**, **i**, **j** and **k**, the quantitative data are the mean $\pm$ s.e.m. In **d**, the statistical significance was determined by a one-sample *t*-test. In **g**, **j** and **k**, the statistical significance was determined by a two-sided unpaired *t*-test. In **i**, the statistical significance was determined by a one-way ANOVA, Tukey post hoc test. n.s., not significant ($P > 0.05$), $*P \leq 0.05$, $**P < 0.01$, $***P < 0.001$.



gene expression. We performed a single-sample gene set enrichment analysis (ssGSEA) to calculate the enrichment scores of the EMCS gene signature, based on the recently available database of EMCS-associated proteins⁵⁵, on the cohort of TNBC patients⁵⁴ (Fig. 7a). Remarkably, TNBC patients with the LAR subtype⁵⁴ expressed an enrichment in the set of genes encoding for EMCS components (EMCS^{high} gene signature) when compared with the MES, IM or BLIS subtypes (Fig. 7a). This association was also confirmed by a manually curated gene expression dataset (Supplementary Table 1) of functionally characterized EMCS regulators, including PERK, MFN2 and SIR (Extended Data Fig. 10c) and corroborated through analysis of the transcriptomic data of TNBC from the TCGA cohorts (Fig. 7b and Extended Data Fig. 10d).

In line with the expression data, when compared with the ferroptosis-vulnerable MDA-MB-157 cells of the LAR subtype, the non-LAR TNBC cell lines, Hs578T, HCC1143 and HCC1937, exhibited reduced protein levels of PERK, MFN2 and SIR (Fig. 7c,d and Extended Data Fig. 10e–h), suggesting an impairment in ER–mitochondria tethering and communication. Consistently, these non-LAR TNBC cell lines exhibited reduced ER–mitochondria appositions when compared with the MDA-MB-157 cells (Extended Data Fig. 10i). Previous metabolomic analysis indicated that the LAR phenotype, harbouring heightened expression of multiple ferroptosis-related molecules, had a higher PEOx/PUFA-PE ratio when compared with other TNBC subtypes⁵⁴. Using both EMCS gene data sets, we observed that the LAR subtype with the heightened EMCS gene signature (EMCS^{high}) was also associated with a robust enrichment in several PEOx species (Fig. 7e). Together, these data suggest a link between the functional status of the EMCSs and the heterogeneous response to ferroptosis of the different TNBC subtypes.

To test this assumption, we first perturbed EMCS formation in the MDA-MB-157 cell line by expressing the 9xL spacer (Extended Data Fig. 5d,e) or by CRISPR–Cas9-mediated PERK deletion (Extended Data Fig. 10j) and tested the effects of these genetic manipulations on RSL3-induced ferroptosis. Transient expression of the 9xL spacer (Fig. 7f,g) or loss of PERK (Fig. 7h,i) caused a similar resistance to RSL3 (Fig. 7f–i). Rescuing EMCS integrity in PERK-deficient MDA-MB-157 cells by the expression of the OMM–ER linker, restored their ferroptosis responses (Fig. 7h,i).

We then postulated that expression of the OMM–ER linker could sensitize the non-LAR TNBC cells to ferroptosis. Consistent with this, expression of the synthetic linker in Hs578T and HCC1143 cells improved their ferroptosis response to RSL3 (Fig. 7j,k), albeit to a reduced extent when compared with the ferroptosis-primed MDA-MB-157 cells (Fig. 7f,i).

Altogether, these findings indicate that EMCS integrity is a critical determinant of the ferroptosis heterogeneity of TNBC and suggest that an EMCS^{high}-gene signature could be a predictor of TNBC vulnerability to PLOx-induced cell death.

Discussion

Despite accumulating knowledge of the mechanisms governing ferroptosis, the primary intracellular sites of lipid-ROS generation and subsequent damage remain elusive^{56,57}.

In this study, by combining super-resolution microscopy in live cells with subcellular oxidative phospholipidomics, we provide several lines of evidence showing PLOx occurs at the ER–mitochondria interface within minutes of exposure to FINs. Following its onset at the EMCSs, which consequently expand, PLOx propagates to the mitochondria, leading to mitochondrial ROS production and dysfunction. The subsequent buildup of cellular lipid peroxides precipitates ferroptosis.

By probing the lipidome of the EMCSs in unstressed conditions, we demonstrated that EMCSs are particularly enriched in several PUFA-containing PL species, with an abundance of PUFA-containing PEs, with a recognized role in ferroptosis^{10,45}. Tempering with EMCS integrity and functions modifies their PL landscape and halts peroxidation of ferroptosis-prone PLs, including several PUFA-containing PEOx. A recent study reported that di-PUFA-PC is incorporated in the

mitochondria membranes and interacts with complex I of the electron transfer chain, driving the generation of mtROS, while PLOx would occur secondarily at the ER¹⁵. Other studies unravelled that beyond the most abundant redox-sensitive, single PUFA-sn-2-arachidonoyl(AA)-PE and sn-2-adrenoyl(AdA)-PE subspecies, the minor populations di-PUFA-PLs can be oxidized during the early phases of ferroptosis. Among them, the di-PUFA-PE species oxidized by 15LOX at high rates in a Fer-1-inhibitable manner are considered potent proferroptotic mediators¹⁷. We found that EMCSs also house the minor populations of di-PUFA-PE involved in early peroxidation¹⁷, and their amount is reduced by genetically impairing EMCS integrity. Hence, our data strongly advocate the role of EMCSs as a primary shuttling platform for the bidirectional transfer of lipid peroxides between the ER and the mitochondria, which ultimately amplify the initial ferroptosis signals. The distinguished PL composition of the EMCSs explains why these subdomains of the ER membranes are particularly prone to generating ferroptosis peroxidized PUFA-PLs early in cell death.

The ER can connect with other organelles with an essential role in ferroptosis, such as the iron-rich lysosomes or lipid droplets, via the formation of three-way contact sites^{58,59}. This suggests that following the initial formation at the EMCSs, PLOx can propagate to membranes of other organelles in close contact, a possibility that warrants further studies.

Furthermore, we demonstrated that preventing EMCS expansion, by depleting key EMCS tethering regulators both at the side of the ER (PERK, SIR and ERMIT2) or mitochondria (MFN2)⁶⁰ or by synthetically spacing EMCSs, curbed ferroptosis. We recently found that PERK serves as a scaffold for the recruitment of the ER-associated lipid transfer protein extended-synaptotagmin 1 (E-Syt1) at the EMCSs, forming a complex that favours the transfer of key PLs to the mitochondria³⁷. Disrupting endogenous tethers may destabilize molecular complexes and impair lipid transfer function, even when other EMCS components are present. However, whether disturbance in EMCS-associated lipid transfer proteins^{37,61,62} or PL biosynthesis enzymes⁶³ modulates ferroptosis requires future studies.

Notwithstanding, all genetic approaches we used to re-establish or impair EMCS interactions, consistently promoted or inhibited ferroptosis in both murine fibroblasts and human TNBC cell lines.

Notably, the ferroptosis-vulnerable TNBC subtype LAR⁵⁴, hosts an enrichment in the expression of EMCS-associated genes along with a higher amount of oxidized-PE species. By contrast, the ferroptosis-insensitive non-LAR TNBC subtypes harbour reduced gene and protein expression of critical EMCS tethers and dysfunctional ER–mitochondria juxtapositions. Expressing the synthetic linker in the non-LAR TNBC cells or distancing EMCSs in the LAR TNBC subtype, respectively sensitized or inhibited ferroptosis of these cancer cells, demonstrating the functional link between EMCS integrity and the heterogeneous responses of TNBC to this type of cell death. This suggests that EMCS gene expression may serve as a potential biomarker to predict sensitivity to ferroptosis in TNBC.

Hence, while EMCSs serve as critical hubs for cancer cells metabolism and plasticity⁵², their unique biochemical environment also creates vulnerability to ferroptosis. These tantalizing perspectives have potential clinical and therapeutic implications and warrant future studies.

Our study unravels EMCSs as prime hotspots for PLOx driving ferroptosis and suggests that manipulation of ER–mitochondria contacts could be harnessed to regulate cell vulnerability to ferroptosis in diseased conditions.

Online content

Any methods, additional references, Nature Portfolio 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/s41556-025-01668-z>.

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Methods

Cell lines

Simian virus 40 (SV40)-immortalized MEFs proficient or deficient for PERK (PERK^{WT} and PERK^{KO} cells respectively) were a kind gift from Dr David Ron (Cambridge Institute for Medical Research). All MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g l⁻¹ glucose and 0.11 g l⁻¹ sodium pyruvate and supplemented with 2 mM glutamine, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 10% foetal bovine serum (FBS). The triple-negative breast cancer cell lines, MDA-MB-157 and HCC1937 purchased from ATCC, were a gift from Professor Marcus Conrad (Helmholtz Zentrum Munich) and Professor Colinda Scheele (VIB-KU Leuven Center for Cancer Biology), respectively. The breast cancer cell lines HCC1143 and Hs578t were purchased from ATCC and authenticated by BMR genomics⁶⁴.

MDA-MB-157 efficiently integrated the negative control non-targeting single-guide RNA (sgRNA) (MDA-MB-157 sgCTRL) and the EIF2AK3 (MDA-MB-157 shPERK) human sgRNA and Cas9 using 4D-Nucleofector SE solution (Lonza Bioscience). The HCC1937 cells were maintained in Roswell Park Memorial Institute supplemented with 2 mM glutamine, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 10% FBS; MDA-MB-157 (wild type and PERK^{KO}), HCC1143 and Hs578T were maintained in DMEM containing 4.5 g l⁻¹ glucose and 0.11 g l⁻¹ sodium pyruvate and supplemented with 2 mM glutamine, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 10% FBS. MEFs eIF2α wild type (S/S) and eIF2α (S/A) were a kind gift from Professor Frans Schuit (KU Leuven).

Plasmids

PERK.K618A.9E10 pcDNA-myc-tagged plasmid (Addgene plasmid #21815), BFP-KDEL (Addgene plasmid #49150) and Sec61β-mCherry (Addgene plasmid #121160) were obtained from Addgene. AKAP1-mRFP, AKAP1 (34-63)-9x-mRFP-yUBC6 (9xL spacer) and AKAP1 (34-63)-mRFP-yUBC6 (OMM-ER linker) were a kind gift from Professor György Hajnóczky (Thomas Jefferson University). KDEL-HRP-myc was previously reported⁴⁰. SIR-GFP was a kind gift from Professor Paolo Pinton (University of Ferrara). CMV-mito-R-GECO1 was a gift from Dr Robert Campbell (University of Alberta) (Addgene plasmid #46021). FEMP, HA-tag-empty vector (HA-tag-EV), MFN2-HA and ERMIT2-HA were previously reported^{24,49}.

Western blotting

The samples were separated by SDS-polyacrylamide gel electrophoresis on the Criterion system (Bio-Rad Laboratories) on a 4%–12% Bis-Tris gel and electrophoretically transferred to Protran 2-µm-pored nitrocellulose paper (PerkinElmer). The blots were blocked for 1 h at room temperature (RT) in TBS-T buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk and then incubated with selected antibody solutions processed and analysed as described in ref. 37.

Antibodies chemicals and reagents

Anti Mouse Alexa Fluor 488, Thermo Fisher Scientific, A11001, 1:200; anti-rabbit 488 Alexa Fluor (Thermo Fisher Scientific, A-27034, 1:200); anti-mouse Alexa Fluor 647 (Thermo Fisher Scientific, A-21235, 1:200); anti-rabbit Alexa Fluor 647 (Thermo Fisher Scientific, A-21244, 1:200); mouse monoclonal anti ACTIN (Sigma-Aldrich, A5441, clone AC-15, lot no. 0000126949, 1:1,000); rabbit monoclonal anti-ACSL4 (Santa Cruz Biotechnology, sc-271800, clone A-5, lot no. H0923, 1:1,000); mouse monoclonal anti c-myc, (Sigma-Aldrich, M4439, clone 9E10, lot no. 0000184338, 1:1,000); rabbit polyclonal anti calnexin, (Enzo, ADI-SPA-865-F, 1:1,000); mouse monoclonal anti CYTC (BD Bioscience, 556433, clone 7H8.2C12 (RUO), lot no. 9157540, 1:1,000); Duolink In Situ Detection Reagents Red (Sigma-Aldrich, DUO92008, 1:40); Duolink In Situ PLA Probe anti-mouse MINUS (Sigma-Aldrich, DUO92004, 1:5); Duolink In Situ PLA Probe Anti-Rabbit PLUS (Sigma-Aldrich,

DUO92004, 1:5); Duolink In Situ Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, DUO82040); rabbit monoclonal anti GAPDH (Cell Signaling, 2118S, clone 14C10, lot no. 16, 1:1,000); rabbit polyclonal anti GPX4 (Cell Signaling, 52455 1:1000); rabbit polyclonal anti HA-Tag (C29F4) (Cell Signaling, 3724S, 1:1,000); mouse monoclonal anti-IP3R3 (BD Bioscience, 610312, clone 2:IP3R3 (RUO), lot no. 2329334, 1:100); rabbit polyclonal anti-MFN2 (Abcam, ab50838, 1:1,000); rabbit polyclonal anti PERK, Cell Signaling, 3192S, 1:1,000); mouse monoclonal anti TOMM20 (Abcam, ab186735, Abcam, EPRI5581-54, lot no. 1011221-48, 1:100); rabbit polyclonal VDAC1 (Abcam, ab15895, 1:100); rabbit polyclonal VDAC1 (Cell Signaling, 4866S; 1:1,000); rabbit polyclonal anti SLC7A11 (Thermo Fisher Scientific, PA1-16893, 1:1,000).

The reagents used were: Bodipy 581/591 C11 (Thermo Fisher Scientific); Liperfluo (Dojindo), D-glucose (Sigma-Aldrich, G7021-1KG); DAPI (Thermo Fisher Scientific, 62248); DAPI (Thermo Fisher Scientific, D1306); DMEM-high glucose (Sigma-Aldrich, D0422); Gibco DMEM/F-12 (Thermo Fisher Scientific, 11320074); Duolink In Situ Mounting Medium with DAPI (Sigma-Aldrich, DUO82040); GSK2606414 PERK Inhibitor (Toronto Research Company, G797800); MitoPerox (Abcam, ab146820); MitoTracker Far Red (Thermo Fisher Scientific, M22426); MitoTracker Green (Thermo Fisher Scientific, M7514); SE Cell Line 4D-NucleofectorTM X Kit L (Lonza Bioscience, V4XC-1024); P3 Cell Line 4D-NucleofectorTM X Kit L (Lonza Bioscience, V4XP-3024); penicillin and streptomycin (Sigma-Aldrich, P0781); Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, 32106×4); Pierce Protease Inhibitor Tablets, EDTA-free (Thermo Fisher Scientific, 88266); potassium chloride (Janssen Chimica, 7447407); protease inhibitor (Thermo Fisher Scientific, A32953); saponin (Sigma-Aldrich, 8047152); Gibco, Roswell Park Memorial Institute Sodium Chloride, (Sigma-Aldrich, A0431796); SYTOX Green (Sigma-Aldrich, S7020); TransIT-X2 Dynamic Delivery System (Mirus Bio, MIR 6000); Tris base (Sigma-Aldrich, 77861); Triton (Sigma-Aldrich, T9234); Tween (Sigma-Aldrich, P4780).

The plates used for microscopy were: glass-bottom dishes, 35 mm uncoated, glass no. 1.5 (Case Mattek corporation, P35G-1.5-14-C); 6-well glass-bottom plates (Cellvis, P06-1.5H-N); 24-well glass-bottom plate with high performance no. 1.5 cover glass P24-1.5H-N; CultureWell Chambered Coverglass for Cell Culture, 16 wells per coverglass, C37000.

The chemical used were: BME (21985023); BAPTA-AM (B1205); Cas9 (2NLS Biolegio); CDDP (Sigma, CAS 15663-27-1); DFO (Sigma-Aldrich, D9533-1G); Fer-1 SML0583-5MG (Sigma-Aldrich, SML0583-5MG); IKE (Selleckchem, S8877); Nec1s (Sigma-Aldrich, 2263-1MG); staurosporine (Sanbio, 81590-1); rapamycin (Adipogen, AG-CN2-0025); RSL3 (Selleckchem, S8155-5MG); 1R,3R-RSL3 (Biotechnie, 6687/10); sg EIF2AK3 human (Biolegio); ZVAD (Bachem, 4027403.0005).

Cell transfection

The MEFs were transiently transfected with PERK.K618A.9E10 pcDNA-myc tagged, BFP-KDEL, mt-GECO1-RFP, AKAP1-mRFP, AKAP1 (34-63)-9x-mRFP-yUBC6 (9xL spacer), AKAP1 (34-63)-mRFP-yUBC6 (OMM-ER linker), KDEL-HRP, HA-tag-EV, MFN2-HA, ERMIT2-HA and SIR-GFP using TransIT-X2 transfection reagent accordingly to the manufacturer's instructions or electroporated with 4D-Nucleofector (Lonza Bioscience) using P3 Cell Line kit (V4XP-3024, Lonza Bioscience) for MEFs (CA-137 Program) or SE Cell line Kit (V4XC-1024, Lonza Bioscience) for MDA-MB-137 cells (Program DN-100). A total of 24 h after transfection, the cells were replated to microscopy culture dishes or collected for lysate after 48 h.

CRISPR-Cas9 gene knockout

The genes were deleted by nucleofecting (SE Cell line 4D-Nucleofector kit Lonza V4XC-1024). Ribonucleoprotein complexes (RNPs) consisting of pooled sgRNAs conjugated with Cas9. The RNPs were prepared by

coincubating 500 pmol of sgRNA pools (for EIF2AK3) with 100 pmol of Cas9 2NLS (Synthego) and introducing the RNPs into MDA-MB-157 using the DN-100 program in Lonza 4D-Nucleofector. A total of 48 h after transfection, the cells were collected for western blot analysis and expanded.

Electron microscopy

MEF PERK^{WT} and PERK^{KO} expressing HRP-KDEL-myc were fixed on coverslips with 1.3% glutaraldehyde in 0.1 M cacodylate buffer and washed in 0.1 M ammonium phosphate (pH 7.4) buffer for 1 h, and HRP was visualized with 0.5 mg ml⁻¹ DAB and 0.005% H₂O₂ in 0.1 M ammonium phosphate (pH 7.4) buffer, following the same procedures as described in ref. 37. The untreated MEFs, the MEFs pretreated with RSL3 for 15 min or RSL3 cotreated with Fer-1 for 15 min were fixed with 1.25% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate at pH 7.4 for 1 h at RT. Thin sections were imaged on a Tecnai-20 electron microscope (Philips-FEI), at the Bioimaging facility of the Department of Biology, University of Padova.

PLA

A PLA was performed using Duolink In Situ Red Started Kit Mouse/Rabbit (Duolink In Situ Detection Reagents Red (Sigma-Aldrich, DUO92008); Duolink In Situ PLA Probe Anti-Mouse MINUS (Sigma-Aldrich, DUO92004); Duolink In Situ PLA Probe Anti-Rabbit PLUS (Sigma-Aldrich, DUO92004); Duolink In Situ Mounting Medium with DAPI (Sigma-Aldrich, DUO82040)) according to the manufacturer's instructions. Briefly, after treatment with RSL3 at the different timepoints, MEFs were fixed with 4% PFA and permeabilized with 0.1% Triton and further processed as described in ref. 37. The PLA images were acquired with an Olympus IX73 (Olympus), cellSens Dimension acquisition software and 63× magnification.

Subcellular fractionation

The MEFs were treated with RSL3 or cotreated with Fer-1 to block lipid peroxidation, and after the specified period (30 min or 6 h), the cells were collected and the resulting pellet after centrifugation (600–800g for 5 min at RT) was resuspended in 5 ml of starting buffer 1 containing 225 mM mannitol, 75 mM sucrose and 30 mM Tris–HCl and 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), pH 7.4 and homogenized. The unbroken cells and nuclei were removed by centrifugation of the cell homogenate at 600g for 5 min (at 4 °C). The crude mitochondrial fraction (mito crude) was pelleted by centrifuging the supernatant at 7,000g for 10 min at 4 °C. The crude mitochondria were resuspended in starting buffer (SB) (225 mM mannitol, 75 mM sucrose and 30 mM Tris–HCl, pH 7.4) and after other two sequential centrifugations (7,000g and 10,000g for 10 min at 4 °C), the obtained pellet was resuspended in 1 ml of mitochondria resuspending buffer MRB (250 mM mannitol, 5 mM HEPES buffer and 0.5 mM EGTA), layered on top of a percoll gradient (30% and 15%) and spun down at 95,000g for 40 min (using a Beckman ultracentrifuge, rotor SW41), to separate EMCSs from the pure mitochondria (mito pure). Further ultracentrifugation (70Ti rotor, 100,000g for 1 h at 4 °C) was required to obtain the pellet of the EMCS fraction.

Lipidomics and redox lipidomics

After isolation of the subcellular fractions from 6 h RSL3 (0.125 μM) treatment or RSL3 (0.125 μM) cotreated with Fer-1 (1 μM) MEFs, we analysed through liquid chromatography–mass spectrometry lipidomics the presence of oxidative PL forms. This procedure was conducted according to the published protocol cited in ref. 65.

After transient transfection of MEFs with AKAP1–mRFP control or 9xL spacer, the cell pellets were analysed through mass spectrometry-based lipidomic using the Lipometrix platform (KU Leuven). This procedure was conducted according to the published protocol in ref. 37.

MitoPerox and Bodipy C11 FACS analysis

After ferroptosis treatments at different timepoints, the cells were washed, trypsinized, collected and then stained for MitoPerox (400 nM) or Bodipy 581/591 C11 (2.5 μM) for 30 min. The samples were then washed two times with cold PBS and processed using BD FACS Canto II BD Canto II or Attune flow cytometer. An analysis was performed using FlowJo software.

Cell death detection

The cells were incubated with RSL3 or IKE at the concentrations and for the time indicated in the legends. When transiently or virally transfected, MEF and MDA-MB-157 cells were treated with 0.5 μM RSL3 while HCC1143 and Hs578T cells were treated with 3 μM and 1 μM RSL3, respectively. To assess the specificity of the cell death, the cells were preincubated with the pan-caspase inhibitor ZVAD (30 μM) or the necroptosis inhibitor or Nec1s (30 μM) 2 h before the treatments, while the iron chelator DFO (50 μM), the radical-trapping antioxidant Fer-1 (1 μM) or the reducing agent BME (50 μM) were added in the medium together with RSL3/IKE. The cell death was measured after 24 h of staurosporine (1 μM) treatment and 48 h after CDDP (100 μM) treatment of MEFs. The cell death was evaluated by the fluorescent viability dye Sytogreen and quantified either by using the Flexstation plate reader at 488/523 nm (FlexStation; Molecular Devices) or by counting the number of SYTOX-Green-positive cells normalized on the total amount of cells before the treatment using an Olympus IX73 (Olympus) or by the Operetta High Content imaging system (PerkinElmer, University of Padova) with incubator and CO₂ chamber. When the cells were manually counted, multiple areas in the same well were acquired and analysed at each timepoint per condition and biological replicate.

Live cell imaging

Time-lapse 3D live cell imaging was acquired with the high-resolution confocal Zeiss LSM 880–Airyscan (Cell and Tissue Imaging Cluster), ZEN 2.3 SP1 acquisition software, objective 63×, numerical aperture 1.4 oil, equipped with temperature (37 °C), CO₂ and a humidity-controlled incubator. The cells were imaged in Krebs solution (150 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 11.6 mM HEPES (pH 7.3), 11.5 mM glucose and 1.5 mM CaCl₂). For mitochondrial and EMCS live imaging, the cells were incubated with MitoTracker green or Far Red 20 nM (M7514, Thermo Fisher Scientific) for 30 min and washed in the physiological Krebs solution (39.47 g NaCl, 2.3 g KCl, 11.88 g glucose monohydrate, 14.3 g HEPES, 1.26 g MgCl₂·6aq, 3.97 g EGTA, 1.15 g CaCl₂·2aq and 5,220 ml MQ, pH 7.4) and then imaged with a Zeiss LSM 880–Airyscan (Cell and Tissue Imaging Cluster). RSL3 or IKE were added directly to the dish after images of the untreated condition were taken. The same imaging procedure was conducted for MEFs transfected with AKAP1–mRFP control or 9xL spacer or BFP–KDEL and acquired as well with the Zeiss LSM 880–Airyscan (Cell and Tissue Imaging Cluster, 63× magnification, equipped with temperature, CO₂ and humidity-controlled incubator. MitoSOX staining 5 μM, Bodipy 581/591 C11 2.5 μM or Liperfluo (10 μM) were preincubated for 30 min and then costained with MitoTracker Far Red 20 nM following the same staining and imaging procedure as described above.

Immunostaining

After inducing ferroptosis, the cells were fixed in PFA 4% for 10 min. After washing, the cells were permeabilized in 0.1% saponin in 5% normal goat serum for 1 h at RT. The primary antibodies were incubated 1:100 diluted in 0.1% saponin and incubated ON at 4 °C. The secondary antibodies were incubated in anti-rabbit/mouse–488 nm– and anti-mouse/rabbit–647 nm– for 1 h at RT. The cells were incubated with DAPI (1:1,000) in PBS for 10 min and successively added prolong gold in PBS and stored at 4 °C. The images were taken with the Zeiss LSM 880–Airyscan (Cell and Tissue Imaging Cluster, 63× magnification).

Ca²⁺ imaging

MEFs transiently transfected with mito-R-GECO1 were loaded with 3 μ M Cal520 at RT in modified Krebs solution (150 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 11.6 mM HEPES (pH 7.3), 11.5 mM glucose and 1.5 mM CaCl₂) for 30 min. This was followed by a de-esterification step in the absence of extracellular Cal520 for 30 min at RT. The cells were washed and were added to Krebs solution without CaCl₂ and EGTA 3 mM to chelate the extracellular Ca²⁺. The cytosolic Ca²⁺ release (Cal520, excitation 493, emission 515) and mitochondrial Ca²⁺ uptake (mito-R-GECO1, excitation 560, emission 584) were monitored in live cell imaging using a Zeiss Axio Observer Z1 inverted microscope. The baseline was recorded for 60 s before the addition of RSL3 for 15 min followed by Ca²⁺ triggers (ATP, 100 μ M).

FEMP imaging and high-throughput EMCS measurements

PERK^{WT} and PERK^{KO} MEFs were infected with an adenovirus carrying the FEMP probe complementary DNA at a multiplicity of infection of 10. After 24 h, the cells were imaged using an Operetta CLS High Content imaging system (Revvy) with the following filter settings: CFP (excitation 410–430, emission 460–500), YFP (excitation 490–510, emission 520–560) and YFP FRET (excitation 410–430, emission 520–560). RSL3/RSL3 + Fer-1/RSL3 + DFO were added to the wells after measuring the baseline FRET intensity of the untreated samples. To image the maximum FRET intensity (FRETmax), the cells were treated with 100 nM rapamycin for 15 min. The images were analysed using Harmony 3.5 image analysis software (Revvy). The YFP channel was chosen to mark the region of interest, and around each region of interest, a second boundary was drawn to measure the background intensity. The cells pretransfected with AKAP1-mRFP, AKAP1 (34–63)-linker, 9x-mRFP (9xL spacer) or AKAP1 (34–63)-mRFP-yUBC6 (OMM-ER linker) were selected on the basis of RFP-positive cells. FRETbasal, FRETmax and FRET ratio were calculated as previously described²⁴. The MDA-MB-157 cells of the LAR subtype and the non-LAR TNBC cells, Hs578T, HCC1143 and HCC1937 were seeded in a 384-well microplate (PhenoPlate, PerkinElmer) and the next day were transfected with the Sec16 β -EGFP plasmid using the jetOPTIMUS transfection reagent (Polyplus). A total of 24 h after transfection, the cells were incubated with 100 nM MitoTracker Far Red for 30 min at 37 °C in serum-free media, washed once and imaged at 37 °C and 5% CO₂ using the Operetta CLS High Content Analysis System (Revvy) with a 63 $\times$ water flow objective. The images were analysed in high-throughput mode using Harmony (Revvy).

Gene set expression analysis for EMCS from a publicly available dataset

The bulk RNAseq expression matrix for 360 samples from FUSCC TNBC cohort⁶⁶ was downloaded from The National Omics Data Encyclopedia (NODE: OEP000155; GEO: [GSE118527](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118527)). A total of 258 samples matched with lipidomics analysis were used for further analysis. The lipidomic data (log₂ transformed abundance of MS2 annotated lipids in TNBC samples) were downloaded from the supplemental information of the article⁵⁴. The bulk RNAseq expression counts were downloaded for 158 The Cancer Genome Atlas Breast Invasive Carcinoma (TCGA-BRCA), samples of patients with TNBC using TCGA biolinks package in R (TCGA breast cancer data: (<https://portal.gdc.cancer.gov>); TCGA-BRCA, dbGaP accession phs000178). The patient samples were labelled as BLIS, IM, LAR and MES, representing distinct subtypes of TNBC, as outlined in the previously published article⁵⁴. To calculate the EMCS score, we downloaded a set of gene from this work⁵⁵ and used only genes annotated as EMCS. We calculated two sets of score, one using a full gene list and the other using a manually curated gene list based on previously available literature. We calculated the enrichment score for the signature gene set using an ssGSEA for each patient sample using the R Bioconductor package GSVA (version 1.49.0). The enrichment score for EMCS was analysed across different subtypes of

TNBC as defined by Yang et al.⁵⁴, and the statistical significance was calculated using Wilcoxon's test.

Image processing, analysis and statistics

For two-dimensional (2D) and 3D live imaging, an EMCS area or volume analysis was performed using ImageJ/Fiji software (<http://imagej.nih.gov/ij/>). The masked area and volume for the ER and mitochondria were created using IJ IsoData threshold method, and their area and volume were analysed. The EMCS mask was created by the overlapping region between ER and mitochondria (EMCS) (ImageCalculator).

The area outside ER and mitochondria was created by Bodipy C11 Red mask minus ER plus mitochondria masks. The per cent of EMCS was measured as a ratio of EMCS area or volume and ER area or volume. We measured the intensity of oxidized Bodipy 581/591 C11 (green) on total Bodipy 581/591 C11 signal (red) or for Liperfluo the integrated density (IntDen) and normalized on total cellular volume. A colocalization analysis was performed using Mander's overlap coefficient above the threshold value defined using Otsu on the oxidized Bodipy 581/591 C11 (green) and Liperfluo in the EMCSs, mitochondrial and ER masks (Mander's coefficient, Jacob plugin, Fiji). For a 3D rendering representation, Imaris 10.1 software was used to visualize the surfaces of the different channels (Liperfluo, ER and mitochondria) together with the EMCS mask. For live imaging of MEFs costained with MitoSOX green and MitoTracker Far Red, the intensity of MitoSOX on MitoTracker Far Red was measured. The mitochondrial morphology was measured with Imaris 10.1. The mitochondrial fragmentation was calculated using the volume statistic features and measured as a fragmentation index (from 0 to 1), equal to the sum of small volumes normalized on the total volume. The PLA images were analysed manually with 'Threshold' and 'Analyze particles' commands from ImageJ/Fiji Software. The number of EMCSs per mitochondria in HRP-stained Epon sections of PERK^{WT} and PERK^{KO} cells were quantified using ImageJ/Fiji Software. TEM morphometric measurements in MEFs untreated and after treatment or cotreatment with RSL3 or RSL3 + Fer-1 were carried out using ImageJ/Fiji Software. For calculations of mitochondria-ER distance, 70 images per condition were considered, and the minimal distance of the ER located in a 30 nm radius from the considered mitochondria was computed. The ERMICC was calculated as previously described²⁴.

All data are represented as the mean $\pm$ standard error of the mean (s.e.m.). The statistical significance between two groups was determined by a standard unpaired two-sided *t*-test with *F*-testing or a one-sample *t*-test. The data distribution was assumed to be normal, but this was not formally tested. The statistical significance between multiple groups was determined by a one-way analysis of variance (ANOVA) to ensure comparable variance, then individual comparisons were performed by Tukey's or Sidak's post hoc test. The analysis was done in Prism v9.0f, GraphPad. The asterisk represents a *P* value < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001, where a *P* value < 0.05 is considered significant. No statistical methods were used to predetermine the sample sizes, but our sample sizes are similar to those reported in previous publication³⁷. The investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary

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

Data availability

Data supporting the findings of this work are available in this study and its Extended data figures and the Supplementary Information. All the data generated and analysed in this study are available from the corresponding author upon request. Source data are provided with this paper.

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Author contributions

M.L.S. and P.A. designed the experiments. M.L.S. performed the majority of all the experiments presented in the manuscript. LC–MS/MS lipidomic analysis and data interpretation were carried out by Y.T., V.T., H.B. and V.K. J.S. helped with lipidomic analysis on the Lipometrix platform (KU Leuven). A.D. created the FEMP adenovirus. FRET imaging was performed and interpreted by M.L.S. with the assistance from A.D. and L.S. E.V. helped with the execution of part of the experiments. S.M. performed the gene set expression analysis for EMCS from publicly available dataset. The Ca²⁺ measurements were performed and interpreted by M.L.S. with the assistance of R.L.R. and G.B. F.G. acquired the HRP–KDEL-stained EM images. The imaging analysis was performed by M.L.S. with the assistance of B.P. B.P. wrote the scripts for the 2D image analysis. The EM images were acquired at the Bioimaging facility of the Department of Biology, University of Padova and the Institute of Integrative Cell Biology, Paris. P.A. supervised the research and interpreted the results with M.L.S. Both P.A. and M.L.S. wrote the manuscript. P.A. oversaw the project and acquired funding. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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