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TEX2 controls mitochondrial dynamics at ER-mitochondria MCSs

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Abstract

Membrane contact sites (MCSs) are specialized regions where organelles come into close apposition to promote the exchange of lipids, ions, and metabolites without exploiting any fusion events (Csordás et al., 2006; Prinz et al., 2020; Scorrano et al., 2019). Among them, ER-mitochondria contact sites, also known as mitochondria-associated membranes (MAMs), represent important hubs for metabolic coordination, controlling calcium signaling, phospholipid synthesis, autophagy initiation, and mitochondrial dynamics (Vance, 2014; Rowland & Voeltz, 2012; Voeltz & Carvalho, 2024). The lipid composition of MAMs is a critical determinant of their function, influencing membrane curvature, protein recruitment, and the activity of the mitochondrial fission machinery (Friedman et al., 2011; Nagashima et al., 2020). Yet, the molecular mechanisms that control lipid transfer at these interfaces remain far to be fully unravelled.

In this study, we identify TEX2, a conserved ER-resident protein containing a Synaptotagmin-like Mitochondrial-lipid-binding Protein (SMP) domain, as a novel tethering factor at MAMs. TEX2 is the mammalian ortholog of the yeast SMP protein Nvj2p, an ER protein enriched at CSs between the nucleus and vacuole, that senses the ceramide levels increase under stress conditions and moves to the the ER-Golgi MCSs promoting the non-vesicular transport of ceramide from the ER to the Golgi (Liu et al., 2017). Building on this evolutionary link, we show that TEX2 localizes to tubular ER subdomains enriched at ER-mitochondria interfaces, where it contributes to the structural and functional integrity of MAMs. TEX2 depletion in human cells leads to reduced ER-mitochondria contacts, mitochondrial elongation, and defective DRP1 activation, phenocopying the loss of canonical MAM tethers such as PDZD8 or ORP5/8 (Hirabayashi et al., 2017; Monteiro-Cardoso et al., 2022). These data indicate that TEX2 is required for efficient mitochondrial fission and organelle coupling. Mass spectrometry analyses of co-immunoprecipitation experiments revealed that TEX2 interacts with FUNDC1, a mitochondrial outer-membrane protein that recruits DRP1 and regulates mitochondrial fission and mitophagy in response to hypoxia (Wu et al., 2016; Chen et al., 2016; Cai et al., 2021). FUNDC1 depletion phenocopied TEX2 knockdown in the absence of stress, resulting in impaired ER-mitochondria association and perinuclear clustering of lysosomes, suggesting that TEX2 and FUNDC1

cooperate within the same pathway to sustain MAM structure and mitochondrial remodeling under basal conditions.

Mechanistically, we propose that TEX2 mediates ceramide transfer to mitochondria through its SMP domain, generating lipid microdomains that favor membrane constriction and DRP1 recruitment. Ceramide, due to its conical shape and high hydrophobicity, induces local negative curvature and facilitates mitochondrial scission (Smith et al., 2013; Ausman et al., 2018; Fugio et al., 2020). Thus, by coupling lipid transport with structural tethering, TEX2 may provide the lipid context necessary for FUNDC1–DRP1 complex formation and activation.

Beyond the ER-mitochondria interface, TEX2 also localizes to ER-lysosome contact sites, where it interacts with the phosphoinositide phosphatase TMEM55B (Du et al., 2023). We demonstrate that TEX2 is also a tether between the ER and the lysosome, and that TEX2 depletion increases mitochondria–lysosome contacts, a phenotype typically associated with impaired mitochondrial fission (Wong et al., 2018; Boutry & Kim, 2021). These findings support a model in which TEX2 coordinates lipid flux and structural communication between multiple organelles, acting as a hub at tripartite ER–mitochondria–lysosome junctions.

Altogether, our results identify TEX2 as a novel tethering molecule acting at MAMs interface, regulating mitochondrial dynamics by interacting with FUNDC1. TEX2 may integrate lipid transfer, through the activity of its SMP domain, and tethering mechanisms to ensure efficient DRP1-dependent fission and organelle turnover, possibly by regulating ceramide flux. This work unveils a novel TEX2–FUNDC1 axis that may couple sphingolipid flux to mitochondrial remodeling, advancing our understanding of how lipid-driven signals govern the architecture and plasticity of inter-organelle contact sites.

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List of abbreviations

ABC — ATP-Binding Cassette

ALS — Amyotrophic Lateral Sclerosis

AP-MS — Affinity Purification–Mass Spectrometry

APP — Amyloid Precursor Protein

ATAD3A — ATPase Family AAA Domain-Containing Protein 3A

ATCC — American Type Culture Collection

ATG — Autophagy-Related

ATG14 — Autophagy-Related Protein 14

ATG2 — Autophagy-Related Protein 2

ATG5 — Autophagy-Related Protein 5

ATP — Adenosine Triphosphate

BAP31 — B-Cell Receptor-Associated Protein 31

BECLIN1 — Autophagy Protein Beclin-1

BFP — Blue Fluorescent Protein

BOK — BCL-2-Related Ovarian Killer

BRET — Bioluminescence Resonance Energy Transfer

CANX — Calnexin

CEPIA — Calcium-Measuring Organelle-Entrapped Protein Indicators

CERT — Ceramide Transfer Protein

CFP — Cyan Fluorescent Protein

CK2 — Casein Kinase 2

CLEM — Correlative Light And Electron Microscopy

CLNX — Calnexin

CRISPR — Clustered Regularly Interspaced Short Palindromic Repeats

DAG — Diacylglycerol

DGK — Diacylglycerol Kinase

DMEM — Dulbecco’s Modified Eagle Medium

DMSO — Dimethyl Sulfoxide

DNA — Deoxyribonucleic Acid

DNM1L — Dynamin-1-Like Protein

DRP1 — Dynamin-Related Protein 1

ECL — Enhanced Chemiluminescence

EDTA — Ethylenediaminetetraacetic Acid

EGFP — Enhanced Green Fluorescent Protein

EGFR — Epidermal Growth Factor Receptor

EGTA — Ethylene Glycol-Bis(β -Aminoethyl Ether)-N,N,N',N'-Tetraacetic Acid

EM — Electron Microscopy

ER — Endoplasmic Reticulum

ERK — Extracellular Signal-Regulated Kinase

ERMES — ER–Mitochondria Encounter Structure

ESCRT — Endosomal Sorting Complex Required For Transport

ESYT1 — Extended Synaptotagmin 1

ESYT2 — Extended Synaptotagmin 2

ESYT3 — Extended Synaptotagmin 3

FAPP2 — Four-Phosphate Adapter Protein 2

FAST — Fluorescence-Activating And Absorption-Shifting Tag

FBS — Fetal Bovine Serum

FCCP — Carbonyl Cyanide-p-Trifluoromethoxyphenylhydrazone

FDR — False Discovery Rate

FFAT — Two Phenylalanines In An Acidic Tract

FIS1 — Mitochondrial Fission 1 Protein

FKBP — FK506-Binding Protein

FKBP15 — FK506-Binding Protein 15

FKBP8 — FK506-Binding Protein 8

FRET — Förster Resonance Energy Transfer

FUNDC1 — FUN14 Domain-Containing Protein 1

FUNDC2 — FUN14 Domain-Containing Protein 2

FYCO1 — FYVE And Coiled-Coil Domain-Containing Protein 1

GAP — GTPase-Activating Protein

GDP — Guanosine Diphosphate

GFP — Green Fluorescent Protein

GRAMD1 — GRAM Domain-Containing Protein 1

GRP75 — Glucose-Regulated Protein 75

GRPEL2 — GrpE-Like Protein 2

GTP — Guanosine Triphosphate

HEPES — 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid

HRP — Horseradish Peroxidase

IMM — Inner Mitochondrial Membrane

INF2 — Inverted Formin 2

IP3 — Inositol 1,4,5-Trisphosphate

IP3R — Inositol 1,4,5-Trisphosphate Receptor

IP3R2 — Inositol 1,4,5-Trisphosphate Receptor Type 2

IPC — Inositol Phosphorylceramide

KIDINS220 — Kinase D-Interacting Substrate Of 220 kDa

KD — Knockdown

KO — Knockout

LAMP1 — Lysosome-Associated Membrane Protein 1

LC3 — Microtubule-Associated Proteins 1A/1B Light Chain 3

LDL — Low-Density Lipoprotein

LIR — LC3-Interacting Region

LTP — Lipid Transfer Protein

LTX — Cationic Lipid Transfection Reagent

MAM — Mitochondria-Associated Membrane

MAPPER — Membrane-Associated Peripheral ER–PM Tether

MCS — Membrane Contact Site

MCU — Mitochondrial Calcium Uniporter

MERCS — Mitochondria–ER Contact Sites

MERLIN — Moesin-Ezrin-Radixin-Like Protein (Merlin/NF2)

MFF — Mitochondrial Fission Factor

MFN1 — Mitofusin 1

MFN2 — Mitofusin 2

MID49 — Mitochondrial Dynamics Protein Of 49 kDa

MID51 — Mitochondrial Dynamics Protein Of 51 kDa

MIGA2 — Mitoguardin 2

MSA — Multiple Sequence Alignment

MYC — Myc Epitope Tag

NMY2 — Non-Muscle Myosin II

NVJ — Nucleus–Vacuole Junction

OCRL-1 — Oculocerebrorenal Syndrome Of Lowe Protein 1

OMM — Outer Mitochondrial Membrane

OPA1 — Optic Atrophy Protein 1

ORP — Oxysterol-Binding Protein-Related Protein

ORP1L — Oxysterol-Binding Protein-Related Protein 1L

ORP5 — Oxysterol-Binding Protein-Related Protein 5

ORP8 — Oxysterol-Binding Protein-Related Protein 8

PA — Phosphatidic Acid

PACS-2 — Phosphofurin Acidic Cluster Sorting Protein 2

PAGE — Polyacrylamide Gel Electrophoresis

PBS — Phosphate-Buffered Saline

PCR — Polymerase Chain Reaction

PDK4 — Pyruvate Dehydrogenase Kinase 4

PDZD8 — PDZ Domain-Containing Protein 8

PE — Phosphatidylethanolamine

PFA — Paraformaldehyde

PGAM5 — Phosphoglycerate Mutase Family Member 5

PI — Phosphatidylinositol

PI(4,5)P2 — Phosphatidylinositol 4,5-Bisphosphate

PI3K — Phosphoinositide 3-Kinase

PI3P — Phosphatidylinositol 3-Phosphate
PI4K — Phosphatidylinositol 4-Kinase
PI4P — Phosphatidylinositol 4-Phosphate
PI5P — Phosphatidylinositol 5-Phosphate
PINK1 — PTEN-Induced Kinase 1
PIP — Phosphatidylinositol Phosphate
PKA — Protein Kinase A
PLA — Proximity Ligation Assay
PM — Plasma Membrane
PS — Phosphatidylserine
PSS1 — Phosphatidylserine Synthase 1
PTPIP51 — Protein Tyrosine Phosphatase–Interacting Protein 51
RAS — Rat Sarcoma
REEP1 — Receptor Expression-Enhancing Protein 1
REEP5 — Receptor Expression-Enhancing Protein 5
RNA — Ribonucleic Acid
ROI — Region Of Interest
ROS — Reactive Oxygen Species
RPRD2 — Regulation Of Nuclear Pre-mRNA Domain-Containing Protein 2
RTK — Receptor Tyrosine Kinase
RTN4 — Reticulon-4
SAM50 — Sorting And Assembly Machinery Component 50
SDS — Sodium Dodecyl Sulfate
SH3GL2 — SH3 Domain-Containing GRB2-Like Protein 2
SIM — Structured Illumination Microscopy
SMP — Synaptotagmin-Like Mitochondrial-Lipid-Binding Protein Domain
SNARE — Soluble N-Ethylmaleimide–Sensitive Factor Attachment Protein Receptor
SNX14 — Sorting Nexin 14
SNX2 — Sorting Nexin 2
SPLICS — Split-GFP-Based Contact Site Sensor

STARD1 — StAR-Related Lipid Transfer Protein 1

STARD3 — StAR-Related Lipid Transfer Protein 3

START — StAR-Related Lipid Transfer Domain

STED — Stimulated Emission Depletion

STORM — Stochastic Optical Reconstruction Microscopy

T2A — Thosea Asigna Virus 2A Peptide

TAG — Triacylglycerol

TBC1D15 — TBC1 Domain Family Member 15

TCEP — Tris(2-Carboxyethyl)phosphine

TDP-43 — TAR DNA-Binding Protein 43

TEX2 — Testis-Expressed Protein 2

TF-PE — TopFluor-Phosphatidylethanolamine

TF-PS — TopFluor-Phosphatidylserine

TGN — Trans-Golgi Network

TGN46 — Trans-Golgi Network Protein 46

TMEM192 — Transmembrane Protein 192

TMEM24 — Transmembrane Protein 24

TMEM55B — Transmembrane Protein 55B

TOM20 — Translocase Of Outer Mitochondrial Membrane 20

TOMM40 — Translocase Of Outer Mitochondrial Membrane 40

TTBS — Tris-Buffered Saline With Tween-20

TULIP — Tubular Lipid-Binding Protein

ULK1 — Unc-51 Like Autophagy Activating Kinase 1

UNC-26 — Uncoordinated Protein 26

VAPA — Vesicle-Associated Membrane Protein-Associated Protein A

VAPB — Vesicle-Associated Membrane Protein-Associated Protein B

VDAC1 — Voltage-Dependent Anion Channel 1

VPS13 — Vacuolar Protein Sorting 13

VPS13A — Vacuolar Protein Sorting 13A

VPS13C — Vacuolar Protein Sorting 13C

VPS34 — Vacuolar Protein Sorting 34

YFP — Yellow Fluorescent Protein

Chapter 1 – Introduction

1.1 Compartmentalization and inter-organelle communication in eukaryotic cells

Eukaryotic cells have a highly organized internal structure where specialized membrane-bound organelles perform distinct, essential functions. This compartmentalization is a key feature of eukaryotic evolution, enabling cells to spatially and temporally separate incompatible biochemical processes, enhance metabolic efficiency, and maintain stricter control over complex molecular pathways (Gabaldón *et al*, 2015). Among the most important organelles are the endoplasmic reticulum (ER), mitochondria, the Golgi apparatus, endosomes, lysosomes, and peroxisomes. Each organelle is defined not only by its unique morphology, protein and lipid composition but also by its dynamic interactions with other parts of the cell.

Despite their physical separation, these organelles do not operate independently. Cellular homeostasis relies on the precise coordination of metabolic flux, ion exchange, lipid transfer, and signal transduction between cellular compartments. Traditional models of inter-organelle communication focused on vesicular transport and diffusible signaling molecules; however, it is now increasingly clear that direct physical contacts between organelles are essential mediators of cellular function (Eisenberg-Bord *et al*, 2016). These interactions enable efficient and localized exchange of metabolites and information, with temporal and spatial resolution that cannot be achieved through diffusion or trafficking alone.

One of the most striking examples of such coordination is observed in the communication between the ER and mitochondria. These two organelles cooperate in vital processes, such as lipid biosynthesis, calcium signaling, redox balance, and apoptosis regulation (Vance, 2014). However, the ER interacts not only with mitochondria but also with endosomes, lysosomes, the plasma membrane, and other organelles, forming a vast network of contact sites that underlie many aspects of cellular physiology (Wu *et al.*, 2018).

1.2 Membrane contact sites (MCSs)

Membrane contact sites (MCSs) are regions where membranes of two or more organelles come into close apposition (typically 10–30 nm) without fusing, enabling biochemical exchange (Csordás *et al.*, 2006). Once regarded as mere ultrastructural curiosities occasionally observed under the electron microscope, MCSs are now recognized as fundamental structures for inter-organelle communication, coordinating a variety of key cellular processes (Scorrano *et al.*, 2019; Venditti *et al.*, 2021).

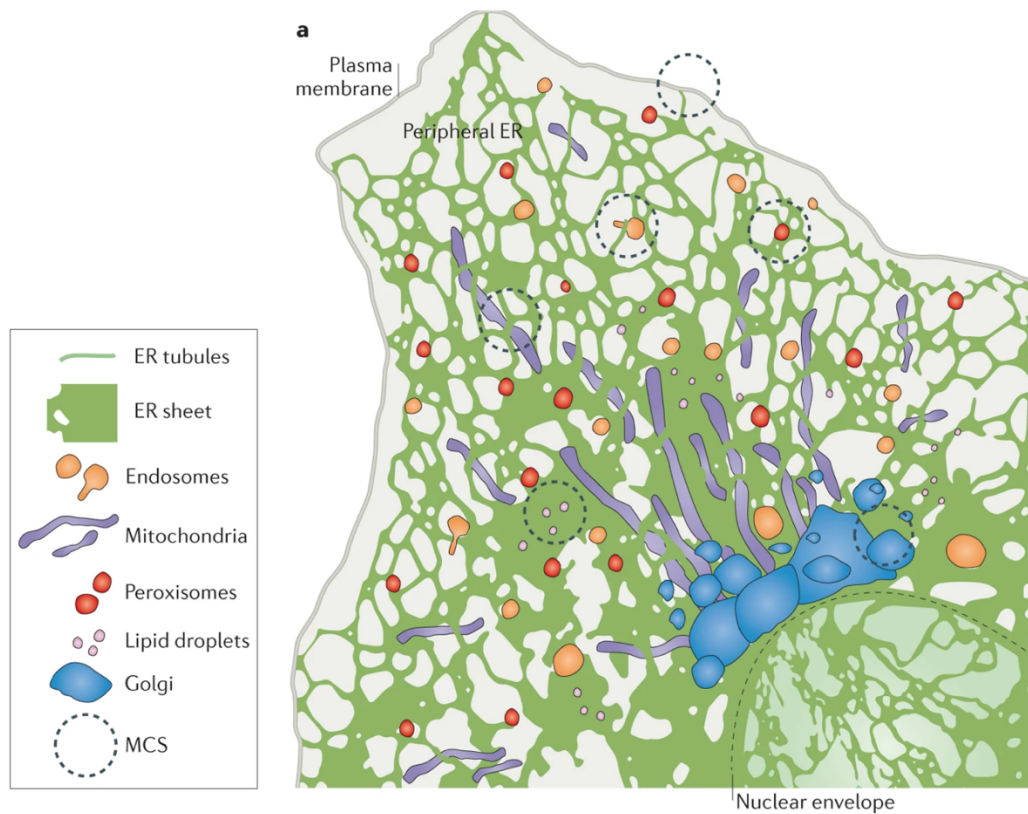


Figure 1.1 – ER-organelles MCSs. (a) ER forms MCSs with PM, mitochondria, peroxisomes, endosomes, lipid droplets and Golgi Apparatus. Adapted from Philips and Voletz, 2016.

In eukaryotic cells, MCSs form between nearly every organelle pair — including the endoplasmic reticulum (ER), mitochondria, endosomes, lysosomes, Golgi apparatus, lipid droplets, and peroxisomes (**Fig. 1.1**)— highlighting their widespread presence and essential role in maintaining cellular organization and homeostasis (Valm *et al.*, 2017). Far from being static or isolated contact points, these interfaces serve as dynamic platforms where molecules, metabolites, and signals are exchanged to coordinate organelle functions and cellular responses.

Functionally, MCSs act as multifunctional hubs that orchestrate diverse biological processes (**Fig. 1.2**). These include non-vesicular lipid transfer, calcium and ion exchange, redox signaling, reactive oxygen species (ROS) regulation, metabolic coordination, membrane remodeling during organelle fission and fusion, and even the inheritance and biogenesis of organelles (Prinz *et al.*, 2020; Reinisch & Prinz, 2021; Hamasaki *et al.*, 2013). A classic example is represented by ER–plasma membrane and ER–mitochondria contacts, where calcium ions are transferred through physically tethered channels to generate localized Ca^{2+} microdomains. These microdomains are critical for fine-tuning cellular signaling and for sustaining mitochondrial metabolism (Rizzuto *et al.*, 1998; Csordás *et al.*, 2010).

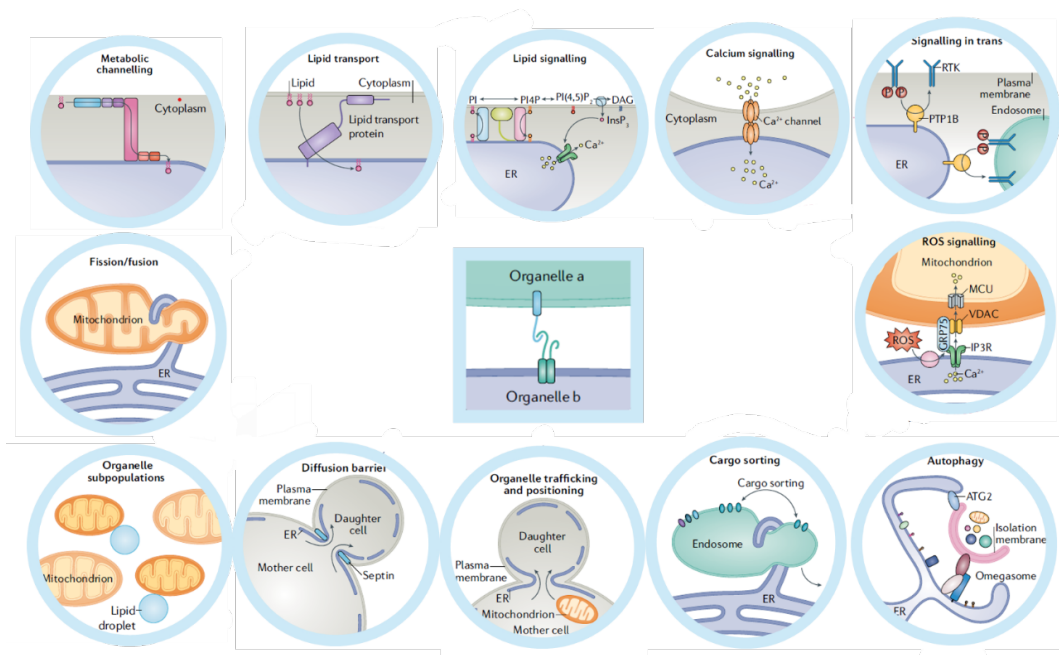


Figure 1.2 – Different functions of membrane contact sites. Some of the functions of MCSs in signalling, regulation of organelle dynamics, lipid transport and calcium signalling are shown. ATG2, autophagy- related protein 2; DAG, diacylglycerol; ER , endoplasmic reticulum; GRP75, 75-kDa glucose- regulated protein; InsP3, inositol 1,4,5- trisphosphate; IP3R, inositol 1,4,5-trisphosphate receptor; MCU, mitochondrial calcium uniporter protein; P, phosphate group; PI, phosphatidylinositol; PI4P, phosphatidylinositol 4-phosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PTP1B, protein- tyrosine phosphatase 1B; ROS, reactive oxygen species; RTK , receptor tyrosine kinase; VDAC, voltage- dependent anion- selective channel proteins. Adapted from Prinz *et al.*, 2020.

Structurally, MCSs are stabilized by tethering proteins or complexes that physically bridge the membranes of distinct organelles. These tethers can be integral membrane proteins or large multiprotein assemblies that often serve additional functions beyond mere anchoring. Many tethers incorporate enzymatic or regulatory domains, ion channels, or scaffolding motifs, allowing them to couple physical tethering with metabolic or signaling functions. Importantly, several tethers display functional versatility, relocating between different contact sites depending on cellular state or environmental cues, which highlights the remarkable plasticity and adaptability of MCSs in supporting cell physiology (Friedman *et al.*, 2011; Di Mattia *et al.*, 2020).

Among the most studied molecular components at MCSs are lipid transfer proteins (LTPs). These proteins play a vital role in mediating the direct, non-vesicular transfer of lipids—such as phospholipids, sterols, and ceramides—between membranes. Unlike vesicle-mediated transport, which involves membrane budding and fusion, LTP-mediated transfer provides a rapid and tightly regulated redistribution of lipids, helping to maintain membrane identity, lipid composition, and signaling ability (Reinisch & Prinz, 2021). Structurally, LTPs often contain lipid-binding domains that protect the hydrophobic lipid molecules during transfer through the aqueous cytosol, effectively acting as molecular shuttles between neighboring membranes. In doing so, they contribute not only to lipid homeostasis but also to the remodeling of organelle membranes under specific metabolic or stress conditions.

Recently, the traditional idea of binary MCSs has expanded to include multi-organelle or tripartite contacts, where three or more organelles interact at the same microdomain. These complex networks form highly integrated hubs that enable coordinated regulation of metabolic pathways, organelle behavior, and signal transduction, especially during stress responses or when cells adapt to changing metabolic needs.

Given their central role in maintaining cellular homeostasis, it is not surprising that MCS dysfunction has been implicated in numerous human diseases. Genetic mutations affecting tethering proteins or MCS-resident components can disrupt organelle communication, leading to pathophysiological consequences such as neurodegeneration (e.g., amyotrophic lateral sclerosis, Parkinson's disease), hereditary spastic paraplegias, metabolic disorders including type 2 diabetes and obesity, and even tumorigenesis (Paillusson *et al.*, 2016; Kim *et al.*, 2022). Furthermore, several pathogens, including viruses and intracellular bacteria, have evolved mechanisms to hijack host MCS machinery, exploiting these contact sites to access lipids and metabolites essential for their replication while evading immune detection (Paul & Bhandari, 2023).

From an evolutionary perspective, MCSs likely represent one of the most ancient mechanisms for inter-compartmental communication, predating the evolution of vesicular trafficking systems. The high conservation of core components across species — from yeast to mammals — underscores their fundamental role in eukaryotic cell organization and metabolic integration (Wideman, 2013).

1.2.1 Tethering molecule at MCSs

Membrane contact sites (MCSs) are regions where two organelles come into close proximity (typically within 10 to 30 nanometers) without fusing (*Venditti et al*, 2019). A fundamental component that defines these structures is the presence of tethering proteins, or “tethers”, molecular bridges that physically hold the opposing membranes together (Eisenberg-Bord *et al*, 2016; Prinz *et al*, 2020).

Tethers are either individual proteins or multi-subunit complexes that localize specifically to contact sites and mediate direct or indirect interactions between organelles. To date, numerous tethers have been identified, and several of them have been well characterized (**Fig. 1.3**). Nevertheless, the discovery of new tethering proteins remains a key objective for fully understanding the regulation and functional roles of membrane contact sites (Eisenberg-Bord *et al.*, 2016; Bohnert, 2020).

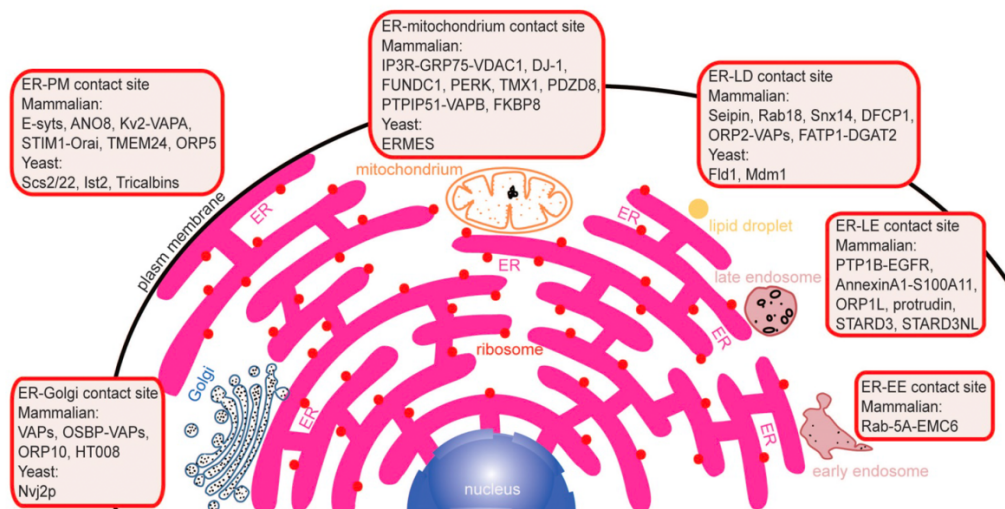


Figure 1.3 – Schematic representation of known and proposed tethering proteins.

These proteins mediate inter-organelle interactions through protein–protein or protein–lipid binding. Altering their expression levels—either through overexpression or genetic deletion—can disrupt contact site formation via distinct mechanisms, leading to a wide range of cellular phenotypes. Adapted from Lin *et al.*, 2020.

Although the idea of a tether may seem simple, demonstrating that a protein truly has tethering activity experimentally is quite difficult. Several factors contribute to this challenge: difficulties in measuring membrane proximity in live cells, the

dynamic and often redundant nature of tethering processes, and the multifunctionality of many proteins at contact sites.

To overcome these challenges, researchers have proposed a set of minimal criteria for defining a bona fide tether (Eisenberg-Bord *et al*, 2016).

First, the protein must be localized to the contact site, a requirement typically addressed through fluorescence microscopy or immunogold electron microscopy. However, localization alone is insufficient, as many proteins reside at MCSs without necessarily functioning as tethers.

Second, the protein must possess structural features that enable it to interact with both membranes. This often involves two distinct membrane-binding domains—such as transmembrane regions or lipid-binding motifs—that enable the protein to bridge the gap between organelles physically. Mutational analysis disrupting one of these domains typically results in a loss of focal localization at MCSs, supporting the protein's role in tethering (Giordano *et al*, 2013; Di Mattia *et al*, 2020).

Finally, a tether must actively mediate membrane proximity. This functional requirement can be directly tested using advanced biophysical techniques, such as optical tweezers or femtosecond laser ablation. Alternatively, researchers can evaluate the effects of tether depletion or overexpression. Removing a key tether often decreases contact between organelles or changes the spacing between them. However, the impact of loss-of-function experiments may be hidden by compensatory mechanisms involving redundant tethers. Conversely, overexpressing a tethering protein can cause an abnormal increase in the contact site. Rescue experiments using synthetic tethers—engineered constructs that artificially bridge organelles—can also provide strong evidence of tethering activity if they restore the morphology or function disrupted by the loss of an endogenous tether (Andersson *et al*, 2007; Kornmann *et al*, 2009; Csordás *et al*, 2010).

Beyond their structural role, tethers exhibit remarkable diversity in terms of function and regulation. Some are indispensable and sufficient for forming contacts on their own, while others act as accessory factors, stabilizing or modulating pre-existing contacts. Some tethers are constitutively active, whereas others respond

dynamically to environmental stimuli, such as changes in calcium levels or lipid composition. Moreover, many tethers are multifunctional, as they not only bridge membranes but also frequently participate in lipid transport, signal transduction, or metabolic regulation. In such cases, tethering may be a secondary feature that evolved alongside the protein’s primary function, yet it has become critical for maintaining organelle communication (Eisenberg-Bord *et al.*, 2016) (**Fig.1.4**).

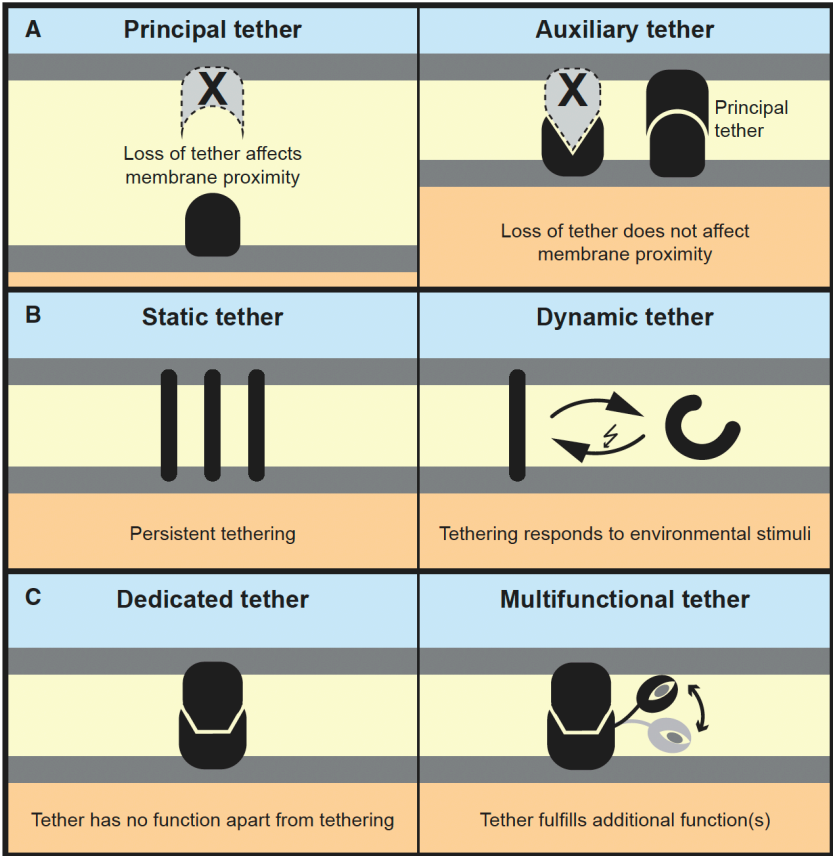


Figure 1.4 – Different classes of tethers.

Illustration summarizing the main functional categories of membrane contact site tethers, classified based on three criteria: (A) primary versus accessory, (B) constitutive versus stimulus-dependent, and (C) exclusively structural versus multifunctional. The symbol “X” indicates loss-of-function mutations, while the lightning bolt represents activation in response to environmental cues. Yellow shading denotes the cytosol; blue and orange represent the lumens of two different organelles. Adapted from Eisenberg-Bord *et al.*, 2016.

Interestingly, the concept of tethering extends beyond MCSs to other cellular processes, such as vesicle docking and membrane fusion. Proteins like extended synaptotagmins (E-Syts), mitofusins, and SNARE complexes have roles in both tethering and membrane merger, highlighting the mechanistic similarities between contact sites and pre-fusion intermediates. (Giordano *et al*, 2013; de Brito & Scorrano, 2008; Jahn & Scheller, 2006) While MCSs are functionally distinct from fusion events, they share overlapping molecular components and architectural principles (Eisenberg-Bord *et al*, 2016; Saheki & De Camilli, 2017).

In summary, tethering proteins are key structural and functional elements of MCSs. They not only establish the physical continuity needed for inter-organelle interactions but also act as regulators of dynamic cellular processes. Understanding their role is crucial for deciphering the spatial and functional architecture of eukaryotic cells, providing insights into a range of physiological and pathological mechanisms.

1.2.2. ER-Lys/late endosome MCSs

Among the many organelle interactions, contacts between the endoplasmic reticulum (ER) and late endosomes (LEs) have recently gained attention, especially in mammalian cells, despite being highly abundant and structurally conserved.

ER–LE MCSs are stabilized by specific tethers, which anchor the ER to endosomal membranes. A central component of these tethers is the Vesicle-associated membrane protein-associated proteins (VAPs), which reside in the ER membrane and bind FFAT motifs (two phenylalanines FF in an acidic tract) on endosomal proteins. For example, Oxysterol-binding protein–related protein 1L (ORP1L) and STARD3/STARD3NL (StAR-related lipid transfer domain-containing protein 3), both sterol-binding proteins, interact with VAPs to promote or modulate ER–endosome contact formation, particularly in response to changes in cholesterol levels (**Fig. 1.5**) (Rocha *et al*, 2009; Alpy *et al*, 2013; Wilhelm *et al*, 2017).

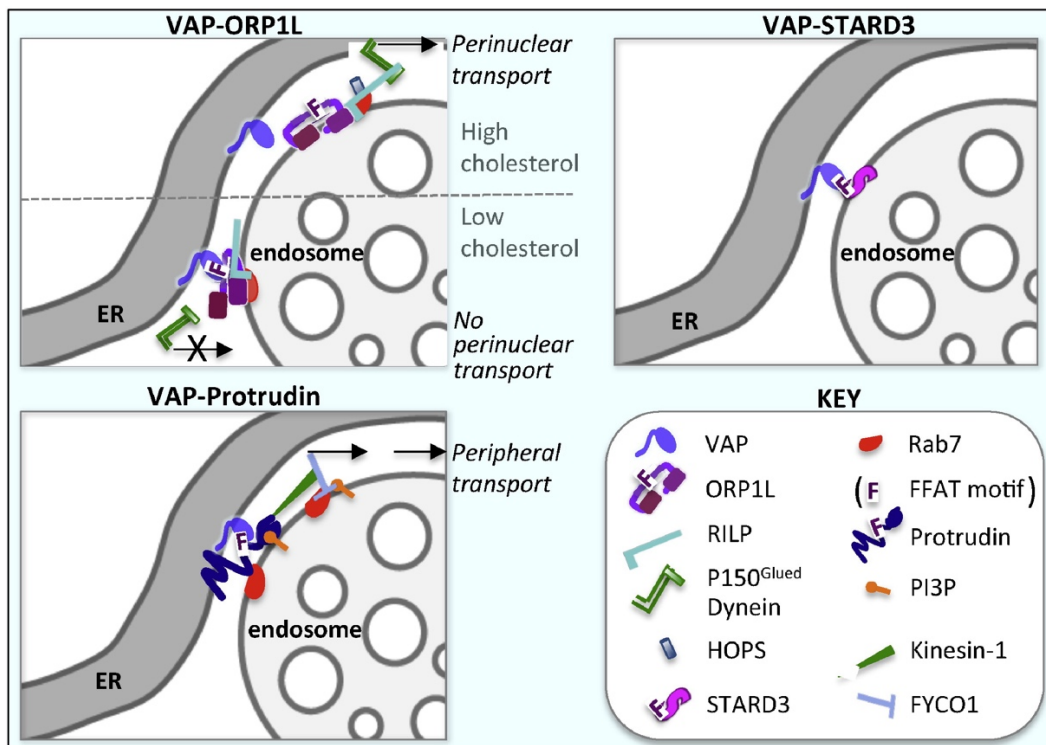


Figure 1.5 – VAP-mediated protein interactions at ER–endosome membrane contact sites. At ER–endosome contact regions, the ER-resident protein VAP engages with multiple FFAT motif-containing proteins to regulate endosomal positioning. Under low LDL-cholesterol conditions, VAP binds to the FFAT motif of ORP1L, disrupting the

ORP1L/RILP/dynein complex and thereby halting minus-end directed transport toward the perinuclear region. VAP also associates with STARD3, another endosomal sterol-binding protein, facilitating the formation of stable contact sites. In parallel, VAP interacts with the ER-anchored protein protrudin, which coordinates with Ras-related protein 7 (Rab7) and Phosphatidylinositol 3-phosphate (PI3P) on the endosomal membrane to support kinesin-1-mediated transport toward the cell periphery. Illustration summarizing the main functional categories of membrane contact. Adapted from Eden *et al.*, 2016

One well-characterized ER-resident protein, Protrudin, binds both the FFAT motif of VAPA and endosomal markers, such as PI3P and Rab7, further reinforcing these contacts. Overexpression of Protrudin causes ER reorganization and the formation of extensive MCSs, highlighting its role as a structural and functional scaffold at ER–LE interfaces (Raiborg *et al*, 2015).

Rab7, a small GTPase and a key marker of late endosomes, plays a central role in coordinating ER–LE contact formation, particularly through its interactions with ORP1L and Protrudin. Likewise, phosphoinositides such as PI3P assist in directing the recruitment and assembly of tethering complexes.

Although these molecular players are important, genetic depletion of individual components (e.g., VAPs, ORP1L, or STARD3) often fails to completely abolish contact site formation, suggesting a high degree of redundancy and plasticity in the tethering machinery. Indeed, multiple redundant tethers likely cooperate to ensure the robustness and dynamic regulation of these MCSs.

Functionally, ER–LE MCSs regulate a wide array of cellular processes:

- *Endosome fission and positioning*: the ER wraps around endosomes at budding sites, controlling membrane fission and defining whether vesicles recycle or mature. ORP1L-VAP interactions influence dynein-mediated transport (Rocha *et al*, 2009, Van der Kant *et al*, 2013), while Protrudin supports kinesin-mediated movement toward the cell periphery, promoting neurite outgrowth (Raiborg *et al*, 2015).
- *Growth factor receptor signalling*: ER–LE contacts serve as platforms for Protein Tyrosine Phosphatase 1B (PTP1B)-mediated dephosphorylation of internalized receptors like the Epidermal Growth Factor Receptor (EGFR) and

Granulocyte Colony-Stimulating Factor Receptor (G-CSFR), thereby contributing to receptor downregulation. PTP1B also modulates intraluminal vesicle (ILV) formation by dephosphorylating the Endosomal Sorting Complex Required for Transport (ESCRT) components (Eden *et al*, 2012; Tomas *et al*, 2015).

- *Non-vesicular lipid transport*: Though direct evidence is still emerging, several sterol transfer proteins, including ORP1L, STARD3, and ORP5 (Oxysterol-binding protein–related protein 5), are believed to mediate lipid exchange across these contacts. It is estimated that up to 30% of LDL-derived cholesterol may reach the ER through ER–LE MCSs in a vesicle-independent manner (Rocha *et al*, 2009; Alpy *et al*, 2013; Wilhelm *et al*, 2017)..
- *Calcium signaling*: The ER and endosomes act as intracellular Ca^{2+} stores. Local Ca^{2+} signals at ER–endolysosomal contacts have been proposed to facilitate endosome–lysosome fusion and modulate tether stabilization. Disruption of endosomal Ca^{2+} storage, as seen in Niemann-Pick type C1 disease, mimics cholesterol trafficking defects (Lloyd-Evans *et al*, 2008; Garrity *et al*, 2016).
- *Cargo sorting and recycling*: ER–LE contacts contribute to the sorting of cargo within multivesicular bodies (MVBs) and toward the Golgi. VAP–SNX2 (Sorting Nexin 2) interactions help direct retrograde traffic, while ER-localized PTP1B regulates the sorting of EGFR into ILVs (Eden *et al*, 2012; Tomas *et al*, 2014).
- *Disease relevance*: Dysfunction in ER–LE MCSs has been linked to neurodegenerative disorders, including hereditary spastic paraplegia. For instance, spastin, an ER–endosome tether, is mutated in this condition. Similarly, SNX14, the mammalian ortholog of yeast Mdm1 (Mitochondrial distribution and morphology protein 1), participates in ER-endolysosomal contact formation and has been implicated in neurological disease (Hariri *et al*, 2022; Bryant *et al*, 2018).

In summary, although ER–LE MCSs remain less well understood than other inter-organelle contacts, their molecular complexity and involvement in vital cellular functions such as lipid metabolism, signal transduction, and organelle dynamics underscore their biological significance.

1.2.3 ER-Mitochondria MCSs (MAM)

Among the various types of membrane contact sites, those formed between the endoplasmic reticulum (ER) and mitochondria have emerged as some of the most functionally versatile and biologically relevant. These specialized regions of close apposition, often referred to as mitochondria-associated membranes (MAMs), constitute a highly conserved and dynamic platform that facilitates the bidirectional communication between the ER and mitochondria (Rizzuto *et al.*, 2009; Rowland and Voeltz, 2012).

Structurally, MAMs are defined by a close connection between the outer mitochondrial membrane and ER tubules, maintained by specific tethering proteins and lipid microdomains. In yeast, the best-characterized MAM tethering complex is ERMES (ER-mitochondria encounter structure)—composed of Mmm1, Mdm10, Mdm34, Mdm12, with SMP domains (Synaptotagmin-like Mitochondrial-lipid-binding Protein domain) that facilitate non-vesicular lipid transfer (Kornmann *et al.*, 2009; AhYoung *et al.*, 2015; Kawano *et al.*, 2018). Although ERMES is absent in mammals, functional analogs have been identified.

In mammals, multiple proteins contribute to the formation and regulation of MERCSs (Mitochondria-ER contact sites). For instance, PDZ domain-containing protein 8 (PDZD8), an ER-localized protein with a lipid-binding SMP domain, is required for the formation of MERCSs and calcium flux from the ER to mitochondria in mammalian cells (Nakamura *et al.*, 2025). Mitofusin 2 (Mfn2), which localizes to both the ER and mitochondria, has been proposed to tether these organelles, although its precise role remains debated (de Brito & Scorrano, 2008; Naon *et al.*, 2016; Filadi *et al.*, 2015). Additional proteins involved include components of the mitochondrial fission machinery, such as Fis1 (Fission 1 protein) and Drp1 (Dynamin-related protein 1), which localize to MERCSs and help coordinate mitochondrial division (Friedman *et al.*, 2011; Korobova *et al.*, 2013). Other key complexes include VDAC1–GRP75–IP3R, which facilitates Ca^{2+} transfer, and VAPB–PTPIP51, whose interaction influences both calcium homeostasis and contact site formation. Proteins such as ORP5, ORP8, VPS13A, and SLC25A46 also participate in lipid exchange at these sites (Galmes *et al.*, 2016;

Monteiro-Cardoso *et al*, 2022; Guillén-Samander *et al*, 2021; Janer *et al*, 2016) (Fig.1.6).

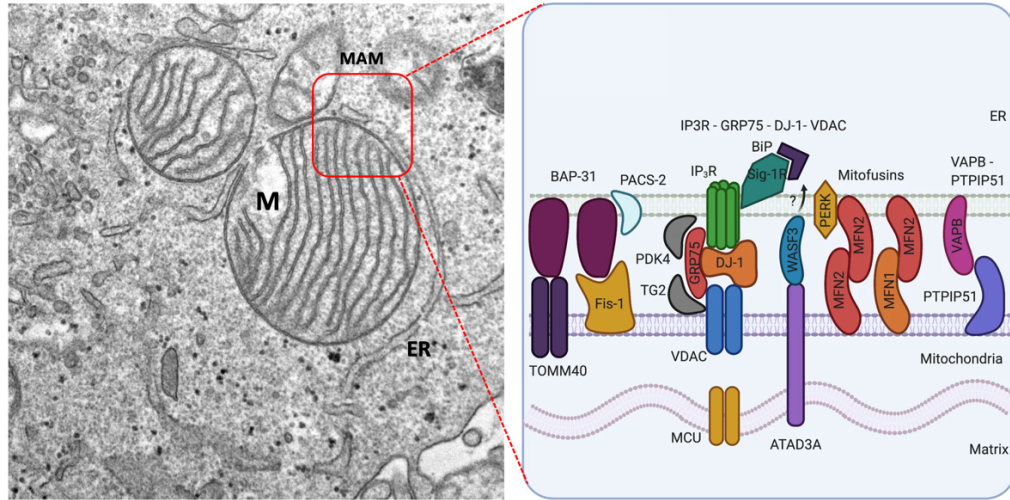


Figure 1.6 – Molecular players at ER-mitochondria MCSs. Image of electronic microscopy in which the contact (MAM) can be seen between the mitochondria (M) and the tubule of endoplasmic reticulum (ER). A diverse class of proteins contributes to the structural and functional integrity of ER-mitochondria contact sites (MERCs). Among them, BAP31, an ER-resident chaperone, plays a central role by physically interacting with Fis1 and TOMM40 on the mitochondrial outer membrane (OMM), forming a tethering platform at MERCs. This interaction is regulated by PACS-2 (phosphofurin acidic cluster sorting protein 2). Calcium signaling at MERCs is tightly controlled by a multiprotein complex composed of IP3 receptors (IP3R) on the ER membrane and voltage-dependent anion channels (VDACs) on the OMM. These proteins form a functional tether via the molecular chaperone GRP75, which physically bridges IP3R and VDAC, ensuring efficient calcium transfer from the ER to mitochondria. Additional regulators, including DJ-1, TG2 (transglutaminase 2), and PDK4 (pyruvate dehydrogenase kinase 4), bind to this complex and modulate its activity, thereby fine-tuning calcium fluxes at the contact sites. (EM performed by Lena Polishuck; illustration adapted from Wilson *et al*, 2021)

MERCs have a number of vital cellular functions. One of their primary roles is in calcium homeostasis. The ER serves as a major intracellular Ca^{2+} store and releases calcium through IP3Rs or RyRs into microdomains at MERCs, from where mitochondria rapidly absorb the ion via the mitochondrial calcium uniporter

(MCU). This localized transfer of Ca^{2+} supports mitochondrial metabolism but must be tightly regulated to prevent calcium overload and cell death.

Another critical function of MERCSs is the non-vesicular transfer of lipids, particularly phosphatidylserine (PS), phosphatidylethanolamine (PE), and cholesterol. This is facilitated by lipid transport proteins such as those containing SMP domains (e.g., PDZD8) or the VPS13 family, which can span the distance between membranes and shuttle lipids across the contact sites (Kawano *et al*, 2018; Galmes *et al*, 2016; Monteiro-Cardoso *et al*, 2022; Guillén-Samander *et al*, 2021)

MERCSs are also implicated in mitochondrial dynamics. These contact sites often mark the location of mitochondrial fission, serving as platforms for the recruitment of fission proteins like Drp1. Furthermore, MERCSs have been linked to mitochondrial DNA replication and, conversely, mitochondrial fusion. They additionally provide membrane material for autophagosome formation during nutrient deprivation, connecting MERCSs to the regulation of autophagy (Friedman *et al.*, 2011; Lewis *et al.*, 2016; Hamasaki *et al.*, 2013).

Disruption of MERCSs has been associated with various human diseases, especially neurodegenerative disorders. For example, mutations in REEP1 impair MERCS formation and are linked to hereditary spastic paraplegia, while defects in SLC25A46, ATAD3A, or C12orf67 affect MERCS integrity and contribute to Leigh syndrome, optic atrophy, or Wolfram syndrome, respectively (Lim *et al*, 2015; Janer *et al*, 2016; Zhao *et al*, 2022; Chen *et al*, 2009).

Interestingly, many MERCS-resident proteins are not exclusive to this contact site but also function in other MCSs, suggesting a broader role in organelle communication networks. VAPB, for instance, interacts with FFAT motif-containing proteins at multiple MCSs, including ER–Golgi and ER-endolysosome contacts. Similarly, PDZD8 is involved in ER-endosome contacts, and ORP5/ORP8 also function at ER–plasma membrane interfaces. Moreover, recent evidence has highlighted that ER-mitochondria contacts are not isolated structures but may also serve as scaffolds for the formation of multi-organelle junctions, involving lysosomes, endosomes and lipid droplets. These tripartite contact sites may

orchestrate more complex signalling and trafficking events (Wong *et al.*, 2018; Valm *et al.*, 2017).

Altogether, ER-mitochondria contact sites are now recognized as critical regulatory hubs, whose dynamic nature and functional versatility make them central players in cell physiology.

1.2.3.1. Mitochondrial dynamics

Mitochondrial dynamics refer to the continuous remodelling of the mitochondrial network through *fission* and *fusion* events. These processes are essential for maintaining mitochondrial function, ensuring proper distribution of mitochondrial DNA (mtDNA), supporting bioenergetics, and regulating apoptosis, calcium signalling, and metabolic adaptation. Disruptions in mitochondrial morphology are implicated in various pathological conditions, including neurodegenerative and metabolic diseases.

Mitochondrial fission is a tightly regulated cellular process by which a single mitochondrion divides into two daughter organelles. This dynamic remodeling of the mitochondrial network is essential for mitochondrial quality control, distribution during cell division, and adaptation to metabolic demands or stress. A key feature of mitochondrial fission is its close spatial and functional coupling with the endoplasmic reticulum (ER), particularly through ER–mitochondria contact sites (MERCs), which mark and orchestrate mitochondrial division events (Friedman *et al.*, 2011; Kleele *et al.*, 2021).

The first phase of fission begins with the physical pre-constriction of mitochondria, which is initiated at MERCs. These contact sites serve as platforms for actin filament nucleation and cytoskeletal remodeling. ER-localised inverted formin 2 (INF2), in cooperation with mitochondria-associated Spire1C, drives localised actin polymerisation at the contact site (Korobova *et al.*, 2013; Chakrabarti *et al.*, 2018). The generated actin network recruits and activates non-muscle myosin II (NMIIA), which provides the contractile force necessary to constrict the mitochondrial membrane before the recruitment of the key fission protein DRP1 (Kleele *et al.*, 2021) (**Fig. 1.7**).

DRP1 (dynamin-related protein 1), a cytosolic GTPase, is then recruited to the outer mitochondrial membrane (OMM) by specific adaptors such as MFF, MiD49, and MiD51. Upon recruitment, DRP1 oligomerizes into ring-like structures that encircle and further constrict the mitochondrial tubule. GTP hydrolysis by DRP1 leads to

conformational changes that complete the membrane scission (Kalia *et al.*, 2018; Kraus *et al.*, 2021). Structural studies have demonstrated that DRP1 filaments can co-assemble with MiD49 to form linear scaffolds, which then transition into closed rings capable of generating mechanical force (Kalia *et al.*, 2018).

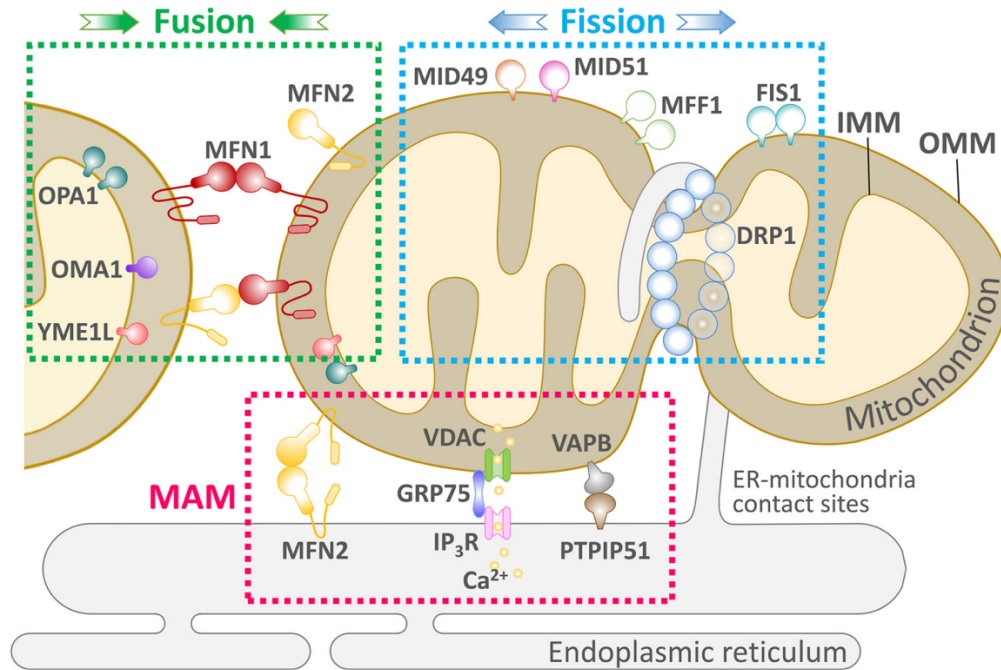


Figure 1.7 – Illustration of mitochondrial dynamics and ER-mitochondria crosstalk. Mitochondria undergo continuous fission and fusion to adapt to cellular conditions. Mild stress favours elongation and networking, while severe stress leads to fragmentation. These processes are coordinated at ER-mitochondria contact sites (MAMs), which facilitate Ca^{2+} transfer via IP₃R, VDAC, and MCU. Proteins like Fis1 and Mff contribute to Drp1 recruitment at the ER, underscoring the ER’s role in regulating mitochondrial division. Adapted from Chiu *et al.*, 2021

Significantly, mitochondrial inner membrane (IMM) constriction may precede DRP1 action and is regulated by local Ca^{2+} and K^{+} fluxes. This inner membrane remodelling is thought to be critical for mtDNA segregation and for determining the site of fission (Kleele *et al.*, 2021). MERCSs thus not only serve as mechanical platforms but also as hubs for localized signalling, particularly calcium signalling. ER-resident IP₃ receptors (IP₃R) release Ca^{2+} into microdomains that are rapidly taken up by mitochondria through the VDAC–GRP75–MCU complex, which helps shape IMM morphology and bioenergetics (Csordás *et al.*, 2010).

The final steps of mitochondrial fission may involve contributions from additional organelles. For instance, lysosomes and Golgi-derived vesicles have been shown to assist in the final scission step. Lysosome–mitochondria contacts, mediated by FIS1 and TBC1D15, are involved in segregating damaged mitochondrial fragments for degradation, a process known as mitophagy (Wong *et al.*, 2018). Furthermore, the presence of phosphoinositide-enriched vesicles from the trans-Golgi network (TGN), particularly those containing PtdIns4P, has been proposed to facilitate membrane scission, possibly by altering local lipid composition (Nagashima *et al.*, 2020).

Two distinct types of mitochondrial fission have been described: midzone fission, which is associated with mitochondrial biogenesis, and peripheral fission, which often marks dysfunctional mitochondrial segments for elimination via autophagy. While midzone fission requires DRP1, MFF, actin, and ER involvement, peripheral fission can occur independently of these components and involves lysosome-mediated signals instead (Kleele *et al.*, 2021).

Mitochondrial fusion, on the other hand, is mediated by mitofusins (MFN1 and MFN2) on the outer mitochondrial membrane (OMM) and OPA1 on the inner membrane (IMM). MFN1/MFN2 form homo- and hetero-oligomeric assemblies that tether opposing mitochondria prior to outer-membrane merger, while OPA1 governs inner-membrane fusion and cristae architecture (Chen *et al.*, 2003; Cipolat *et al.*, 2004; Detmer and Chan, 2007; Ishihara *et al.*, 2006). MFN2 also localises to MERCSs and can modulate ER–mitochondria juxtaposition and Ca²⁺ transfer, although its precise tethering role remains debated (de Brito and Scorrano, 2008; Naon *et al.*, 2016; Filadi *et al.*, 2015) (**Fig. 1.7**)

Recent studies have highlighted the presence of specialized subdomains along the mitochondrial network, called mitochondrial *nodes*, which act as focal points for various mitochondrial functions (Abrisch *et al.*, 2020; Nguyen *et al.*, 2022). These nodes are rich in molecular machinery involved in mitochondrial division and fusion, including the ER-mediated recruitment of the GTPase DRP1, and often align with membrane contact sites between the ER and mitochondria. At these hubs, dynamic coordination of mitochondrial dynamics, lipid exchange, and mitophagy

can occur simultaneously, indicating that mitochondrial nodes serve as spatially organized platforms that integrate signaling and membrane remodeling activities.

MERCSs play a central role in coordinating these dynamics. Beyond serving as physical platforms for division and fusion, they regulate local calcium flux and phospholipid transfer, key modulators of mitochondrial shape. Phospholipids such as cardiolipin and phosphatidic acid directly affect membrane curvature and are involved in the recruitment and activation of DRP1 and OPA1.

1.2.3.2 MAMs as platforms for mitophagy: focus on FUNDC1

Among their multiple functions, MAMs serve as a central hub for regulating mitochondrial quality control through mitophagy, particularly during stress conditions such as ischemia or hypoxia (Jia *et al.*, 2024).

MAMs have been shown to host essential mitophagy-regulating proteins, including PINK1, Parkin, Beclin1, and mitofusin 2 (Mfn2), which gather at these contact sites and facilitate the initiation and progression of mitochondrial autophagy (Gelmetti *et al.*, 2017; Barazzuol *et al.*, 2020). One such protein, FUNDC1 (FUN14 domain-containing protein 1), has emerged as a key regulator of MAM-resident mitophagy under hypoxic conditions.

Under normoxia, FUNDC1 is present at low levels at MAMs; however, upon hypoxic stress, its levels at the ER-mitochondria interface significantly increase (Wu *et al.*, 2016a). This relocalization is mediated through its interaction with the ER protein calnexin (CANX). FUNDC1 directly engages with LC3, a key autophagy effector, to promote the formation of mitophagosomes, although this interaction is tightly regulated by phosphorylation. Phosphorylation at Tyr18 by Src and at Ser13 by CK2 impairs LC3 binding, thereby suppressing mitophagy. Notably, ischemic conditions lead to hyperactivation of Src, which phosphorylates FUNDC1 and attenuates its mitophagic function. Inhibition of Src restores FUNDC1's activity and confers neuroprotective effects via mitophagy (Chen *et al.*, 2016; Tang *et al.*, 2023).

In addition to autophagy, FUNDC1 also influences mitochondrial dynamics by interacting with both OPA1, a mitochondrial fusion protein, and Drp1, a fission regulator. These interactions suggest that FUNDC1 plays a crucial role in coordinating fission and mitophagy processes, particularly during ischemia/reperfusion injury, where excessive mitochondrial fragmentation and dysfunction are common (Chen *et al.*, 2016; Grohm *et al.*, 2012).

Mechanistically, FUNDC1 also promotes calcium transfer across MAMs by binding to the ER calcium channel IP3R2, reinforcing ER-to-mitochondria calcium

signaling during stress, a feature that further impacts mitochondrial function and autophagic flux (Wu *et al.*, 2017).

The physiological relevance of FUNDC1 has been underscored in several ischemia models. For instance, treatments that enhance FUNDC1 expression or prevent its inactivation have been shown to mitigate brain injury and improve neurological outcomes following ischemic stroke (Cai *et al.*, 2021; Li *et al.*, 2021). Conversely, downregulation of FUNDC1 results in impaired mitophagy and worsened cellular damage under ischemic conditions (Tian *et al.*, 2023).

Given its dual role in mitophagy and mitochondrial dynamics, and its precise localization to MAMs, FUNDC1 represents a promising therapeutic target in neurological disorders characterized by mitochondrial dysfunction, such as ischemic stroke. More broadly, the study of FUNDC1 within the MAM context underscores the significance of membrane contact sites as regulatory platforms for maintaining organelle homeostasis and adapting to stress.

1.2.3.3 Pathological implications of mitochondrial dynamics

Mitochondrial dynamics—comprising fission, fusion, mitophagy, and trafficking—are essential processes that maintain mitochondrial quality control, energy production, and cellular adaptation to stress. The ability of mitochondria to divide and fuse enables cells to preserve mitochondrial shape, isolate damaged regions for degradation, allocate mitochondria to areas of high energy demand, and adjust to changing metabolic conditions. Dysregulation of these processes has increasingly been linked to the development of various human diseases, from neurodegenerative and metabolic disorders to cardiovascular diseases and cancer (Chen *et al.*, 2023).

In neurodegenerative diseases, such as Alzheimer's, Parkinson's, Huntington's disease, and amyotrophic lateral sclerosis (ALS), mitochondrial fragmentation is a hallmark. Excessive fission driven by DRP1 hyperactivation and reduced fusion capacity, resulting from the downregulation or mutation of MFN2 or OPA1, leads to defective energy production, impaired calcium buffering, and increased oxidative stress, all of which contribute to neuronal degeneration. For example, mutations in OPA1 cause dominant optic atrophy by disrupting inner membrane fusion and mtDNA stability (Alexander *et al.*, 2000). In ALS, VAPB–PTPIP51 tethers at MERCSs are disrupted, leading to altered Ca^{2+} transfer and mitochondrial dysfunction (De Vos *et al.*, 2012; Bernard-Marissal *et al.*, 2015) (**Fig. 1.8**).

Similarly, metabolic disorders such as type 2 diabetes, obesity, and non-alcoholic fatty liver disease (NAFLD) are associated with increased mitochondrial fission and defective fusion. In insulin-resistant skeletal muscle, DRP1 overactivation correlates with mitochondrial fragmentation and impaired oxidative phosphorylation, contributing to metabolic inflexibility (Jheng *et al.*, 2012). Moreover, defective mitochondrial dynamics compromise fatty acid oxidation and enhance lipid accumulation, exacerbating liver steatosis and metabolic syndrome.

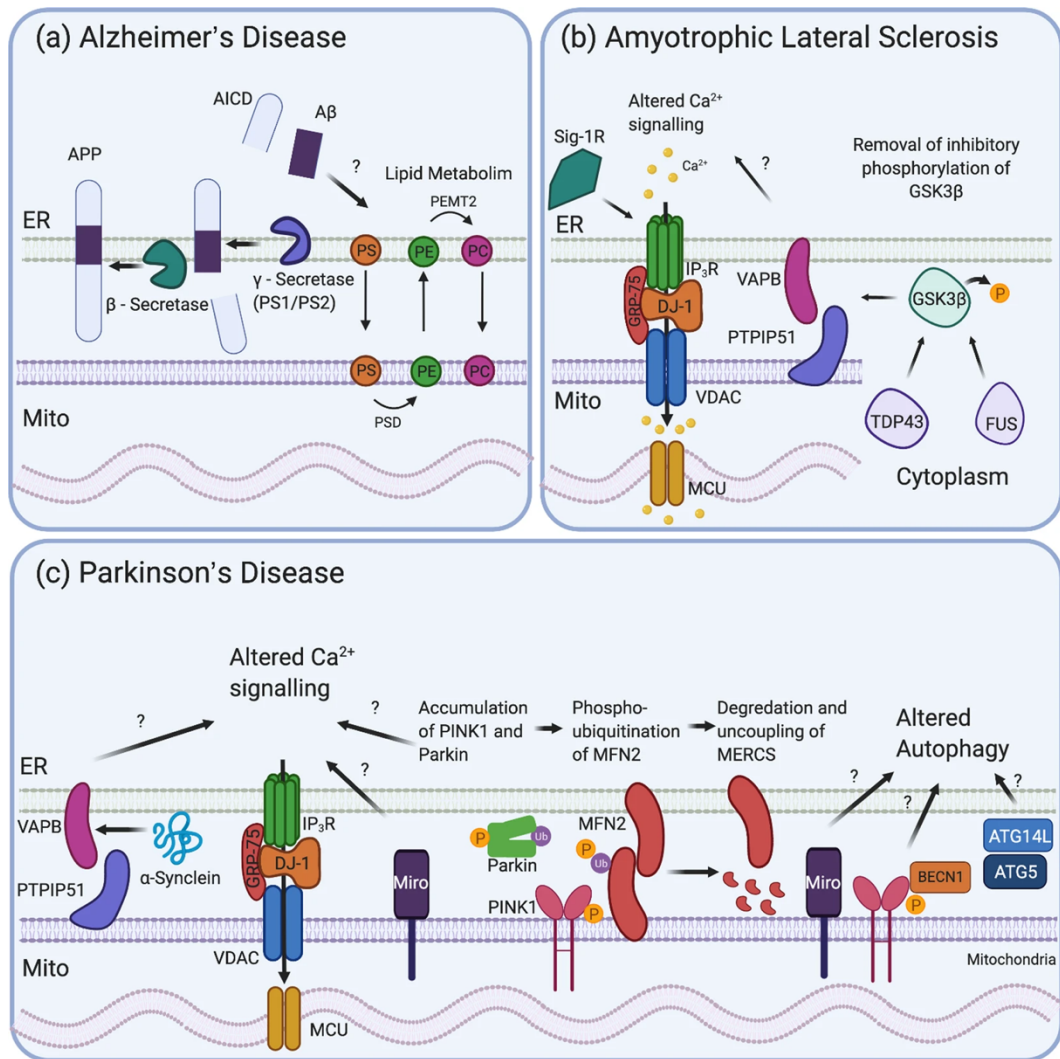


Figure 1.8 - MERCS involvement in neurodegenerative diseases. (a) AD: APP and secretases localize to MERCSs, where Aβ production alters lipid metabolism. (b) ALS: TDP-43 and FUS activate GSK3β, disrupting VAPB–PTPIP51 interaction and ER-mitochondria coupling. Loss of Sig-1R destabilizes IP3R, impairing Ca²⁺ signaling. (c) PD: α-Synuclein interferes with VAPB–PTPIP51 binding, affecting MERCS integrity. Miro, PINK1, and Parkin regulate Ca²⁺ signaling and mitophagy at MERCSs, with MFN2 degradation contributing to ER-mitochondria uncoupling. Adapted from Wilson *et al*, 2021.

In the cardiovascular system, alterations in mitochondrial morphology have been implicated in myocardial ischemia-reperfusion injury and heart failure. Excessive DRP1 activity in cardiomyocytes leads to fragmented mitochondria with reduced membrane potential and increased ROS production. Conversely, promoting

mitochondrial fusion via MFN2 or OPA1 overexpression protects against ischemic damage and supports cardiac function (Ong *et al.*, 2010; Wai & Langer, 2016).

In the context of cancer, mitochondrial fission is often hijacked to favor tumor progression. Increased DRP1 expression promotes mitochondrial fragmentation, which supports glycolytic metabolism, enhances cellular proliferation, and inhibits apoptosis. Several oncogenes, including RAS and MYC, upregulate fission machinery, while inhibition of DRP1 has been shown to reduce tumor cell invasiveness and restore apoptotic sensitivity (Zhao *et al.*, 2013; Serasinghe *et al.*, 2015).

The pathological effects of dysregulated fission and fusion are therefore dependent on the context but ultimately lead to mitochondrial dysfunction, energy failure, and disrupted communication between organelles. Because of their key coordinating role, MERCSs are now seen not just as structural junctions but as signaling centers that coordinate metabolic, apoptotic, and stress responses throughout the cell. As a result, targeting mitochondrial dynamics—by modifying DRP1 activity, boosting fusion, or stabilizing MERCSs—offers a promising therapeutic approach for various diseases.

1.2.4 Mitochondria–Lysosome contact sites (MLCs)

In recent years, direct physical contacts between mitochondria and lysosomes have emerged as a new type of membrane contact site, adding complexity to the organelle communication network. Originally described as transient interactions, mitochondria–lysosome contacts (MLCs) are now recognized as regulated and functionally important junctions, separate from vesicular trafficking events (Wong *et al.*, 2018; Cabukusta & Neefjes, 2018). High-resolution live-cell imaging has shown that lysosomes dynamically “kiss” mitochondria, pausing at specific subdomains of the mitochondrial network before disengaging, a behavior that suggests functional specialization.

Functionally, MLCs play a central role in mitochondrial quality control and dynamics. Lysosomes act as downstream effectors of mitophagy, but beyond degradation, they also contribute to the earlier decision-making steps of mitochondrial fission. Indeed, several studies have demonstrated that MLCs are enriched at sites of mitochondrial division and often colocalize with ER-mitochondria contacts, indicating a cooperative role in coordinating fission (Wong *et al.*, 2018; Kleele *et al.*, 2021). Mechanistically, the lysosomal GTPase Rab7 and its effectors mediate tethering to mitochondria, while FIS1 and TBC1D15 are involved in coordinating local fission and recruiting autophagic machinery (Peng *et al.*, 2020) (**Fig 1.9**).

Besides dynamics, MLCs serve as platforms for metabolic and signaling exchange. Lysosomes regulate cellular nutrient status via mTORC1, and their interaction with mitochondria influences the integration of metabolic cues with mitochondrial bioenergetics (Valm *et al.*, 2017). Moreover, Ca²⁺ microdomains at MLCs have been proposed to fine-tune local signaling, while lipid transfer events—though less well understood—may contribute to membrane remodeling during mitophagy (Todkar *et al.*, 2021).

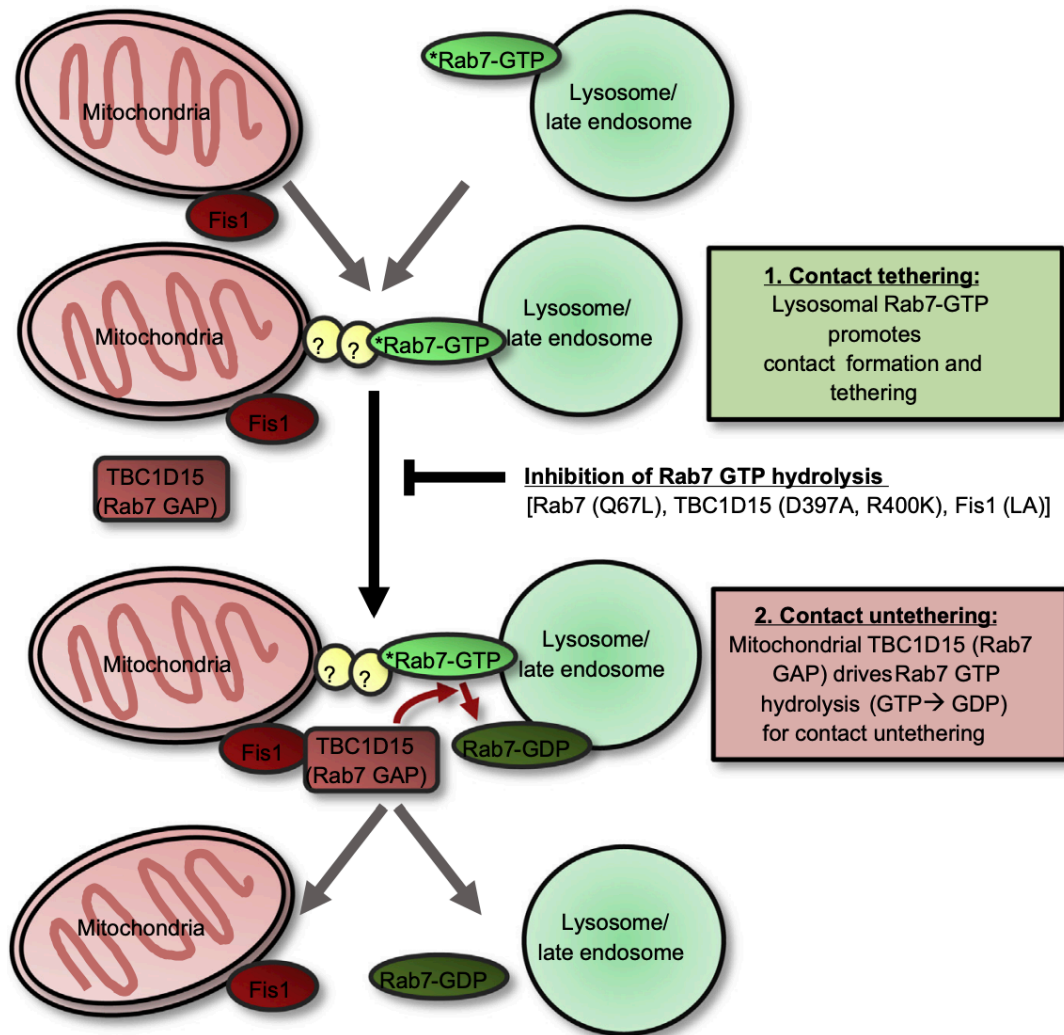


Figure 1.9 - Rab7-dependent regulation of mitochondria–lysosome contact dynamics.

The establishment and resolution of mitochondria–lysosome contacts are tightly controlled by the state of the small GTPase Rab7, which tightly controls lysosome contacts. In its active GTP-bound form, Rab7 promotes tethering of lysosomes to mitochondria, likely through interactions with Rab7 effector proteins. Contact disassembly requires the recruitment of the GAP TBC1D15, which is targeted to mitochondria by the outer membrane protein Fis1. At these sites, mitochondrial TBC1D15 stimulates GTP hydrolysis on lysosomal Rab7, converting it into the GDP-bound form. This inactivation prevents Rab7 from binding its effectors and from remaining anchored to lysosomal membranes, ultimately leading to the release of contact. Mutations that impair Rab7 hydrolysis (Rab7 Q67L), abolish TBC1D15 GAP activity (D397A, R400K), or disrupt Fis1-mediated recruitment of TBC1D15 (Fis1-LA) all result in defective untethering and stabilization of mitochondria–lysosome contacts.

Importantly, dysregulation of MLCs has been linked to disease. Genetic or pharmacological interventions that increase or decrease mitochondria–lysosome coupling impact mitochondrial turnover and morphology, with implications in neurodegenerative diseases such as Parkinson’s disease, where defective mitophagy and lysosomal dysfunction co-occur (Osellame *et al.*, 2013). Additionally, pathogenic mutations in Rab7, associated with Charcot–Marie–Tooth type 2B neuropathy, modify MLC stability and dynamics, highlighting their physiological importance.

Overall, MLCs are now seen as regulated hubs where mitochondria and lysosomes collaborate on fission, signaling, and quality control. Their frequent interaction with ER contacts emphasizes the idea that organelles rarely function alone but instead operate within complex contact networks that coordinate multiple signals for maintaining cellular homeostasis.

1.2.5 Tripartite membrane contact sites: emerging hubs of organelle coordination

The concept of membrane contact sites (MCSs) has expanded significantly over recent decades, evolving from simple two-organelle interactions to more complex three-way associations involving three different organelles (**Fig 1.10**). These tripartite MCSs are now recognized as essential hubs for regulating lipid metabolism, organelle movement, and cellular stability, particularly in situations where vesicular trafficking is limited or too slow to meet the cell's needs (Guyard & Giordano, 2025; Voeltz *et al.*, 2024).

- *Mitochondria–ER–lipid droplet contacts*

One of the most well-defined tripartite MCSs is the mitochondria–ER–LD contact. Mitochondria-associated ER membranes (MAMs) act as central hubs for phosphatidylserine (PS) and phosphatidic acid (PA) trafficking to mitochondria, where they are converted into cardiolipin (CL) and phosphatidylethanolamine (PE)—key components of mitochondrial inner membranes (Guyard & Giordano, 2025; Ma *et al.*, 2021). Proteins such as ORP5/8, seipin, and MIGA2 localize at these sites, coordinating both lipid transport and LD formation. Specifically, ORP5/8 interact with mitochondrial proteins like SAM50 and PTPIP51, forming a tripartite tethering structure essential for PS transport across membranes (Prinz *et al.*, 2020). Additionally, peridroplet mitochondria (PDM)—a distinct mitochondrial subset associated with LDs—exhibit a metabolic profile favoring lipid synthesis over β -oxidation. Their recruitment is triggered by fatty acid treatment and depends on MAM integrity (Yang *et al.*, 2022). This indicates that mitochondria-ER-LD contacts serve as anabolic platforms where mitochondria facilitate LD growth rather than energy production (Guyard & Giordano, 2025).

- *Peroxisome–ER–LD contacts*

Tripartite contacts involving peroxisomes, ER, and LDs have also been documented, particularly in yeast. The ER-shaping protein Pex30, together with Nem1 and Sei1 (yeast seipin), promotes LD biogenesis at ER-peroxisome interfaces (Joshi *et al.*, 2018). Its mammalian analog MCTP2 also supports LD growth in HeLa cells and *C. elegans*, although LDs can still form independently of these structures, indicating functional redundancy (Guyard & Giordano, 2025).

- *Mitochondria–ER–Golgi contacts*

Tripartite contacts between mitochondria, ER, and the Golgi have emerged as regulators of mitochondrial fission. After Drp1 is recruited to ER-mitochondria MCSs, Golgi-derived vesicles enriched in phosphatidylinositol-4-phosphate (PI4P) localize at these sites and promote actin polymerization, driving the final step of mitochondrial division (Nagashima *et al.*, 2021). The involvement of small GTPases such as Arf1 and PI4KIII β further links Golgi activity to mitochondrial morphology (Prudent *et al.*, 2021; Guyard & Giordano, 2025)

- *Mitochondria–ER–endosome/lysosome contacts*

PDZD8, a SMP-domain lipid transfer protein, plays a key role at ER–endosome–mitochondria contact sites. PDZD8 binds Rab7-positive late endosomes and localizes to three-way contacts involving mitochondria, where it likely facilitates phosphatidylserine transfer or modulates endosomal maturation (Elbaz-Alon *et al.*, 2020; Guillén-Samander *et al.*, 2019). The protein ORP1L, another lipid transfer protein, has been shown to mediate these tripartite contacts during mitochondrial fission, indicating that lysosomes contribute to mitochondrial division through lipid remodeling (Boutry & Kim, 2021).

- *Mitochondria–ER–plasma membrane contacts*

In yeast, the protein Num1 establishes ER–mitochondria–plasma membrane contacts (termed MECA), anchoring mitochondria to the cell cortex. This structure regulates mitochondrial distribution, inheritance, and PI4P polarization at the plasma membrane (Casler *et al.*, 2024). The ER protein Scs2 binds Num1 via a FFAT-like domain, forming a tether that integrates lipid signaling with organelle positioning.

Tripartite membrane contact sites are increasingly appreciated as multifunctional platforms for organelle communication, coordinating lipid flux, calcium signaling, organelle morphology, and metabolic reprogramming. They exhibit context-dependent specialization. Their study is crucial for understanding how cells integrate structural and metabolic information, and the dysregulation of this process may underlie diverse pathologies, ranging from lipid storage disorders to neurodegeneration.

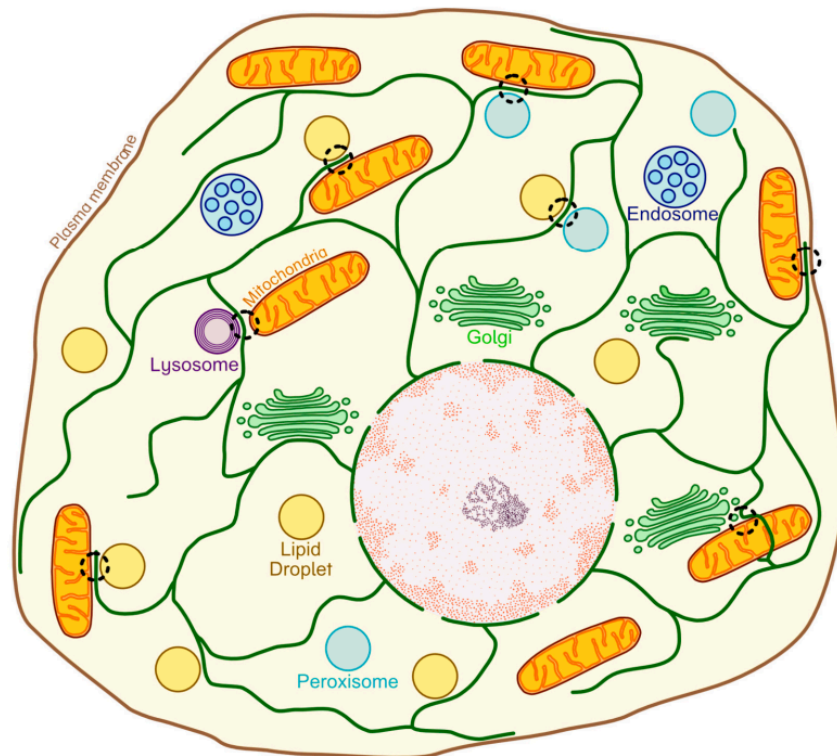


Figure 1.10 – Tripartite membrane contact sites in mammalian cells. Schematic representation of the currently identified three-way membrane contact sites involving the endoplasmic reticulum (ER) and two additional organelles. Documented configurations

include ER–mitochondria–lipid droplet, ER–mitochondria–lysosome, ER–mitochondria–Golgi, ER–mitochondria–endosome, ER–Golgi–endosome/lysosome, and ER–lipid droplet–lysosome contacts. These specialized junctions enable the coordinated transfer of lipids, signaling, and organelle dynamics across multiple compartments. Adapted from Guyard & Giordano, 2025.

1.3 Methods to study membrane contact sites

Investigating membrane contact sites (MCSs) presents a methodological challenge because these structures are nanoscopic, dynamic, and often heterogeneous. Their typical intermembrane distance of 10–30 nm makes them invisible to conventional light microscopy, and their transient nature complicates static ultrastructural analyses. Therefore, studying MCSs requires a multi-level methodological approach that combines ultrastructural imaging, live-cell fluorescence techniques, proximity labeling, proteomics, and functional assays to assess the physiological effects of organelle coupling (Scorrano *et al.*, 2019; Prinz *et al.*, 2020) (**Fig 1.11**).

Electron microscopy (EM) has traditionally been the primary method for providing direct evidence of MCSs by visualizing closely apposed membranes. Transmission EM and electron tomography enable precise measurements of gap distances and contact lengths, while volume EM techniques such as serial block-face or focused ion beam scanning EM reconstruct entire cellular volumes at nanometer resolution, offering a three-dimensional view of organelle networks (Wu *et al.*, 2018; Ching *et al.*, 2024). Recently, cryo-electron tomography of lamellae prepared through focused ion beam milling has facilitated the visualization of native, unfixed organelle contacts, revealing not only the actual spacing between membranes but also the presence of tethers connecting them (Keller & Fernández-Busnadiego, 2024; Nakamura *et al.*, 2025). Although EM is vital for understanding the ultrastructure of contacts, its molecular specificity remains limited, which can be partly addressed by combining it with immunogold labeling or correlative light and electron microscopy (CLEM). The latter technique is potent because it superimposes fluorescence signals from tagged proteins or reporters with ultrastructural data, linking molecular identity to membrane organization (Lees *et al.*, 2017).

Fluorescence microscopy remains the method of choice to investigate MCS dynamics in living cells. Confocal microscopy, although diffraction-limited, enables the visualization of apposed organelles and the quantification of contact

frequency, especially when coupled with object-based image analysis. Advanced confocal modalities such as Airyscan further improve resolution and sensitivity, providing a reliable balance between spatiotemporal resolution and phototoxicity (Wu *et al.*, 2018). Super-resolution techniques have significantly expanded our capacity to study MCSs. Structured illumination microscopy (SIM), including lattice-SIM, allows live-cell imaging at ~100 nm resolution, and has been widely used to monitor ER-mitochondria or ER-lysosome contacts (Cieri *et al.*, 2018). STED microscopy offers even higher resolution, down to 50 nm, and single-molecule localization methods, such as PALM, STORM, or MINFLUX, can resolve nanodomains of tethering proteins, albeit typically in fixed samples (Sigal *et al.*, 2018). As each method carries its own limitations, validation across different imaging platforms remains essential to avoid artefacts and misinterpretations.

In parallel to microscopy, genetically encoded contact reporters have been developed to monitor MCSs *in vivo*. One of the most established tools is SPLICS (split-GFP-based contact site sensor), which reconstitutes fluorescence when two halves of GFP, targeted to different organelles, come into proximity within a defined distance. By engineering short or long variants, it is possible to distinguish tight versus loose contacts (Cieri *et al.*, 2018). SPLICS has been successfully applied in cell culture and zebrafish models, enabling the quantification of contacts under physiological or pathological conditions. More recently, splitFAST, a reversible split-fluorogen system, has been introduced for monitoring ER-mitochondria contacts with fast kinetics. In contrast, BRET-based reporters, such as MERLIN, provide continuous readouts of average organelle spacing, making them particularly suited for pharmacological screens (Vance *et al.*, 2020). Although powerful, these reporters can potentially perturb contact site stability, making validation with orthogonal techniques necessary.

Other immuno-based approaches include proximity ligation assays (PLA), which generate fluorescent puncta when two proteins of interest are within ~30–40 nm of each other. PLA has been widely used to quantify specific tether interactions, such as VAPB–PTPIP51, in neurons and to assess alterations in patient tissues (Gomez-Suaga *et al.*, 2017). However, as PLA detects protein proximity rather than

membrane distance, it does not provide structural information and requires careful validation of the antibodies used.

Over the past decade, proximity labeling combined with proteomics has revolutionized how we study MCS components. Enzymes like APEX2, BioID, and TurboID, targeted to a specific side of a contact, covalently label nearby proteins within approximately 10–20 nm, which can then be identified through mass spectrometry (Hung *et al.*, 2017). More advanced techniques such as Contact-ID or split-TurboID activate enzyme activity only when two membranes are tightly aligned, thereby enriching genuine contact proteins and reducing background noise (Kwak *et al.*, 2020; Cho *et al.*, 2020). Simultaneously, biochemical fractionation methods that isolate mitochondria-associated ER membranes (MAMs) or other contact-rich subdomains remain common, providing samples for western blotting, lipidomics, and proteomics (Wieckowski *et al.*, 2009).

Beyond structural and compositional analysis, functional assays provide essential information on the physiological roles of MCSs. ER–mitochondria calcium transfer, for example, can be monitored using genetically encoded calcium indicators targeted to specific compartments, such as CEPIA or GCaMP variants, allowing simultaneous measurement of ER release and mitochondrial uptake (Suzuki *et al.*, 2014; Csordás *et al.*, 2010). Similarly, lipid exchange at contact sites can be inferred using lipid-specific probes, pulse-chase approaches, or photocaged lipid analogues, often combined with genetic perturbation of tethers or lipid transfer proteins (Reinisch & Prinz, 2021). Metabolic readouts, including mitochondrial ATP or ROS reporters, further complement this view, revealing how MCSs integrate signaling and metabolic flux (Mammucari *et al.*, 2018). Finally, synthetic perturbation tools allow direct testing of causality. Chemically inducible tethers based on FKBP–FRB dimerization can acutely increase contact frequency, revealing their effects on calcium dynamics or autophagy, while optogenetic systems such as iLID or LIT provide spatiotemporal precision, enabling the on-demand recruitment of ER to mitochondria or lysosomes (Bajar *et al.*, 2016). These approaches are particularly informative but require cautious interpretation, as artificial tethering can force non-physiological interactions.

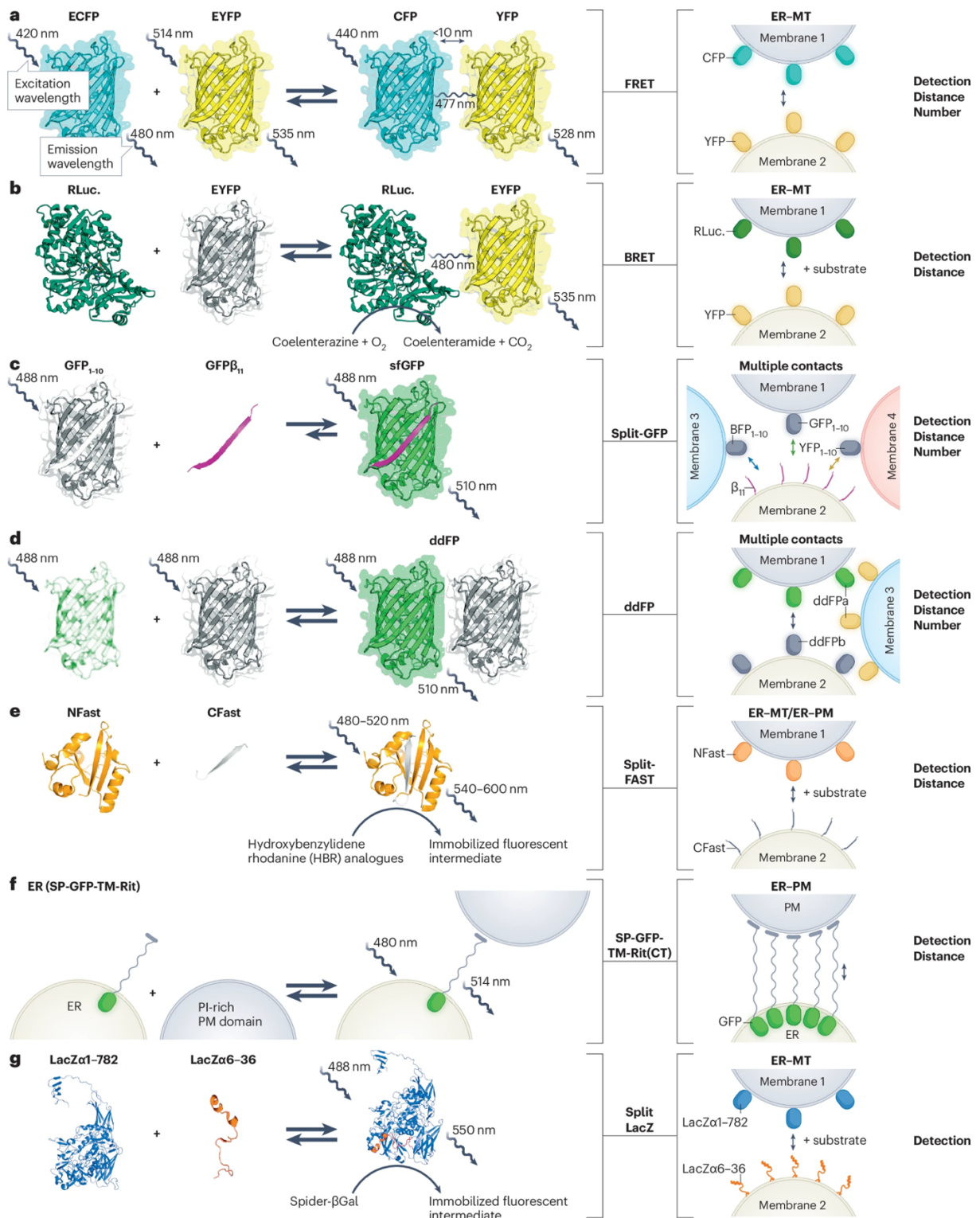


Figure 1.11 - Genetically encoded reporters to study organelle contact sites. On the left are illustrated the main strategies used to design genetically encoded reporters for membrane contact sites, based on either full-length or split fluorescent proteins with their excitation and emission spectra. Fluorescent and split-fluorescent reporters allow detection and quantification of membrane contact sites. Tools such as FRET, BRET, Split-LacZ, Split-FAST and MAPPER are mainly used for binary contacts, while ddFP and split-GFP can visualize multiple types of contacts, even simultaneously, through different fluorophores (e.g. GFP, CFP, YFP, luciferases). Some systems, including BRET, Split-FAST, Split-LacZ and inducible FRET, require exogenous substrates for fluorophore reconstitution. Abbreviations: ER, endoplasmic reticulum; PI, phosphatidylinositol; PM, plasma membrane. Image from Tito Cali *et al.* (2025).

Altogether, the study of MCSs relies on an integrated toolbox that spans ultrastructural imaging, live-cell fluorescence, genetic reporters, proteomics, functional assays, and acute perturbations. No single method suffices to describe these dynamic and heterogeneous structures fully; rather, complementary strategies must be combined to correlate structure, composition, and function. By adopting such multimodal workflows, researchers can dissect how organelle contacts contribute to cellular homeostasis and how their dysregulation underlies disease (Prinz *et al.*, 2020; Scorrano *et al.*, 2019).

1.4 SMP domain-containing proteins: lipid transporters and structural components at membrane contact sites

Among the many protein families enriched at membrane contact sites (MCSs), SMP domain-containing proteins form a structurally and functionally conserved group across eukaryotes. The SMP domain (Synaptotagmin-like Mitochondrial-Lipid-binding) belongs to the tubular lipid-binding (TULIP) superfamily and specializes in the non-vesicular transport of lipids between adjacent organellar membranes. These proteins typically serve as both tethers, physically maintaining organelle proximity at MCSs, and as lipid shuttles, facilitating the exchange of glycerophospholipids in either a directional or bidirectional manner (Reinisch & Prinz, 2021; Jeyasimmana *et al.*, 2024).

Structurally, the SMP domain adopts an elongated barrel-shaped conformation that forms a hydrophobic tunnel. This configuration allows the accommodation of one or more lipid molecules, enabling transfer between membranes spaced only 10–30 nm apart. While the precise mechanism of lipid uptake and release remains under investigation, SMP domains are thought to mediate transport via either a “shuttle” or “tunnel” model, depending on the protein complex and spatial organization (AhYoung *et al.*, 2015; Saheki & De Camilli, 2017). Several protein families containing SMP domains localize to different types of MCSs and are involved in diverse physiological functions (**Fig.1.12**).

One of the best-characterized groups is the Extended Synaptotagmins (E-Syts) in mammals—homologous to the Tricalbins in yeast—which localize to ER–plasma membrane contact sites. E-Syts are anchored to the ER via an N-terminal hairpin and contain a cytosolic SMP domain followed by multiple C2 domains. These proteins tether the ER to the plasma membrane by interacting with PI(4,5)P₂ through their C2 domains in a Ca²⁺-dependent manner. Structurally, the E-Syt SMP domain forms a ~90 Å long dimer with a hydrophobic groove that accommodates glycerolipids without strict headgroup specificity (Schauder *et al.*, 2014). E-Syts are involved in phosphatidylserine (PS) and diacylglycerol (DAG) recycling

between the plasma membrane and the ER, and have been implicated in processes such as insulin secretion, synaptic development, and membrane repair (Giordano *et al.*, 2013; Jeyasimmana *et al.*, 2024). Notably, triple-knockout mice lacking all three E-Syts are viable and fertile, suggesting redundancy or compensation by other LTPs (Sclip *et al.*, 2016).

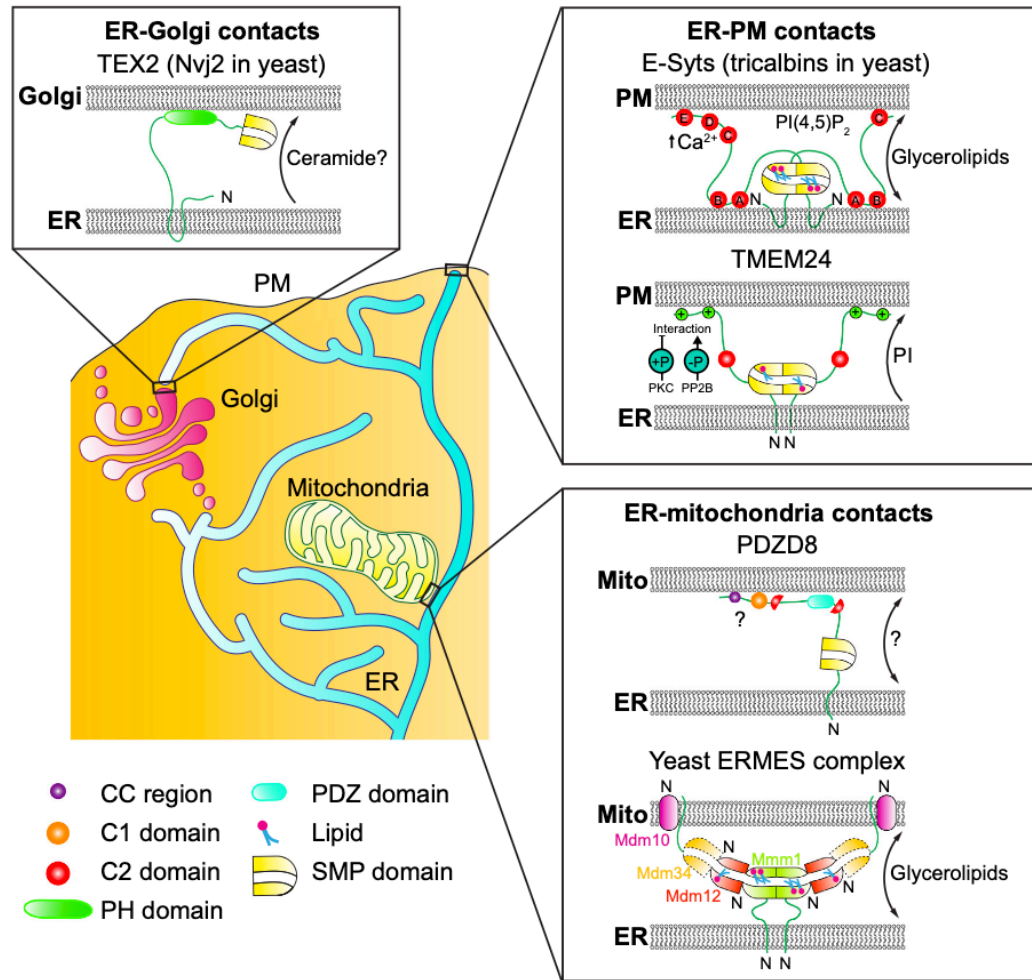


Figure 1.12 - Conserved SMP domain proteins at membrane contact sites. SMP-containing proteins localize to various MCSs and mediate lipid transport. At ER–PM contacts, E-Syts and TMEM24 tether the membranes and transfer glycerolipids or PI in a Ca^{2+} -regulated manner. At ER–mitochondria contacts, PDZD8 and the yeast ERMES complex facilitate tethering and support PS–PE lipid exchange. Nvj2/TEX2 functions at ER–Golgi contacts and transports ceramide via its SMP domain. Adapted from Jeyasimman *et al.*, 2020.

Another SMP-containing tether is TMEM24, an ER-resident protein enriched at ER–plasma membrane MCSs. It binds the plasma membrane via a positively charged domain regulated by phosphorylation. TMEM24 shows a preference for transferring phosphatidylinositol (PI), which is essential for replenishing the PM pool of phosphoinositides, especially in excitable cells like neurons and pancreatic β -cells. It also modulates Ca^{2+} dynamics in dendrites and participates in insulin granule exocytosis (Lees *et al.*, 2017).

In yeast, the ERMES complex (ER–mitochondria encounter structure) represents a prototypical example of an SMP-dependent lipid transfer machinery at ER–mitochondria contact sites. ERMES comprises three SMP-containing proteins—Mmm1 (ER-anchored), Mdm12 (cytosolic), and Mdm34 (OMM-bound)—that form a large bridge-like complex. Cryo-EM studies have shown that Mmm1 and Mdm12 form an elongated heterotetramer with a continuous hydrophobic tunnel, capable of binding phosphatidylserine (PS) and facilitating its transfer from the ER to mitochondria for decarboxylation into phosphatidylethanolamine (PE) (Jeong *et al.*, 2017; Kawano *et al.*, 2018). The disruption of ERMES impairs mitochondrial morphology, mtDNA maintenance, and respiratory growth.

In mammals, where ERMES is absent, the PDZD8 protein has been identified as a functional analog at ER–mitochondria MCSs. PDZD8 contains an SMP domain structurally similar to Mmm1 and E-Syt2 and mediates Ca^{2+} -dependent tethering and lipid transfer. It is highly expressed in neurons, where it regulates rapid mitochondrial Ca^{2+} uptake and may influence synaptic activity (Hirabayashi *et al.*, 2017). While its lipid transfer capacity remains less defined, its structural homology supports a conserved role in lipid shuttling.

Interestingly, the existence of redundant or compensatory lipid transport pathways has been demonstrated in yeast. For example, when ERMES is inactivated, the vCLAMPs (vacuole–mitochondria contact sites) expand and support partial rescue of lipid trafficking. Moreover, the VPS13 family—large LTPs with extended hydrophobic grooves—has emerged as an essential backup system. VPS13A and VPS13C, human homologs of yeast Vps13, are involved in lipid transfer at multiple MCSs including ER–mitochondria, ER–lipid droplet, and mitochondria–endosome

contacts. Mutations in VPS13A cause chorea-acanthocytosis, while VPS13C mutations are associated with early-onset Parkinson's disease, highlighting the physiological relevance of lipid transfer proteins in neurodegeneration (Kumar *et al.*, 2018).

In summary, SMP domain-containing proteins are central players in maintaining organelle communication through both structural tethering and active lipid transport at MCSs. Despite significant advances, open questions remain regarding their regulation, lipid specificity, and coordination with other LTPs and tethering complexes. Given their roles in organelle homeostasis, metabolism, and disease, SMP proteins represent an exciting field of study with growing biomedical relevance.

1.4.1 Nvj2p: an SMP-domain ceramide transporter at ER–Golgi MCSs

Nvj2p (nucleus-vacuole junction protein 2) is an SMP domain-containing protein originally identified in *saccharomyces cerevisiae*, where it plays a crucial role in regulating lipid homeostasis, particularly through the non-vesicular transport of ceramide from the endoplasmic reticulum (ER) to the Golgi apparatus. Structurally, Nvj2p is anchored to the ER membrane via an N-terminal transmembrane segment and contains both a Pleckstrin Homology (PH) domain and a Synaptotagmin-like Mitochondrial-lipid-binding Protein (SMP) domain in its cytosolic region.

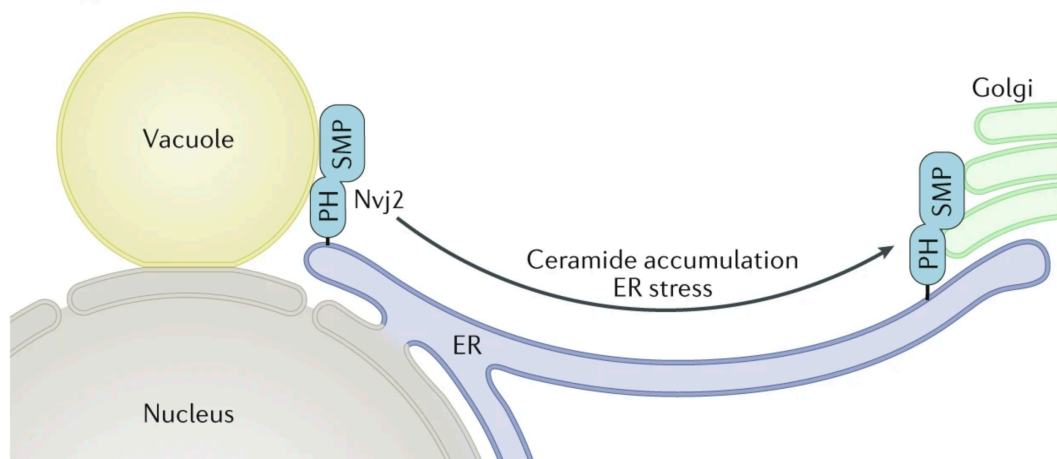


Figure 1.13 - Models of Nvj2p-dependent ER–medial Golgi tethering during stress. Conserved SMP domain proteins at membrane contact sites. Under normal conditions, Nvj2p is primarily localized at the nucleus–vacuole junction (NVJ), contributing only modestly to ceramide transfer toward the Golgi (left panel). Upon ER stress or excess ceramide accumulation, Nvj2p shifts its localization, promoting the formation of ER–medial Golgi contact sites to enhance ceramide export from the ER. Adapted from Prinz *et al*, 2020.

Ceramide is the central precursor in the biosynthetic pathway of complex sphingolipids, including sphingomyelin, glycosphingolipids (such as cerebroside and gangliosides), and ceramide-1-phosphate. It is synthesized *de novo* in the endoplasmic reticulum from serine and palmitoyl-CoA, and subsequently converted in the Golgi apparatus into downstream sphingolipid species that contribute to membrane structure and signaling (Hannun & Obeid, 2008; Castro *et al.*, 2014).

Under normal conditions, Nvj2p mainly localizes to the nucleus–vacuole junction (NVJ), a specialized membrane contact site. However, in response to ER stress or disruption of NVJ integrity, Nvj2p relocates to ER–Golgi contact sites, a process that depends on its PH domain, which mediates interaction with Golgi membranes. Remarkably, this relocation is associated with increased ceramide transfer activity, especially during stress or when ceramide synthesis is elevated (**Fig 1.13**). Overexpressing Nvj2p promotes expansion of ER–Golgi contacts and boosts the biosynthesis of inositolphosphorylceramide (IPC), a Golgi-localized complex sphingolipid, even in mutants lacking vesicular transport, strongly indicating that Nvj2p facilitates a non-vesicular ceramide transport pathway (Liu *et al*, 2017) (**Fig. 1.13**).

Biochemical studies further confirmed this role: cell lysates overexpressing Nvj2p produced significantly higher levels of IPC compared to wild-type, while Nvj2p mutants carrying point mutations in the SMP domain, predicted to impair lipid binding, failed to rescue ceramide transport. These results indicate that the SMP domain is directly responsible for binding and possibly shuttling ceramide molecules between the ER and Golgi (Liu *et al*, 2017).

Functionally, this lipid transport activity has critical physiological implications. Ceramide is a central bioactive sphingolipid that, if accumulated in excess, can become cytotoxic. Nvj2p alleviates this lipotoxicity by facilitating ceramide export from the ER to the Golgi, where it is metabolized into complex, less harmful derivatives. Consistently, overexpression of Nvj2p reduces ceramide-induced stress and enhances cell survival under lipotoxic or ER stress conditions.

Importantly, this function appears to be evolutionarily conserved. The mammalian homolog TEX2, which also localizes to tubular ER, can partially rescue the phenotype of Nvj2 Δ yeast mutants, suggesting functional conservation of ceramide transport activity across species (Liu *et al*, 2017).

1.4.2 What is known about TEX2

TEX2 (Testis-expressed protein 2) is a conserved lipid transfer protein (LTP) characterized by the presence of a Synaptotagmin-like Mitochondrial-lipid-binding Protein (SMP) domain, a pleckstrin homology domain (PH) and localized specifically to the tubular subdomains of the endoplasmic reticulum (ER) (**Fig.1.14**). TEX2 is evolutionarily conserved across metazoans and shares homology with the yeast protein Nvj2p, which is known to participate in ER–Golgi contacts under stress conditions and facilitate ceramide transfer (Liu *et al.*, 2017). In mammals, however, the function of TEX2 had remained largely unexplored until recently.

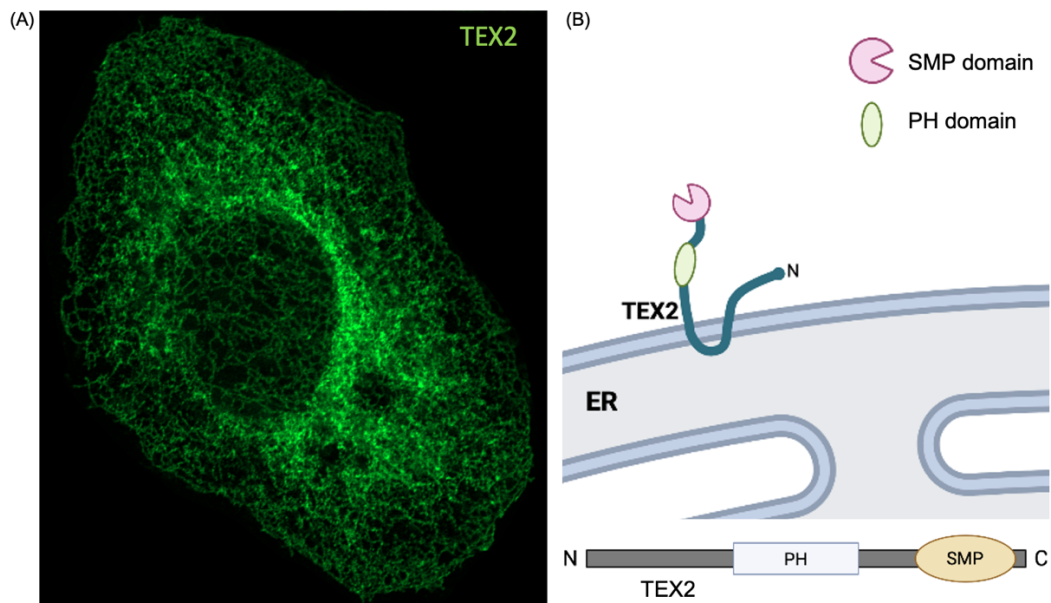


Figure 1.14 – TEX2, an ER-resident SMP protein. (A) Immunofluorescence image of fixed HeLa cells expressing GFP-TEX2, showing its specific enrichment along the tubular ER network. (B) Schematic illustration of TEX2 topology at the ER membrane. Both the N- and C-termini are exposed to the cytosol, with the C-terminal region containing a PH domain and an SMP domain.

1.4.2.1 TEX2 at ER-LE MCSs

In a pivotal study by Du *et al.* (2023), TEX2 was identified as a tubular ER protein that localizes independently of classical ER-shaping proteins such as RTN4 and REEP5. Using CRISPR-Cas9 knock-in and GFP-tagged constructs, TEX2 was shown to localize exclusively to ER tubules via an N-terminal region encompassing residues 277–474. Although this domain mediates targeting to the ER, its localization is not dependent on major ER-shaping proteins, suggesting an autonomous targeting mechanism (Du *et al.*, 2023).

Importantly, TEX2 is recruited to ER–late endosome/lysosome (LE/lys) membrane contact sites (MCSs) through a direct interaction with TMEM55B, a phosphoinositide 4-phosphatase that converts PI(4,5)P₂ to PI5P. The interaction is mediated by the N-terminal region of TEX2 (residues 1–276) and requires the catalytic CX₅R motif within the PTase domain of TMEM55B, although not its full enzymatic activity. TMEM55B-mediated recruitment of TEX2 is spatially restricted to perinuclear regions, where ER tubules closely associate with LE/lys membranes (Du *et al.*, 2023).

Interestingly, this interaction is negatively regulated by type II phosphatidylinositol 4-kinases (PI4KII α/β), which are known to generate PI4P on endosomal membranes. Overexpression of PI4KIIs inhibits the recruitment of TEX2 to ER–LE/lys contacts, suggesting a phosphoinositide-sensitive regulatory mechanism. Notably, depletion of PI4KIIs promotes TEX2 recruitment even in the absence of TMEM55B overexpression, highlighting the sensitivity of these contacts to local lipid composition (Du *et al.*, 2023).

Functionally, TEX2 plays a critical role in lysosomal homeostasis. TEX2 knockout cells display impaired lysosomal digestive capacity, as shown by accumulation of fluorescent pH-sensitive lysosomal reporters and defective autophagic flux. These defects are not due to disrupted autophagosome–lysosome fusion but rather to decreased lysosomal function itself. Complementation assays revealed that both the SMP domain and the TMEM55B-binding N-terminal region are necessary to rescue

lysosomal defects in TEX2-deficient cells. Notably, the deletion of the SMP domain abolishes TEX2's ability to restore lysosomal function, underscoring its essential role in lipid transfer (Du *et al.*, 2023) (**Fig. 1.15**).

In line with this, TEX2 binds multiple lipid species, including phosphatidylcholine (PC), phosphatidylserine (PS), and ceramide, both in vitro and in cells. Lipidomics analysis showed that TEX2 preferentially associates with sphingolipids, particularly sphingosine and ceramide, despite their relatively low abundance in total cellular membranes. TEX2 depletion results in altered lipid composition in LE/lys membranes, with a significant accumulation of sphingosine—an event associated with lysosomal dysfunction in pathologies such as Niemann-Pick disease type C (Du *et al.*, 2023; te Vrugte *et al.*, 2004).

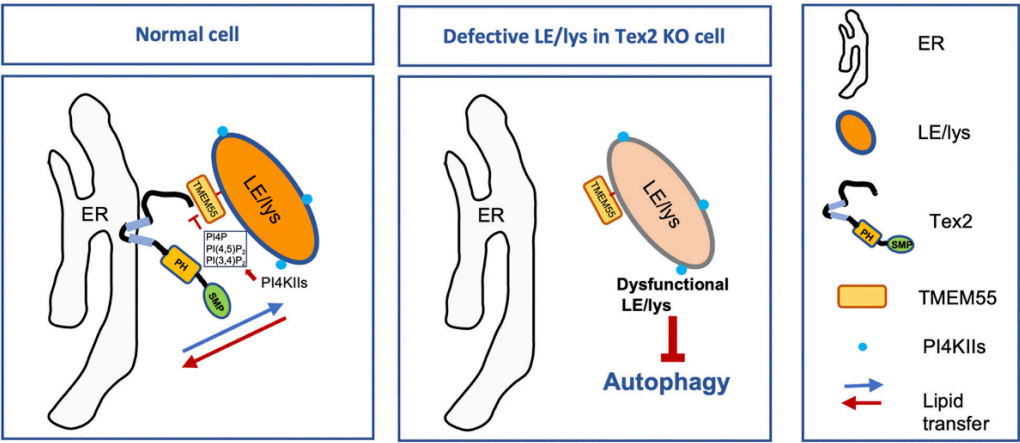


Figure 1.15 – Proposed model of TEX2 function at ER–LE/lysosome membrane contact sites (MCSs). TEX2 is exclusively localized to the tubular ER and is recruited to ER–LE/lys MCSs through interaction with TMEM55, a PI(4,5)P₂ phosphatase on LE/lys membranes. This recruitment is negatively regulated by type II PI4K activity. Loss of TEX2 at these contact sites impairs lysosomal degradation and disrupts autophagic flux, potentially due to altered lipid composition in LE/lys membranes. Adapted from Du *et al.*, 2023.

Altogether, these findings identify TEX2 as a key component of TMEM55-dependent ER–LE/lys MCSs, where it likely mediates non-vesicular lipid transfer crucial for maintaining lysosomal function. The study of TEX2 thus expands our understanding of how lipid homeostasis and organelle communication are orchestrated through ER-resident LTPs at inter-organelle contact sites.

1.4.2.2 The cooperative role of TEX2 and PDZD8 in lipid regulation in *C. elegans*

TEX2 and PDZD8 are SMP domain-containing proteins that play a crucial and redundant role in maintaining endosomal lipid homeostasis during embryogenesis in *C. elegans*. SMP domain proteins are known to facilitate non-vesicular lipid transfer at membrane contact sites (MCSs), and PDZD-8 represents metazoan homologs of the ERMES complex.

In early *C. elegans* embryos, TEX2 and PDZD8 work together to prevent aberrant accumulation of PI(4,5)P₂ (phosphatidylinositol 4,5-bisphosphate) on endosomal membranes. When these proteins are absent, PI(4,5)P₂ accumulates abnormally, causing the misplacement of actomyosin regulators such as non-muscle myosin II (NMY-2), anillin (ANI-1), and F-actin to endosomes. This misplacement interferes with actomyosin contractility at the plasma membrane, ultimately impairing polarity formation, cytokinesis, and embryonic survival (Jeyasimman *et al.*, 2020).

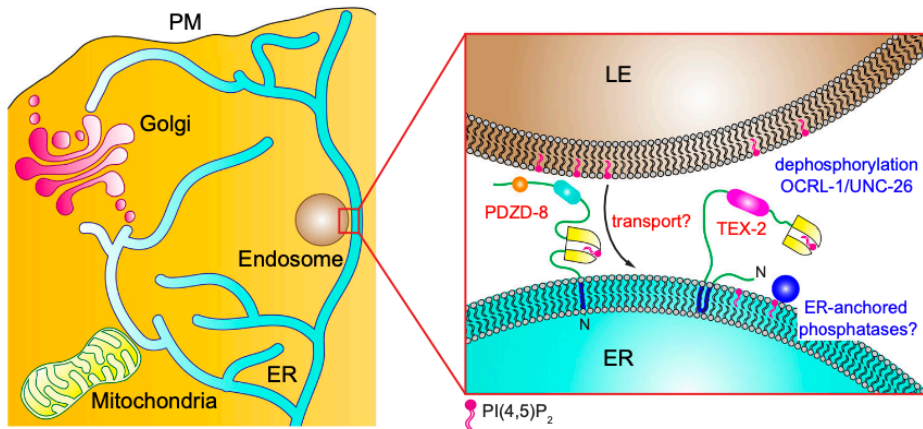


Figure 1.16 – Model of PDZD8 and TEX2 function in endosomal PI(4,5)P₂ regulation.

PI(4,5)P₂ is typically enriched at the plasma membrane, but PDZD8 and TEX2 help maintain its spatial distribution. PDZD8 also localizes to ER–LE contact sites, where it may mediate the removal of PI(4,5)P₂ from endosomal membranes, potentially via lipid transfer through its SMP domain. TEX2 acts redundantly with PDZD8 and associates with ER–LE contact sites. Together with endosomal phosphatases like OCRL-1 and UNC-26/synaptojanin, these SMP proteins cooperate to maintain phosphoinositide homeostasis and prevent aberrant endosomal PI(4,5)P₂ accumulation.

Mechanistically, both TEX2 and PDZD8 are anchored to the endoplasmic reticulum. PDZD8 is additionally enriched at ER–late endosome contacts and is responsible for lipid transfer through its SMP domain. In vitro assays confirmed that the SMP domain of PDZD8 can transfer various phosphoinositides, including PI(4,5)P₂, as well as phosphatidylserine (PS) and phosphatidic acid (PA), supporting its direct role in lipid trafficking. Notably, when both TEX2 and PDZD8 are deleted in combination with the PI(4,5)P₂ phosphatases OCRL-1 and UNC-26/synaptojanin, embryos exhibit massive PI(4,5)P₂ accumulation on endosomal membranes, accompanied by large cytoplasmic vesicles, cortical contractility failure, cytokinesis defects, and lethality, demonstrating the functional synergy between SMP proteins and lipid phosphatases in maintaining phosphoinositide compartmentalization (Jeyasimman *et al.*, 2021) (**Fig 1.16**).

Aims

The main aim of my Ph.D. project was to investigate the role of TEX2, an endoplasmic reticulum (ER)–resident protein containing a Synaptotagmin-like Mitochondrial-lipid-binding Protein (SMP) domain and a Pleckstrin Homology (PH) domain, in the regulation of inter-organelle communication and mitochondrial dynamics. TEX2 is the mammalian ortholog of the yeast ceramide transporter Nvj2p (Liu *et al.*, 2017), suggesting an evolutionarily conserved function in lipid transfer. Despite its localization at ER–late endosome contacts and the presence of lipid-binding domains (Liu *et al.*, 2017), the physiological role of TEX2 in mammalian cells remains poorly understood.

Our main hypothesis was that TEX2 acts as an ER-mitochondria tether and lipid transfer protein, regulating the formation, composition, and activity of MAMs.

Specifically, we proposed that TEX2 plays a role in shaping the lipid microenvironment needed for mitochondrial fission and mitophagy, potentially by facilitating ceramide transfer to mitochondria. Additionally, its reported location at ER-lysosome contact (Liu *et al.*, 2017) suggests that TEX2 may function as a hub coordinating multiple inter-organelle contact sites, including ER-mitochondria and ER-lysosome interfaces, thereby helping to synchronize lipid balance and organelle remodeling.

To address this hypothesis, our project pursued the following main objectives:

1. To define the subcellular localization and molecular partners of TEX2, to establish whether it acts as a tethering component of ER-mitochondria and/or ER-lysosome membrane contact sites.
2. To assess the functional role of TEX2 in mitochondrial dynamics, focusing on its contribution to mitochondrial fission and on the activation of DRP1-dependent scission events.
3. To investigate the lipid transfer potential of TEX2, with particular attention to its SMP domain and possible involvement in ceramide handling at MAMs.

4. To explore the physiological interaction between TEX2 and FUNDC1, a mitochondrial outer membrane protein that connects fission and mitophagy, to determine whether these proteins form a functional tethering complex linking lipid metabolism with mitochondrial remodeling.
5. To evaluate the broader role of TEX2 in coordinating ER–mitochondria–lysosome communication, and how its loss affects organelle organization and the balance of contact sites.

Through this strategy, we aimed to understand how TEX2 integrates structural tethering and lipid transfer activities to regulate the organization and function of MAMs. Ultimately, this work intends to establish TEX2 as a new key regulator of inter-organelle communication, providing insights into how lipid metabolism orchestrates mitochondrial fission, autophagy, and cellular homeostasis.

Chapter 2 – Materials and Methods

2.1 Cell culture

HeLa cell lines were from ATCC. HeLa cells were cultured in Dulbecco's modified Eagle's Medium (DMEM – Gibco) supplemented with 10 % Fetal Bovine Serum (FBS – Euroclone), 1% penicillin/streptomycin (P/S - Thermo Fisher Scientific), and 2 mM L-glutamine (L-Glu - Thermo Fisher Scientific). For the analysis of mitochondria–lysosome contacts, immortalized human fibroblasts (BJ-5ta; ATCC® CRL-4001™) were used. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% GlutaMAX™, and 1% penicillin–streptomycin (Thermo Fisher Scientific), at 37 °C in a humidified atmosphere containing 5% CO₂ and routinely tested for Mycoplasma contamination.

2.2 Antibodies and dyes

The antibodies used in the study are the following: rabbit polyclonal TEX2 (Novus, NBP1-83156, dilution 1:200 in IF; 1:500 for WB); mouse monoclonal anti-TOM20 (BD, 612278, dilution 1:200 in IF; 1:2000 for WB); rabbit polyclonal anti-Calnexin (Abcam, ab22595, dilution 1:200 in IF; 1:2000 for WB); goat polyclonal anti-HA (Bethyl, A190-138A, dilution 1:200); mouse monoclonal anti-DRP1 (BD, 611739, dilution 1:200 in IF; 1:1000 for WB); rabbit polyclonal anti-pDRP1 (Ser616) (Thermo, PA5-106169, dilution 1:200 in IF; 1:1000 for WB); rabbit polyclonal anti-pDRP1 (Ser637) (Thermo, PA5-101038, dilution 1:200 in IF; 1:1000 for WB); rabbit polyclonal anti- α Actin-1 (Sigma, A2066, dilution 1:8000 for WB); rabbit polyclonal anti-FUNDC1 (Enzo Life, ARP53280-P050, dilution 1:200 in IF; 1:1000 for WB); rabbit polyclonal anti-LAMP1 (Abcam, ab24170, dilution 1:200 for IF); sheep polyclonal anti-LAMP1 (R&D, AF4800, dilution 1:200 for IF); rabbit polyclonal anti-TMEM55B (Novus, NBP2-13453, dilution 1:1000 for WB); mouse monoclonal anti-ORP8 (Santa Cruz, 134409, dilution 1:200 for IF); rabbit polyclonal anti-PTPI51 (Sigma, HPA009975, dilution 1:200 for IF). The antibody anti-VAPA rabbit (1:600 for IF) was previously produced in our laboratory by Giuseppe Di Tullio.

Donkey Secondary Antibodies targeting IgG or IgM of different species (mouse, rabbit, goat, and sheep) are Alexa Fluor 405-488-568-647 (ThermoFisher, diluted 1:400).

For cell imaging, cells were stained with LysoTracker™ Red DND-99, LysoTracker™ Deep Red (Thermo Fisher Scientific, 1:1000) to label lysosomes, with MitoTracker™ Orange CMTMRos and MitoTracker™ Deep Red FM (Thermo Fisher Scientific, 1:10.000) to label mitochondria, and with Lipi-Blue™ (1:1000) to visualize lipid droplets.

2.3 Reagents

Rapamycin (Sigma-Aldrich, R0395) and FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazine; Sigma-Aldrich, C2920) were dissolved in DMSO to prepare 2.74 mM and 5 mM stock solutions, respectively, and stored at -20°C . Working dilutions were freshly prepared in complete culture medium immediately before use (final DMSO concentration $< 0.1\%$).

TopFluor-phosphatidylethanolamine (TopFluor-PE; Avanti Polar Lipids, 810282) was used to monitor phospholipid transfer and incorporation into cellular membranes. The original stock (1 mg/mL in chloroform; 1.1 mM) was dried under a gentle nitrogen stream in a chemical hood, and the lipid film was resuspended in absolute ethanol to a final concentration of 0.5 mM, according to the manufacturer's protocol. The solution was vortexed for 5 min, saturated with nitrogen, and stored at -20°C until use. For cell treatment, an aliquot was diluted in pre-warmed culture medium to the desired final concentration and added directly to cells.

2.4 DNA cloning and plasmids

Rossella Venditti and Michele Santoro designed all the plasmids used in the study in our laboratory. To generate the MCS reporter TGN46-FRB-HA-GFP-T2A-mCherry-FKBP-Cb5-pIRES-neo2 (ER-TGN), TMEM192-FRB-HA-GFP-T2A-mCherry-FKBP-Cb5-pIRES-neo2a (ER-LE), and N33(TOM20)-FRB-HA-GFP-T2A-mCherry-FKBP-Cb5-pIRES-neo2a (ER-Mitochondria), a series of sequential subcloning steps was performed using the pIRES-neo2 backbone. The ER membrane-targeting reporter Cb5, which consists of 17 amino acids from the transmembrane domain of rat cytochrome b5 (Bulbarelli et al., 2002), was cloned in-frame downstream of mCherry-

FKBP, and this fusion was inserted into the pIRES-neo2 vector. To enable bicistronic expression, a T2A peptide coding sequence (Osborn et al., 2005) was introduced by in vitro primer annealing upstream of the mCherry-FKBP-Cb5 fragment, creating the intermediate construct T2A-mCherry-FKBP-Cb5-pIRES-neo2. During this cloning step, the AgeI restriction site at the 3' end was removed, and a new AgeI site was engineered at the 5' end of the T2A sequence to allow later insertion of the TGN46 construct.

The TGN46-FRB-HA plasmid was used as the source of the TGN component. This modified construct was cloned upstream of T2A-mCherry-FKBP-Cb5 in the pIRES-neo2 vector to obtain the TGN46-FRB-HA-mCherry-T2A-FKBP-Cb5-pIRES-neo2, generated by Rossella Venditti.

Based on the TGN-targeted MCS reporter construct described above, additional reporter systems were generated to visualize and manipulate other classes of membrane contact sites, including ER-late endosome (ER-LE) and ER-mitochondria (MAM) contacts. These constructs were developed by replacing the TGN46-FRB-HA module with alternative organelle-specific reporters.

For ER-LE contacts, the lysosomal membrane protein TMEM192, fused to a 3xHA epitope tag, was used instead of TGN46, enabling specific targeting to late endosomes and lysosomes. For ER-mitochondria contact sites, the N-terminal 33 amino acids of the mitochondrial protein TOM20, known to be enough for targeting the outer mitochondrial membrane, were inserted instead of TGN46 to ensure precise localization to mitochondria. All constructs were cloned upstream of the T2A-mCherry-FKBP-Cb5 cassette in the pIRES-neo2 backbone, preserving the same bicistronic expression setup as the original ER-TGN reporter.

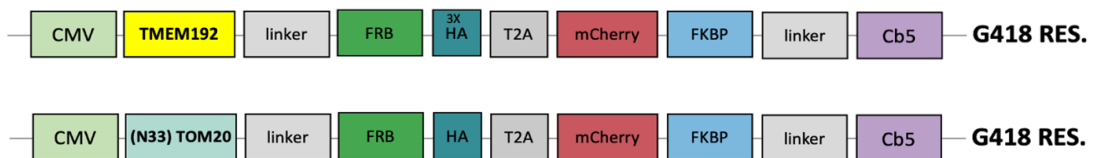


Figure 2.1 - Schematic representation of the ER-LE/Lys and ER-mitochondria reporters.

(Top) The lysosomal membrane-anchored version (TMEM192-FRB-3xHA-T2A-mCherry-FKBP-Cb5) positions the FRB domain on the cytosolic face of lysosomes through the TMEM192 targeting sequence. (Bottom) The mitochondrial outer membrane-anchored module (N33-TOM20-FRB-HA-T2A-mCherry-FKBP-Cb5) targets the FRB domain to the cytosolic side of the mitochondrial surface via the TOM20 N-terminal signal sequence (N33). In both constructs, the FRB and FKBP domains heterodimerize in the presence of rapamycin, inducing the stabilization contacts between the respective organelles and the ER. The Cb5 tail anchors the FKBP moiety to the ER membrane, and the self-cleaving T2A peptide ensures co-expression of both fluorescent and functional components within a single transcriptional unit.

The TEX2-GFP construct used in this study was generated by subcloning the full-length human TEX2 coding sequence (NM_001288733) from a commercial TEX2-turboGFP plasmid (OriGene, RC208694) into a mammalian expression vector containing EGFP. The turboGFP tag was replaced with EGFP to ensure compatibility with other constructs and fluorescence channels used in co-expression assays. Standard restriction-ligation cloning was employed to generate the final TEX2-GFP (wild-type) construct.

Based on this plasmid, two additional SMP-domain mutant variants (L853A/V1098A and L853W/V1098W) were generated to disrupt conserved residues predicted to be critical for lipid binding and transfer. These mutations were selected based on conserved positions within the SMP domains of Extended Synaptotagmins (E-Syts), which were previously shown to affect lipid transfer activity (Giordano *et al.*, 2013).

Site-directed mutagenesis was performed by PCR using complementary primers carrying the desired substitutions (see **Table 2**), followed by DpnI digestion of the parental plasmid and transformation into *E. coli* DH5 α . All plasmids were verified by Sanger sequencing.

Each TEX2-GFP variant, including wild-type, L853A/V1098A, and L853W/V1098W, was subsequently rendered siRNA-resistant by introducing synonymous nucleotide substitutions within the siRNA target region, using specific oligonucleotides listed in Table 1. These silent mutations preserved the amino acid sequence while abolishing siRNA recognition.

PRIMERS	SEQUENCE
Tex2 x GFPn1-fw	AGATCTCGAGCTCAAGCTTCGAATTCATGACAAGTCTGTATGGTCGC CATG
Tex2 x GFPn1-rev	GCTCACCATGGTGGCGACCGGTCTGGTTGATCAGCAGCCTCCACAG
vec-GFP/CHERRY-N1 (+)	GGACCGGTGCGCCACCATGGTGAGC
GFP-C (-)	GAATTCGAAGCTTGAGCTCGAGATCT
Tmem192 x Ires-fw	CTCGGATCGATATCTGCGGCCTAGCTAGCAAAAAGCGCTACCGGTAC CATGGCG
Tmem192 x Ires-rev	GCACCAGCACCTGAATTCGCAGATCTGGCTCCCGTTCTACTTGGCTGA CAG
Frap-3xHA-rev	AAGGGTATCCAGCGCTGCCAGCGTAGTCAGGCACATCATAGGGGTA GGATCCTGTTCTGCGTAATCAGGCACGTCGTATGGATAACTAGTCTT TGAGATTCTGTCG
3xHA x ires-fw	CTATGATGTGCTGACTACGCTGGCAGCGCTGGATACCTTATGATG TGCCTGATTATGCTGGAGGTAGTGGAGCGACCGGTGAGGGCAGGG GCAGCCT
Frap-fw	CCAGATCTGCGAATTCAGGTGCTGGTGC
T2A x N33ofTomm20(-)	TCCACAGACTCCCGCGGCGATGGCGTTGTTCCGACCTACCATACCTG GACCAGGGTTTTCTCCACGTC
IRES (+)	GCGGCATGGACGAGCTGTACAATGATGATCCAGTGTGCTGGAATTA ATTCGCTG
N33 x T2A-fw	GAACAACGCCATCGCCGCGGGAGTCTGTGGAGCGCTCTTTATAGGGT ACTGCATCTACTTTGACCGCAAAAGGAGAAG
FKBP-fw	CTTTGACCGCAAAAGGAGAAGTGATCCTAACGGGGGAATGGGAGTG CAGGTGGAAAC
TEX2-V1098A (+)	GTCATGCCAAACATGGATGATGCTTATATCACTATAATGCACTCAG
TEX2-V1098A (-)	CTGAGTGCATTATAGTGATATAAGCATCATCCATGTTTGGCATGAC
TEX2-V1098W (+)	AAGTTTTTGTGATGCCAAACATGGACGATTGGTATATCACTATAATGC ACTCAGCCATGGACCC
TEX2-V1098W (-)	GGGTCCATGGCTGAGTGCATTATAGTGATATACCAATCGTCCATGTTT GGCATGACAAAACCTT
TEX2- L853A (+)	GATCTGGTGTCTAAGAAGATCCAAATGAAAGCCAGCAAAATAAAGCT CCCCTAC
TEX2- L853A (-)	GTAGGGGAGCTTTATTTTGTGCTGCTTTCATTTGGATCTTCTTAGACAC CAGATC
TEX2- L853W (+)	GGTCTGATCTGGTGTCTAAGAAGATCCAAATGAAATGGAGCAAAATA AAGTCCCCTA
TEX2- L853W (-)	TAGGGGAGCTTTATTTTGTCTCATTTCATTTGGATCTTCTTAGACACCA GATCAGACC
TEX2rnaRST#1(+)	CGGTCAACATTCCACCACCCCAACGGATAGAGTGTGGTATGGTTTC CGAAAGCCACC
TEX2rnaRST#1(-)	GGTGGCTTTCGGAAACCATACCACACTCTATCCGTTGGGGGTGGTGG AATGTTGACCG
TEX2rnaRST#2(+)	GTGCCAAAAATCCTCCAGGCCTTCAAACCGTATGTGGACCATCAGGG ACTCTGGATTGATTTGGAAT
TEX2rnaRST#2(-)	ATTTCCAAATCAATCCAGAGTCCCTGATGGTCCACATACGGTTTGAAG GCCTGGAGGATTTTGGCAC
TEX2rnaRST#3(+)	GACTTCTTAGGAGAGAAATACTGGTCTGATCTGGTTAGCAAAAAAAT CCAAATGAAACTCAGCAAAATAAAGCTCCCCTAC
TEX2rnaRST#3(-)	GTAGGGGAGCTTTATTTTGTGAGTTTCATTTGGATTTTTTGTAAAC CAGATCAGACCAGTATTTCTCTCCTAAGAAGTC

Table 1 – List of primers in cloning experiment

All resulting TEX2–GFP siRNA-resistant constructs were sequence-verified (Eurofins Genomics) and expressed comparable protein levels when transiently transfected into HeLa and BJ5ta cells. Plasmid DNA was purified using the NucleoBond Xtra Midi Kit (Macherey-Nagel) before transfection. Other plasmids used in this study were obtained from Addgene: mCherry-DRP1 (#49152), Mito-BFP (#49151), GFP-MFN1 (#141154).

2.5 Stable cell line generation

To obtain stably expressing clones, HeLa cells were transfected with ER-TGN, ER-LE, and ER-Mito reporter plasmids. Expression was maintained for 48 hours, then cells were selected in full medium supplemented with 2 mg/mL Gentamicin (G418 – Sigma Aldrich). After the initial selection step, cells were expanded and kept in culture with 0,6 mg/mL G418. These cells were then validated through Western blot, immunofluorescence, and wound healing assays to evaluate their migration properties.

2.6 Transfection and RNA Interference

For protein expression, HeLa cells were transfected using Lipofectamine LTX and PLUS Reagent (Thermo Fisher Scientific) or Lipofectamine 2000 (Thermo Fisher Scientific), both following the manufacturer's instructions. Expression was maintained for 16 to 24 hours. For gene silencing experiments, siRNA oligonucleotides were transfected using Oligofectamine (Thermo Fisher Scientific) following the manufacturer's protocol. A final siRNA concentration of 40 nM was used for standard experiments, including immunofluorescence and western blot analyses (**Fig. 2.2**).

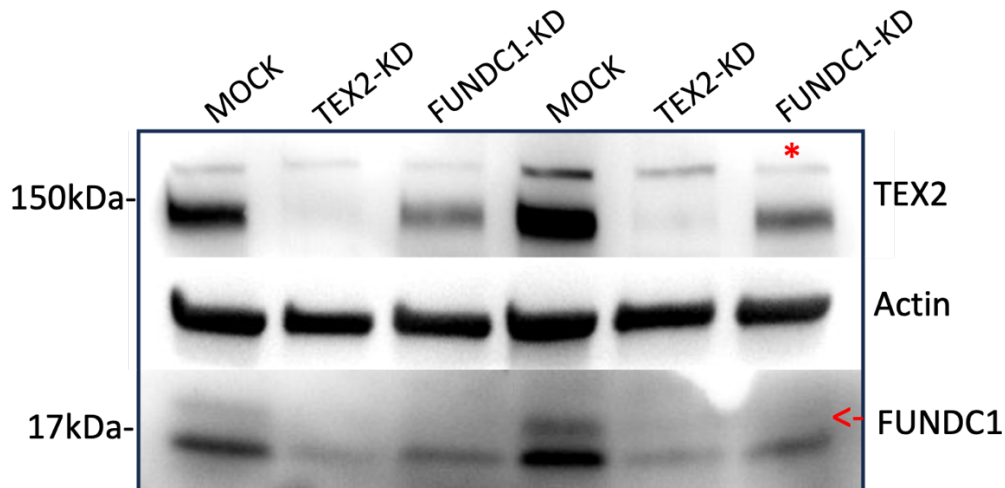


Figure 2.2 - Reciprocal regulation of TEX2 and FUNDC1 protein levels in HeLa cells.

Total protein lysates (60 µg per lane) from HeLa cells transfected with control siRNA (Ctrl), TEX2-targeting siRNA (TEX2-KD), or FUNDC1-targeting siRNA (FUNDC1-KD) were separated on a 4–15% gradient SDS–PAGE gel and analyzed by immunoblotting with antibodies against TEX2 and FUNDC1. Actin was used as a loading control. The anti-TEX2 antibody detected a major band corresponding to the expected molecular weight of TEX2 and a weaker non-specific band (marked with a red asterisk) that was consistently present across all samples. Despite this background signal, TEX2 protein levels were clearly reduced upon TEX2 knockdown, confirming the efficiency of the siRNA treatment. Interestingly, the FUNDC1 immunoblot revealed two close bands, of which the upper band corresponds to the specific FUNDC1 signal (indicated by the red arrowhead), as confirmed by immunoprecipitation assays (see Results). A consistent reduction of this band was observed in FUNDC1-KD samples, confirming antibody specificity and knockdown efficiency. Of note, a reciprocal decrease in protein abundance was observed between TEX2 and FUNDC1: TEX2 depletion led to a noticeable reduction of FUNDC1, whereas FUNDC1 silencing also caused a mild but reproducible decrease in TEX2 signal. This observation suggests that the two proteins may be functionally interconnected or stabilized within the same molecular complex, in line with the hypothesis that TEX2 and FUNDC1 act at common membrane contact sites.

<i>TEX2</i>	#1	5'-CGACUGACCGAGUAUGGUA-3'
	#2	5'-GCCUUACGUUGAUCACCAA-3'
	#3	5'-GUGUCUAAGAAGAUCCAAA-3'
<i>FUNDC1</i>	#1	5'-GCAGCACCUGAAAUCAACA-3'
<i>TMEM55B</i>	#1	5'-GCATCAGCATGTAGTCAAA-3'
	#2	5'-GCCAATCTCTCATCAACGT-3'
	#3	5'-GGGCATTTGTCATCCTGTT-3'

Table 2 – List of siRNAs used in the experiments

2.7 Rapamycin-induced stabilization of membrane contact sites

HeLa cells, expressing TGN46-FRB-HA and mCherry-FKBP-Cb5 for ER–TGN contacts, Rab7-FRB-HA and mCherry-FKBP-Cb5 for ER–late endosome contacts, and TOM20-FRB-HA and mCherry-FKBP-Cb5 for ER-mitochondria contacts, were pretreated with cycloheximide (50 µg/ml, 30 min at 37°C) to inhibit protein synthesis and keep the steady-state expression levels of these constructs. Rapamycin was then added to induce FKBP–FRB heterodimerization, which stabilizes the pre-existing ER–organelle contact sites. Specifically, 200 nM rapamycin was applied for 2 minutes at 37°C for ER–LE assays, while a lower concentration (20 nM) was used for the ER-mitochondria condition to achieve milder tethering compatible with physiological MERCS stability. Following treatment, cells were fixed with 4% paraformaldehyde and immunostained with anti-HA antibody (to verify the expression of FRB-tagged constructs).

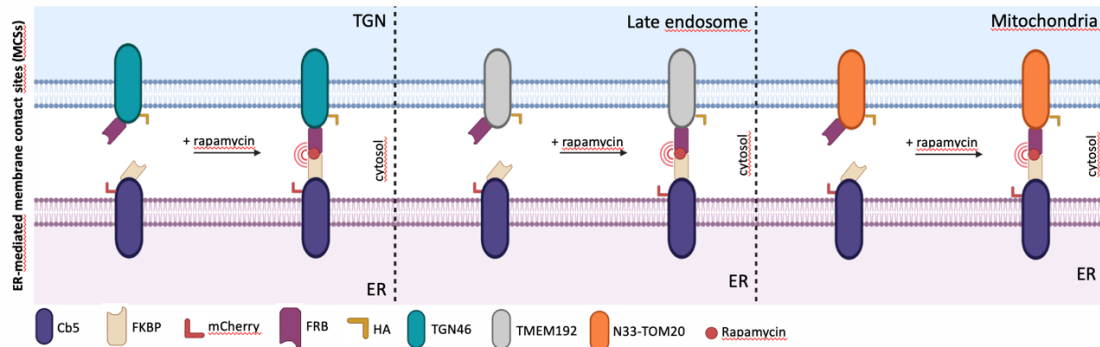


Figure 2.3 - Schematic representation of the rapamycin-inducible split-FRB/FKBP tethering system used to monitor ER-mediated membrane contact sites (MCSs). Schematic diagram showing the experimental setup for the inducible reporter system used to reconstitute and visualize ER contact sites with distinct organelles (TGN, late endosome, and mitochondria). Constructs encoding the ER protein Cb5 fused to FKBP and mCherry (Cb5-FKBP-mCherry) and organelle-specific FRB-tagged proteins (TGN46-FRB-HA, TMEM192-FRB-HA, or N33-TOM20-FRB-HA) were expressed in cells. Upon addition of rapamycin, the heterodimerization of FKBP and FRB domains promotes a rapid and specific tethering between the ER and the indicated organelle, mimicking the formation of an ER-mediated membrane contact site.

2.8 Immunofluorescence

Cells were grown to sub-confluent density on glass coverslips and fixed with 4% paraformaldehyde (PFA) for 10 minutes at Room Temperature and then rinsed twice in Phosphate Buffered Saline (PBS). The blocking and permeabilization steps are done incubating coverslips in Blocking Buffer (BB - 0,05 % Saponin, 0,5 % BSA, 50 mM NH_4Cl in PBS) for 30 minutes.

Coverslips are then incubated with the primary antibody diluted in BB for one hour, rinsed three times in PBS and then incubated with the secondary antibody (1:400 dilution) for three times in PBS and once in sterile water, to remove salt residues.

2.9 Proximity ligation assays (PLA)

Proximity ligation assays (PLA) were performed using the Duolink™ In Situ Red Detection Kit (Sigma–Aldrich) to visualize protein–protein interactions in situ. Cells were seeded on glass coverslips and preincubated with MitoTracker™ (1:10,000 in Opti-MEM) for 30 min at 37 °C in 5% CO₂, then washed and fixed with freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature. Following fixation, cells were quenched with 50 mM NH₄Cl for 15 min and permeabilized/blocking in 0.1% saponin and 1% BSA in PBS (blocking buffer, BB).

For PLA, samples were incubated with primary antibodies recognizing ORP8 (mouse) and PTPIP51 (rabbit), diluted in BB (typically 1:100 and 1:200, respectively), for 1 hour at room temperature in a dark, humidified chamber. After three washes in BB, PLA probes anti-rabbit PLUS and anti-mouse MINUS (Sigma–Aldrich, Duolink™) were diluted 1:5 in BB and preincubated for 20 minutes before use. Coverslips were incubated with 40 µL of probe solution per sample for 1 hour at 37 °C, followed by ligation (30 minutes at 37 °C) and amplification (1 hour 40 minutes at 37 °C) according to the manufacturer's instructions. All steps were carried out in the dark. After final washes with buffers A and B (provided in the kit), cells were mounted in Vectashield™ mounting medium. Samples were imaged using confocal microscopy with identical acquisition settings across conditions. PLA puncta were quantified using ImageJ/Fiji.

2.10 Confocal Microscopy and Image Analysis

Cells were imaged using a confocal laser microscope (ZEN LSM700, LSM800, LSM880 equipped with an AiryScan Module, LSM900 and Zeiss Elyra 7 Lattice SIM²) with a 63x/1.4 NA oil objective and controlled by Zen Blue or Zen Black software (Carl Zeiss Microscopy, LLC). Optical confocal sections were taken at 1 Air Unit. The images used for quantifications were acquired with the same parameters for digital gain, laser power, and magnification.

The acquired images were processed using Fiji software (ImageJ; National Institute of Health (NIH)). Brightness and contrast were adjusted with Fiji software. Panels were assembled using Microsoft Office PowerPoint. Three-dimensional reconstructions

were generated using IMARIS software (Bitplane) during the research period conducted at the laboratory of Dr. Francesca Giordano (I2BC, Gif-sur-Yvette, France).

To quantify ER-Golgi, ER-LE, and ER-Mitochondria MCSs, 15–20 randomly selected fields (containing approximately 150–200 cells) were imaged for each condition using identical confocal microscope settings. The extent of ER redistribution toward the respective organelle (peri-Golgi, perinuclear, or perimitochondrial clustering) was measured with ImageJ, and data from at least three independent experiments were analyzed. Statistical significance was determined based on the mean values of biological replicates.

Quantification of tripartite membrane contact sites (MCSs) among the endoplasmic reticulum, mitochondria, and lysosomes was performed using a custom macro developed in Fiji (ImageJ, NIH). The macro identifies pixels where the three fluorescent signals (ER, mitochondria, lysosomes) converge by generating binary masks for each channel, computing their pairwise overlaps, and extracting the intersection area common to all three. The resulting tripartite contacts were expressed as the number of overlapping voxels or puncta normalized both to the total mitochondrial area and to the number of lysosomes per cell. The same analytical approach was applied to quantify mitochondria–lysosome contacts (MLCs) by assessing the colocalization between the mitochondrial and lysosomal masks only.

In addition, colocalization analyses were performed using the JACoP (Just Another Colocalization Plugin) plugin in Fiji, which provided quantitative coefficients to assess the degree of spatial overlap between organelle markers.

Mitochondrial length and morphology were measured using the Mitochondria Analyzer plugin in Fiji (ImageJ, NIH). This plugin enables semi-automated analysis of fluorescent mitochondrial networks, extracting morphometric data such as length, area, and connectivity. Briefly, mitochondrial channels were pre-processed to reduce background noise and improve contrast, then subjected to adaptive thresholding to create binary masks of the mitochondrial signal. Each mask was skeletonized to produce a one-pixel-wide representation of mitochondrial structures, facilitating accurate measurement of individual branch lengths. The plugin calculated average, median, and total mitochondrial lengths per cell, along with additional metrics of

network complexity. Results were exported as tables and visually verified using the skeleton overlay generated by the plugin.

2.11 Protein extraction and analysis

- *Preparation of cell lysates and quantification*

Total cell lysates were prepared by scraping cells into Lysis Buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA) supplemented with a protease inhibitor cocktail. The lysates were centrifuged at 14,000 rpm for 20 minutes at 4°C, and the resulting supernatant was then collected. The samples were quantified using the Bradford Reagent (Biorad) assay, with BSA as a standard. The samples were mixed with buffer (0.125 M Trizma base, 4% SDS, 20% glycerol, 10% β -mercaptoethanol, pH 6.8) and boiled at 95 °C for 5 minutes before loading.

- *SDS PAGE and Western Blot*

The polyacrylamide gel was prepared using a single solution of the desired polyacrylamide concentration (typically 10% for TEX2). The electrophoresis is carried out in Running Buffer (RB - 0.2 M glycine, 20 mM Trizma base, 4 mM SDS pH 8.3) under constant current of 30 mA.

The transfer is carried out at a constant voltage (100V) for 1-1.5 h in Transfer Buffer (TB – 0.2 M glycine, 20 mM Trizma base, pH 8.3).

After transfer, the membrane was stained with 0.2% Ponceau in 5% acetic acid for 5 min, then rinsed in 5% acetic acid. It was cut into strips and incubated for 30 min at RT in blocking buffer (TTBS with 1% BSA). Strips were then incubated with primary antibodies at working concentrations (see Table 2.1) for 2-3 h at RT or overnight at 4 °C, followed by three TTBS washes (10 min each). Next, strips were incubated for 1 h with HRP-conjugated secondary antibodies (1:10 000 for ECL) and washed three times with TTBS and once with PBS. Finally, strips were treated with ECL (Euroclone, UK) for 1 min at RT and visualized via chemiluminescence.

2.12 Mass spectrometry analysis

Endogenous TEX2 was used as bait for immunoprecipitation, while rabbit IgG served as the negative control. Immunoprecipitations were performed from 3 mg of total protein lysate using 0.1% Triton X-100 and 0.1% NP-40 to preserve potential TEX2-associated complexes. The immunoprecipitated material was precipitated with four volumes of ice-cold acetone (-20°C , overnight), centrifuged at $20,000 \times g$ for 20 min at 4°C , washed with 70% ethanol, and air-dried. Protein pellets were resuspended in 6 M guanidine-HCl containing 5 mM TCEP and 20 mM chloroacetamide, then heated at 95°C for 10 min for reduction and alkylation. Proteins were sequentially digested with Lys-C and trypsin in ammonium bicarbonate buffer (ABC) under decreasing guanidine-HCl concentrations (from 6 M to < 1 M), with incubations at 37°C for 3 hours and overnight, respectively. Digestion was stopped by adding 1% trifluoroacetic acid (TFA), and samples were desalted using in-house packed C18 StageTips equilibrated with methanol, buffer B (80% acetonitrile, 0.1% formic acid), and buffer A (0.1% formic acid). Peptides were eluted with buffer B, concentrated to 2–3 μL by SpeedVac, resuspended in 8 μL of buffer A, and sonicated for 10 minutes before LC–MS/MS analysis.

Peptide separation and analysis were conducted using nano-LC coupled with tandem mass spectrometry (Q Exactive HF, Thermo Fisher Scientific) at the TIGEM Proteomics Core Facility (Paolo Grumati Lab). Protein identification was performed with MaxQuant software and the Andromeda search engine against the human UniProt database. Carmine Cirillo performed downstream statistical analysis: only proteins significantly enriched in the TEX2 IP compared to the IgG control samples were considered specific TEX2 interactors.

2.13 Isolation of mitochondria-associated membranes (MAMs)

Mitochondria-associated membranes (MAMs) were isolated from HeLa cells following a modified version of the protocol described by Montesinos and Area-Gómez (Methods in Cell Biology, 2020). Briefly, cells were cultured to confluence in 150-mm dishes, harvested by gentle scraping in ice-cold phosphate-buffered saline (PBS), and collected by centrifugation at $4,000 \times g$ for 5 min at 4°C . The pellet was

washed twice with PBS and then resuspended in ice-cold homogenization buffer (225 mM mannitol, 25 mM HEPES-KOH (pH 7.4), 1 mM EGTA, and EDTA-free protease inhibitors). Cells were disrupted with 5–10 strokes using a glass–Teflon Potter-Elvehjem homogenizer on ice, ensuring optimal cell breakage while maintaining organelle integrity.

The homogenate was centrifuged at $600 \times g$ for 10 min at 4 °C to remove nuclei and cell debris. The resulting supernatant was then centrifuged again under the same conditions to eliminate any remaining unbroken cells. The clarified supernatant was then centrifuged at $10,000 \times g$ for 20 min at 4 °C to obtain a crude mitochondrial pellet (P2) and a post-mitochondrial supernatant containing cytosol and bulk ER membranes.

The P2 fraction was resuspended in a small volume of homogenization buffer and layered on top of a freshly prepared 30% Percoll solution in the same buffer. Samples were centrifuged at $95,000 \times g$ for 30 min at 4 °C in an ultracentrifuge. Two distinct bands became visible: an upper, lower-density fraction containing MAMs and mitochondria associated with MAM, and a lower band containing purified mitochondria. The MAM-containing fraction was carefully collected, diluted fivefold with homogenization buffer to remove traces of Percoll, and centrifuged at $8,000 \times g$ for 10 min at 4 °C. The supernatant was then ultracentrifuged at $100,000 \times g$ for 1 h at 4 °C to pellet the MAM fraction.

The final MAM pellet was resuspended in a small volume of homogenization buffer and either processed immediately for western blot analysis. The purity of the isolated fractions was verified by immunoblotting using specific subcellular markers.

2.6 Bioinformatic analysis of TEX2

Domain annotation and structural predictions of TEX2 were performed using InterPro, Pfam, and HHpred. The SMP (synaptotagmin-like mitochondrial-lipid-binding) core of TEX2 was aligned with those of representative human SMP-containing tethering proteins — E-SYT1, E-SYT2, E-SYT3, PDZD8, and TMEM24 — using Clustal Omega and manually curated in Jalview. Pairwise sequence identities were calculated from the multiple sequence alignment (MSA), and a distance matrix was generated

using the formula $1 - \text{identity}$, followed by hierarchical clustering to produce a dendrogram representing relative sequence divergence among SMP domains. For reference, lipid-binding preferences of TEX2 were derived from previously published PIP-strip assays performed by Du et al. (2020), which tested the recombinant PH domain of TEX2 against a panel of phosphoinositides.

2.14 Statistics and reproducibility

Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software), applying t-tests to variables that followed a normal distribution. When data were normally distributed, differences between control and treated groups were evaluated using t-tests or ANOVA, with appropriate post-hoc multiple comparison tests depending on the experiment. Each experiment included three replicates for statistical analysis; all replicates were biological rather than technical.

Chapter 3 – Results

3.1 Bioinformatic positioning of TEX2 within the SMP superfamily

Among the variety of tethering molecules acting at the MCS interface, the Synaptotagmin-like mitochondrial-lipid-binding (SMP) domain-containing proteins mark many membrane contact sites tethers that couple physical bridging with local lipid handling. Canonical examples include the ER–PM tethers E-Syts and TMEM24, as well as the ER-lysosome and ER-mitochondria factor PDZD8. In contrast, TEX2 is comparatively under-characterized, despite prior links to ER-lysosome contacts (Du *et al.*, 2023). We therefore asked where TEX2 sits within this landscape to assess whether its SMP core and accessory architecture suggest a distinct operating mode.

To achieve this, we extracted the SMP domains from human TEX2, ESYT1/2/3, PDZD8, and TMEM24, aligned them using Clustal Omega and PRRN, and performed a series of comparative bioinformatic analyses (**Fig. 3.1B**). The multiple sequence alignment (MSA) showed that TEX2 maintains the general β -barrel structure of SMP domains but contains unique insertions and low-complexity regions absent in all other members. Specifically, TEX2 has an extended C-terminal insertion of about 100 amino acids, forming several long loops that disrupt otherwise conserved β -strands. These insertions probably represent structural modifications beyond the canonical TULIP core, possibly providing greater flexibility or a larger lipid-binding cavity. Importantly, analysis of PhosphoSitePlus identified multiple serine and tyrosine residues within this inserted region that are predicted to undergo phosphorylation, indicating that the TEX2 SMP domain may be regulated post-translationally. Additionally, per-column conservation across all SMPs was assessed on a scale of 0 to 1.

Consistent with this divergence, the phylogenetic tree (**Fig. 3.1C**) derived from pairwise distances positions TEX2 within the broader E-Syt clade but on a distinct, elongated branch, reflecting significant evolutionary separation. The three E-Syt paralogs cluster tightly together (pairwise identity > 0.6), indicating a shared, conserved architecture. By contrast, TEX2 shares only ~20–25% identity with the E-Syts, and even less with PDZD8 or TMEM24. PDZD8, which operates at ER–mitochondria and ER–endosome sites, appears more distant (branch length ≈ 5.9),

while TMEM24, a phosphatidylinositol transporter at ER–plasma membrane junctions, forms the outermost, most divergent branch (6.7). This phylogenetic placement suggests that TEX2 evolved from an ancestral E-Syt-like SMP scaffold but diverged early, potentially adapting its lipid-binding groove to hydrophobic substrates such as ceramide rather than to the phospholipids typically handled by E-Syts and PDZD8.

A pairwise identity matrix (**Fig. 3.1D**) corroborates this evolutionary gradient, with high similarity among E-Syts, moderate similarity between TEX2 and E-Syts, and minimal overlap with PDZD8 or TMEM24. Meanwhile, the per-position conservation profile (**Fig. 3.1E**) across all SMP domains highlights a core region of high conservation corresponding to the β -barrel interior, surrounded by variable loops and termini, precisely where TEX2's extended insertions map. These variable regions may determine lipid specificity and partner recognition, suggesting that the extra residues in TEX2 could facilitate contacts with regulatory proteins or enable adaptation to different lipid environments.

Finally, the predicted tertiary structure of the TEX2 SMP domain, modelled by AlphaFold, supports this view (**Fig. 3.1A**): the conserved β -barrel is preserved, but the elongated C-terminal region forms an exposed, flexible appendage with low predicted confidence, consistent with a disordered or regulatory segment. The combination of a stable lipid-binding core and a flexible peripheral tail may enable TEX2 to function as a modular, tunable lipid-transfer protein, capable of sensing or responding to local signals through phosphorylation or partner binding.

In summary, these analyses reveal that TEX2 encodes an atypical SMP domain: evolutionarily related to the E-Syt family yet structurally expanded and potentially regulated. Its unique ~100-residue insertion, together with predicted phosphorylation sites and low sequence conservation, strongly suggests that TEX2 represents a specialized branch of the SMP superfamily. This distinctive architecture raises the possibility that TEX2 mediates non-canonical lipid transfer reactions, potentially involving sphingolipids such as ceramide, whose physicochemical properties would require a broader, more hydrophobic binding groove. These findings establish a structural and evolutionary rationale for investigating TEX2 as a novel lipid-transfer and tethering factor at inter-organelle contact sites.

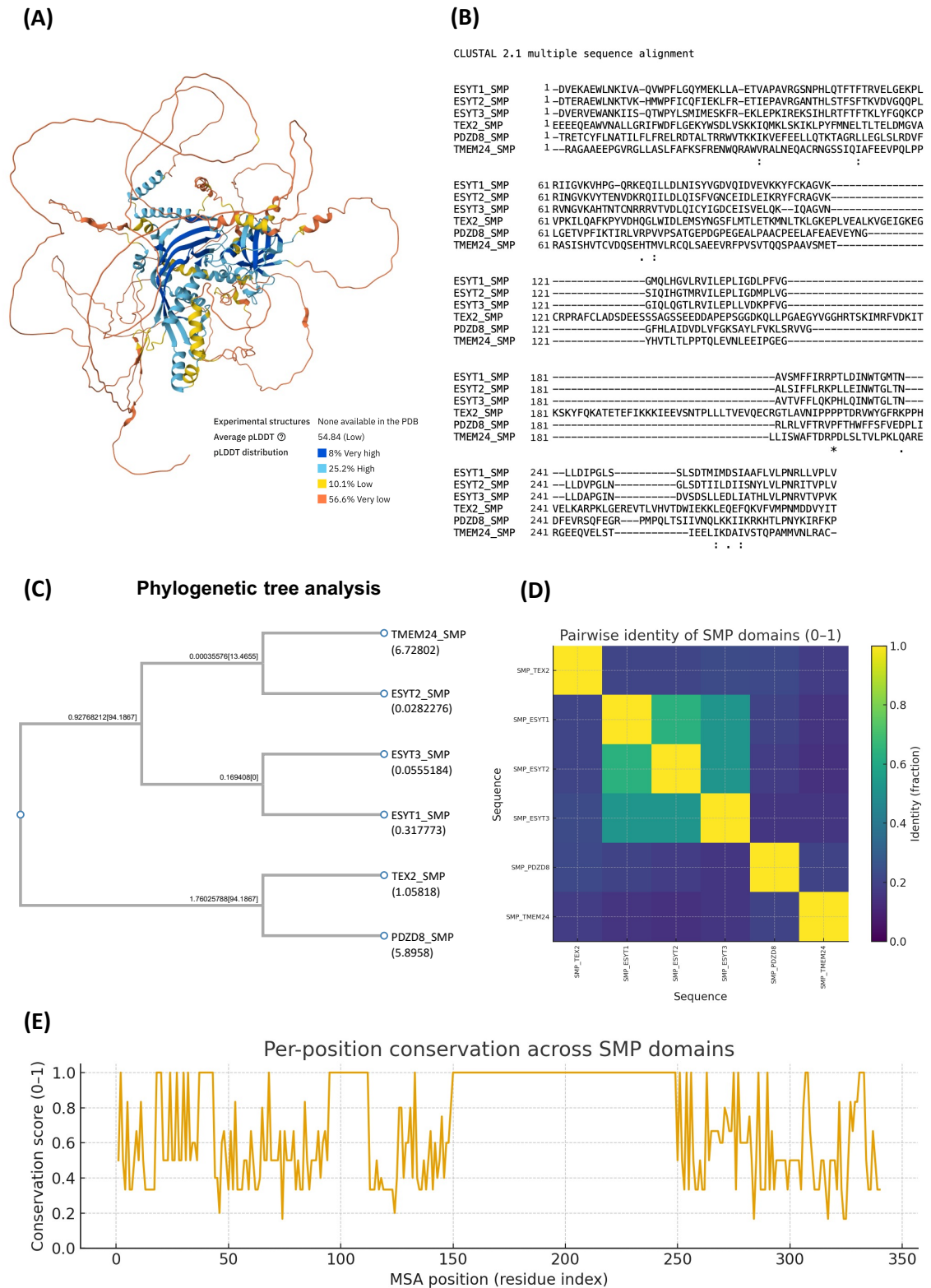


Figure 3.1 – Comparative bioinformatic analysis of the TEX2 SMP domain.

(A) Predicted tertiary structure of the TEX2 SMP domain (residues encompassing the predicted TULIP-like fold), generated using AlphaFold2. The model reveals a conserved β -

barrel core characteristic of SMP domains (blue), extending into long peripheral loops and an unstructured C-terminal region with low predicted confidence. Such poorly ordered extensions, absent in canonical SMPs, may confer enhanced conformational flexibility or serve as regulatory segments for partner binding and post-translational modification. The overall architecture preserves the elongated tubular channel typical of lipid-transfer proteins, consistent with a conserved lipid-binding function.

(B) Multiple sequence alignment (MSA) of SMP domains from human TEX2, ESYT1/2/3, PDZD8, and TMEM24, performed using the PRRN algorithm. Conserved residues forming the SMP β -barrel appear as aligned blocks, while TEX2 displays unique insertions and low-complexity segments absent in the other SMPs. These insertions, particularly a long C-terminal extension, interrupt the canonical secondary structure and represent candidate regions for functional divergence. The consensus line below the alignment indicates positions of strong conservation (*), chemical similarity (:), or weak similarity (.).

(C) Phylogenetic relationship between SMP domains, displayed as a midpoint-rooted phylogram based on pairwise distances (1 – identity). The three E-Syts (ESYT1/2/3) cluster tightly together, reflecting strong sequence conservation and shared function as ER–PM lipid tethers. TEX2 branches next to this cluster but on a distinct, longer branch (distance ≈ 1.05), denoting substantial divergence from E-Syts while maintaining overall relatedness. In contrast, PDZD8 (distance ≈ 5.9) and TMEM24 (distance ≈ 6.7) form outgroups, consistent with their specialized localizations and substrate preferences (ER–mitochondria/endosome and ER–PM, respectively). Bootstrap values (in brackets) indicate high statistical support for the ESYT cluster and TEX2 branching.

(D) Pairwise identity matrix for SMP sequences (range 0–1). High intra-cluster similarity is observed among E-Syts (identity 0.6–0.7), while TEX2 shares only 0.2–0.3 identity with E-Syts and <0.15 with PDZD8 or TMEM24. This pattern mirrors the phylogenetic distances, reinforcing the notion that TEX2 represents an E-Syt-related but functionally distinct SMP subtype.

(E) Per-position conservation score across all SMP domains, calculated from the MSA using a sliding-window approach (scale 0–1). Conserved peaks correspond to β -strand regions forming the SMP hydrophobic channel, whereas variable troughs align with exposed loops and terminal regions. TEX2-specific insertions map precisely within these low-conservation zones, indicating that they contribute to unique structural features not shared by other SMPs.

3.2 Identifying novel TEX2's interactors

Since TEX2 remains one of the least characterized members of the SMP-domain family, we conducted an unbiased affinity purification mass spectrometry (AP–MS) approach to identify its endogenous interactome without assuming a specific membrane contact site. We immunoprecipitated endogenous TEX2 by using a specific antibody and processed matched IgG controls in parallel. The efficiency of immunoprecipitation and bait recovery was confirmed by immunoblotting (**Fig. 3.2B**). Differential enrichment analysis (TEX2 IP versus IgG) identified a high-confidence set of approximately 60 protein interactors (**Fig. 3.2A**), many of which exceeded both fold-change and significance thresholds in the volcano plot (**Fig. 3.2C**).

To achieve an unbiased assessment of compartment-specific trends, we conducted Gene Ontology (GO: Cellular Component) enrichment analysis on the set of significant interactors. This analysis revealed a marked enrichment of membrane-associated categories, with the most prominent signal corresponding to the “integral component of the mitochondrial outer membrane (OMM)” (**Fig. 3.2D**). Notably, this annotation profile was unexpected, given that TEX2 has traditionally been characterized at the endoplasmic reticulum (ER)–lysosome interface.

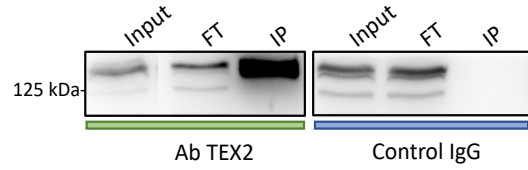
In view of these unanticipated findings, we considered the possibility that TEX2 may also be selectively enriched at endoplasmic reticulum–mitochondria contact sites, known as mitochondria-associated membranes (MAMs). Among the highest-ranking proteins, FUN14 domain-containing protein 1 (FUNDC1) emerged as a prominent hit, accompanied by additional mitochondrial and OMM-localized factors (e.g., FUNDC2).

FUNDC1 is an integral OMM protein well-known to act as a mitophagy receptor via its LC3-interacting region (Chen *et al.*, 2016). Moreover, FUNDC1 has also been found to be enriched at mitochondria-ER contacts (Wu *et al.*, 2016). FUNDC1 can recruit/scaffold DRP1 (DNM1L) at the mitochondrial surface, thereby promoting stress-responsive mitochondrial fission, with its activity tuned by site-specific phosphorylation/dephosphorylation (Chen *et al.*, 2016).

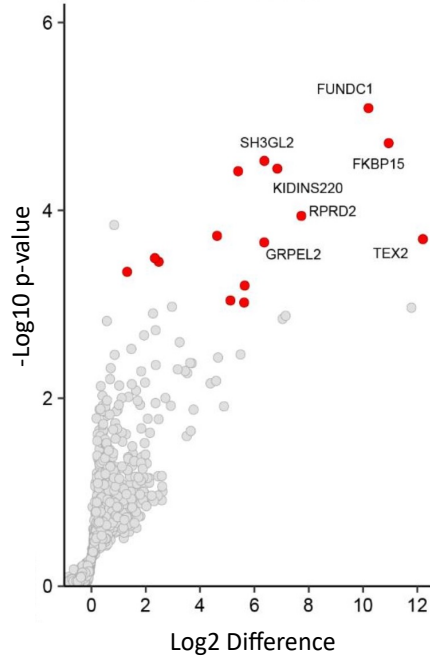
(A)

Protein name	Gene symbol
Angiomotin-like protein 1	AMOTL1
Rho guanine nucleotide exchange factor 12	ARHGEF12
Armstrong repeat-containing protein 8	ARMC8
Chromobox protein homolog 8	CBX8
Coiled-coil domain-containing protein 6	CCDC6
Carboxypeptidase, lysosomal protective protein	CTSA
Protein DEK	DEK
Dystrobrevin; Dystrobrevin beta; Dystrobrevin alpha	DTNB; DTNA
Protein FAM98A	FAM98A
FK506-binding protein 15	FKBP15
Protein flightless-1 homolog	FUJ1
FUN14 domain-containing protein 1	FUNDC1
FUN14 domain-containing protein 2	FUNDC2
Alpha-galactosidase A	GLA
GrpE protein homolog 2, mitochondrial	GRPEL2
Probable E3 ubiquitin-protein ligase HERC1	HERC1
U3 small nucleolar ribonucleoprotein protein IMP3	IMP3
Inosine-5'-monophosphate dehydrogenase 2	IMPDH2
Kinase D-interacting substrate of 220 kDa	KIDINS220
Lipoma-preferred partner	LPP
Leucine-rich repeat-containing protein 16A	LRRCL16A
Leucine-rich repeat flightless-interacting protein 1	LRRFIP1
Macrophage erythroblast attacker	MAEA
Neurofibromin; Neurofibromin truncated	NF1
Protein kinase C and casein kinase substrate in neurons protein 2	PACSLN2
Partitioning defective 3 homolog	PARD3
cGMP-inhibited 3,5-cyclic phosphodiesterase A	PDE3A
DNA-directed RNA polymerase II subunit RPB1	POLR2A
DNA-directed RNA polymerase II subunit RPB2	POLR2B
Serine/threonine-protein phosphatase 4 regulatory subunit 1	PPP4R1
Testis	PRSS21
Poly(U)-binding-splicing factor PUF60	PUF60
ATP-dependent DNA helicase Q5	RECQL5
Regulation of nuclear pre-mRNA domain-containing protein 1B	RPRD1B
Regulation of nuclear pre-mRNA domain-containing protein 2	RPRD2
40S ribosomal protein S25	RP25
Reticulon-3; Reticulon	RTN3
Reticulon-4; Reticulon	RTN4
SEC23-interacting protein	SEC23IP
Endophilin-A2	SH3GL1
Endophilin-A1	SH3GL2
Signal-induced proliferation-associated protein 1	SIPA1
Beta-2-syntrophin	SNTB2
Sorting nexin-1	SNX1
Transforming acidic coiled-coil-containing protein 2	TACC2
T-complex protein 1 subunit alpha	TCP1
Testis-expressed sequence 2 protein	TEX2
Thyroid hormone receptor-associated protein 3	THRAP3
Transducin-like enhancer protein 3	TLE3
Transmembrane and coiled-coil domain-containing protein 1	TMCO1
Tensin-1	TNS1
WD repeat-containing protein 26	WDR26
Xenotropic and polytropic retrovirus receptor 1	XPR1
Zinc finger protein 609	ZNF609

(B)



(C)



(D)

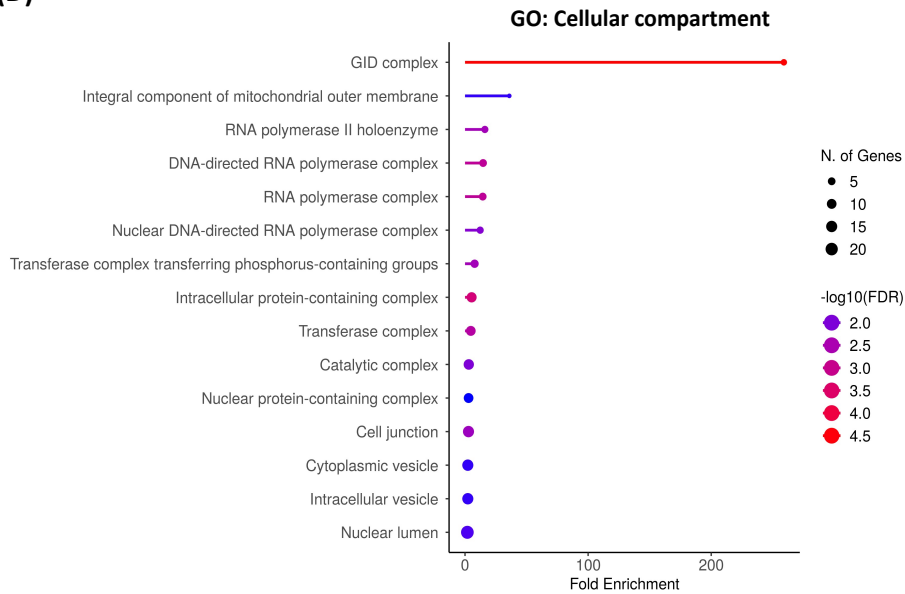


Figure 3.2 - Endogenous TEX2 interactome and functional annotation.

(A) List of the top 60 interactors identified by AP-MS of endogenous TEX2 (protein name and gene symbol) identified through IP experiments. The proteins listed are significantly enriched over the IgG control (see detailed description in the Methods section).

(B) Immunoprecipitation validation by Western blot. Input (60 μ g), FT (flow-through, 60 μ g), and IP (3 mg) from anti-TEX2 and matched IgG control are shown. The ~125-kDa protein recognized by the antibody raised against TEX2 corresponds to TEX2, as validated by siRNA experiments (see Methods).

(C) Volcano plot of the comparison between samples obtained from anti-TEX2 or IgG immunoprecipitation: X-axis, $\log_2$ difference; Y-axis, $-\log_{10}$ p-value. Red dots mark significantly enriched proteins (grey = background). Selected hits are labeled, including FUNDC1, FUNDC2, FKBP15, SH3GL2, KIDINS220, RPRD2, GRPEL2, and TEX2.

(D) GO cellular components analysis of the significant TEX2 interactors. X-axis, fold enrichment relative to the experimental background; dot size, number of genes; dot color, $-\log_{10}(\text{FDR})$ after Benjamini–Hochberg correction. Notably, the term “integral component of the mitochondrial outer membrane (OMM)” is enriched alongside nuclear complexes and other intracellular compartments.

3.3 TEX2 localizes in close proximity to mitochondria

Prompted by the AP–MS analysis of endogenous TEX2 interactors, which identified outer mitochondrial membrane (OMM) proteins among the top interactors, notably FUNDC1, we investigated whether TEX2, that is an ER resident protein, accumulates at ER-mitochondria interfaces.

Confocal imaging of endogenous TEX2 showed a reticular ER pattern with several enriched domains of the protein, in close apposition to the mitochondrial network, stained by the OMM protein TOM20, highlighting a clear enrichment along the mitochondrial network (**Fig. 3.3A**). To quantify this, we calculated the extent of colocalization between TEX2 and TOM20 by using Mander's coefficient. In addition, we measured the same parameter for two more ER proteins that broadly mark the ER and ER-mitochondria MCSs: VAP-A and Calnexin (CLNX). TEX2/TOM20 exhibited a significantly higher overlap coefficient compared to both VAPA/TOM20 and CLNX/TOM20 (**Fig. 3.3B**; statistics in Methods), suggesting that the mitochondria proximity we have observed in TEX2 distribution does not simply reflect a general proximity of the ER to the mitochondrial network. Still, it highlights instead that the protein is preferentially distributed at the ER microdomains adjacent to mitochondria, consistent with MAMs.

Intriguingly, besides the localization of the endogenous TEX2 protein, the overexpression of a fluorescently tagged version of TEX2 (TEX2–GFP) recapitulates perfectly the same pattern. By SIM (Structured Illumination Microscopy) experiments, it is possible to appreciate TEX2-positive ER tubules wrapping around mitochondria (**Fig. 3.3D**). 3D reconstruction and rendering of deconvolved z-stacks, obtained by the IMARIS software processing, clearly highlight tight spatial apposition between TEX2 and TOM20 and visualize ER wrapping around the mitochondrial surface (**Fig. 3.3C**).

Altogether, these data validate our proteomics results, confirming that TEX2 is localized at the ER-mitochondria contact sites interface.

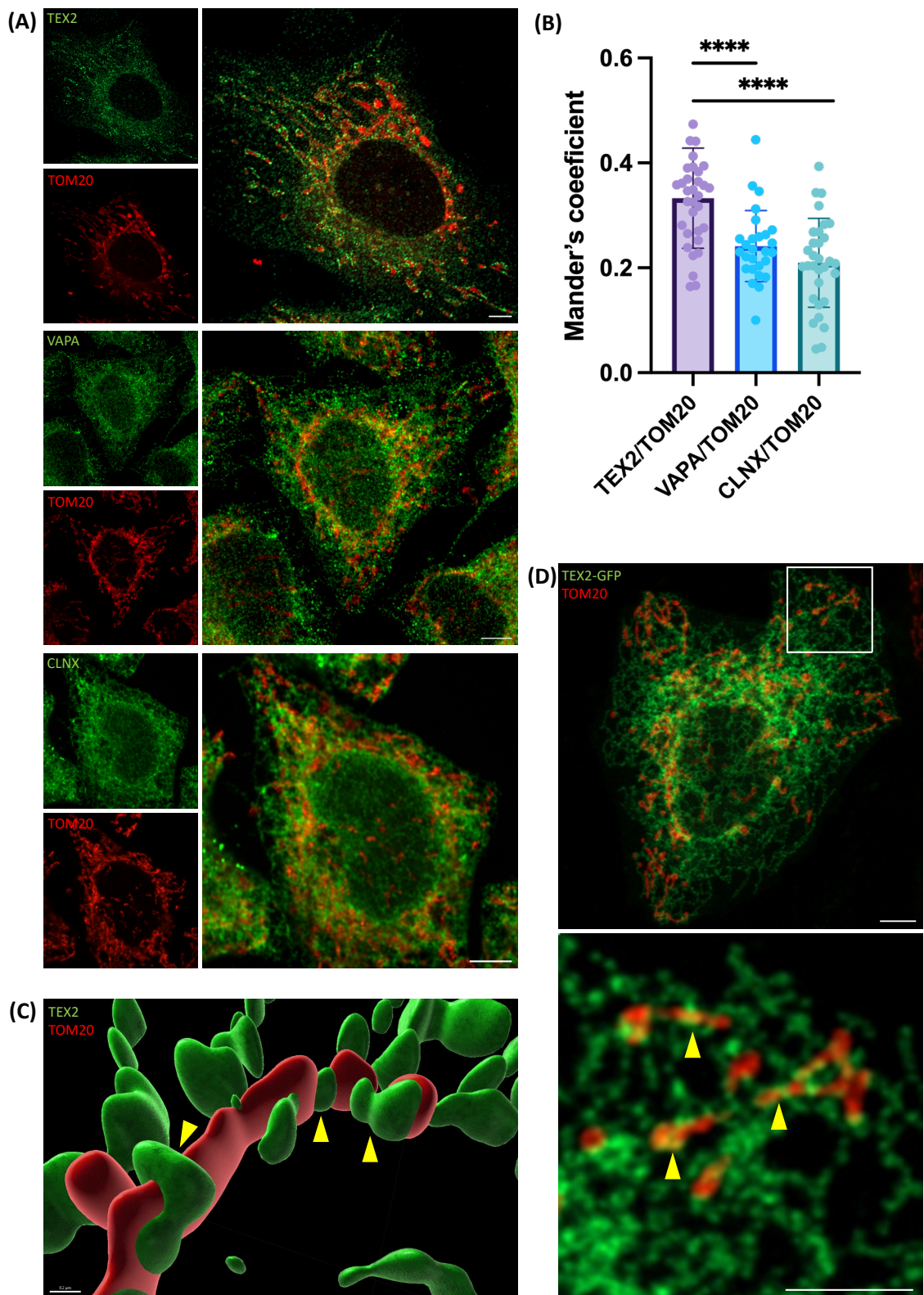


Figure 3.3 - TEX2 localizes in close proximity to mitochondria

(A) Representative confocal images of HeLa cells immunostained for endogenous TEX2 (green) and the mitochondrial outer membrane protein TOM20 (red). For comparison, cells were stained for the ER-resident proteins VAPA or Calnexin (CLNX, both in green) together with TOM20 (red). The right panels show merged images. Cells were fixed in 4% paraformaldehyde and imaged using a Zeiss Elyra 7 Lattice SIM² microscope equipped with a 63×/1.4 NA oil objective. Primary antibodies were used at the following dilutions: anti-TEX2 (rabbit, 1:200), anti-VAPA (rabbit, 1:200), anti-Calnexin (rabbit, 1:200), and anti-TOM20 (mouse, 1:400). Secondary antibodies were Alexa Fluor-conjugated anti-rabbit and anti-mouse IgG (1:400). TEX2 displays punctate accumulations enriched in proximity to mitochondria, more prominently than VAPA or Calnexin. Scale bars, 10 μ m.

(B) Quantification of colocalization between TEX2, VAPA, or CLNX and TOM20, expressed as Mander's coefficient (mean $\pm$ S.D.). Values were derived from n = 30 cells from 3 independent experiments using the JACoP plugin in Fiji/ImageJ. TEX2 shows significantly higher overlap with TOM20 compared to VAPA or CLNX.

(C) Three-dimensional surface rendering of TEX2 (green) and TOM20 (red) from deconvolved confocal stacks generated in IMARIS (Bitplane). Yellow arrowheads indicate regions where TEX2-positive ER membranes wrap around mitochondrial tubules. Scale bar, 0.2 μ m.

(D) Representative confocal images of HeLa cells expressing TEX2-GFP (green) transiently transfected with 0.5 μ g plasmid DNA using Lipofectamine LTX Plus and fixed after 16 h. Cells were immunostained for TOM20 (red) to visualize mitochondria. The boxed area is shown magnified below, highlighting close apposition of TEX2-positive ER segments along mitochondrial tubules (yellow arrowheads). Scale bars, 10 μ m (top) and 2 μ m (bottom).

3.3.1 TEX2 localizes at ER-mitochondria MCSs

To further determine whether TEX2 stably associates with mitochondria-associated membranes (MAMs), we performed biochemical isolation of MAMs using sequential centrifugations and a Percoll gradient, as previously described (**Fig. 3.4 A**, Montesinos *et al.*, 2020). Immunoblotting analyses of the fractions showed that they are properly isolated, as demonstrated by the distribution of the marker for the different compartments: TOM20 for mitochondria, Calnexin (CLNX), and VAP-A for ER/MAM. TEX2 is present in the ER fraction, as expected, but it is also highly enriched in the MAM fraction (3-fold increase), while it is completely absent in the pure mitochondria one (**Fig. 3.4B**). We can conclude that TEX2 is not only localized closely to mitochondria by fluorescence analyses, but it is also highly associated with MAMs, as already suggested by TEX2 interactors identified by mass-spectrometry experiments.

To better visualize TEX2 at the ER-mitochondria interfaces, we took advantage of a rapamycin-inducible stabilization system of reporters. In our lab, over the last years, several systems to visualize different classes of ER-mediated contact sites have been developed (Venditti *et al.*, 2019). These reporter systems rely on transmembrane proteins that act as a proxy for the organellar structure of interest, fused to FKBP and FRB domains, respectively. When rapamycin is added (at a very low dose, 100nM and for a very short period, 4 minutes), FKBP and FRB heterodimerize with high affinity at the nanometer scale, stabilizing existing ER-mediated contacts rather than pulling distant membranes together. This method provides a time-specific snapshot that extends the natural junctions present at the moment of drug application. Similarly, we generated a system of reporters specifically for ER-mitochondria contact sites. We chose an ER anchor, the C-terminal part of the cytochrome B5 (Cb5-mCherry) and an outer-mitochondrial anchor, the N-terminal part of TOM20 (N33-TOM20-FRB-HA), and we added FKBP and FRB domains respectively (see Methods section for details). This FKBP/FRB reporter thus enables stabilization and readout of already existing endogenous contacts with minimal geometric bias, providing a clean entry point to test whether TEX2 selectively accumulates where ER-mitochondria contacts are already formed.

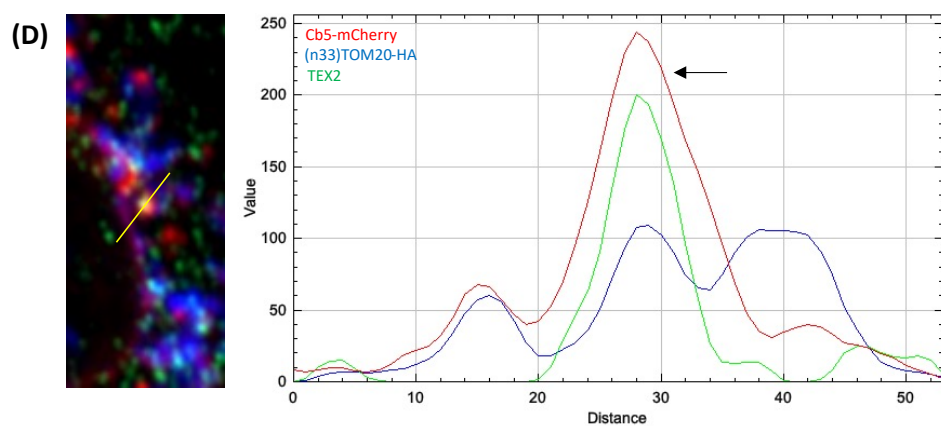
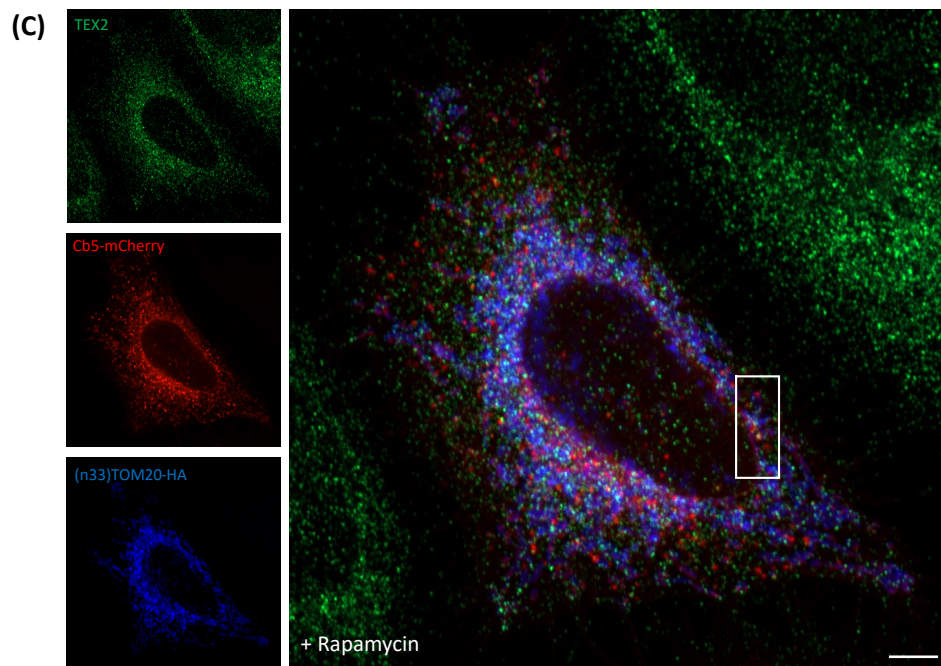
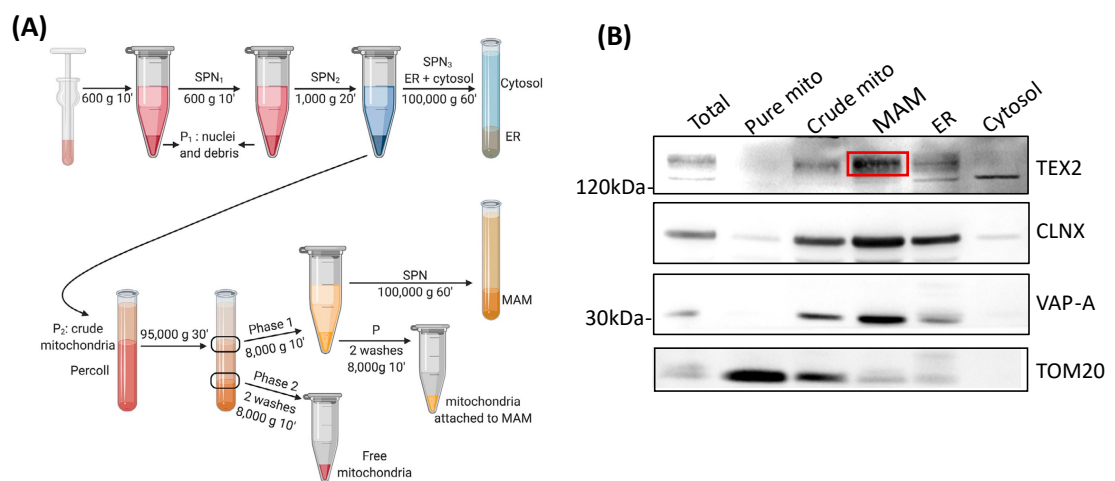


Figure 3.4 - TEX2 is enriched at ER-mitochondria contact sites.

(A) Schematic workflow of MAM isolation from HeLa cells. After cell homogenization, post-nuclear supernatants were subjected to differential centrifugation to obtain crude mitochondrial fractions. These were further separated on a discontinuous Percoll gradient (30%, 23%, and 15%) to isolate pure mitochondria and MAM fractions, while ER and cytosolic fractions were recovered from the supernatant. The workflow was adapted from Montesinos and Area-Gomez (2020).

(B) Representative immunoblots of the isolated fractions probed for TEX2, Calnexin (CLNX), VAPA, and TOM20. TEX2 is highly enriched in the MAM fraction, displays a weak signal in pure mitochondria, and is also present in the ER fraction, consistent with its localization at ER subdomains in contact with mitochondria. Protein samples (60 µg per lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Primary antibodies were used at the following dilutions: anti-TEX2 (rabbit, 1:500), anti-Calnexin (mouse, 1:1000), anti-VAPA (mouse, 1:1000), and anti-TOM20 (mouse, 1:2000).

(C) Confocal images of HeLa cells subjected to rapamycin-induced ER-mitochondria reporter. Cells were transfected with Cb5-mCherry-FKBP (ER anchor, red)-n33TOM20-HA-FRB (OMM anchor, blue), then treated with 20 nM rapamycin for 4 min to induce heterodimerization of FKBP-FRB modules and stable ER-mitochondria apposition. Endogenous TEX2 (green) was detected by immunofluorescence using rabbit anti-TEX2 (1:200) and Alexa Fluor 488 488-conjugated secondary antibody (1:400). Imaging was performed using a Zeiss LSM 800 confocal microscope with a 63×/1.4 NA oil objective. The merged panel shows TEX2 accumulation at induced ER-mitochondria junctions (dashed box). Scale bar, 5 µm.

(D) RGB intensity profile corresponding to the boxed region in (C), showing fluorescence signals of Cb5-mCherry (ER, red), TOM20-HA (OMM, blue), and TEX2 (green). TEX2 peaks coincide with the ER signal and lie adjacent to TOM20, consistent with TEX2 localization on the ER side of the contact site.

Upon rapamycin treatment, we observed a fast relocalization of the ER marker: Cb5–mCherry redistributed into discrete ER domains juxtaposed to mitochondria, consistent with the stabilization of ER close to TOM20-positive mitochondria (**Fig. 3.4C**). In this context, we checked the distribution of the TEX2 fluorescence intensity in the ER-mitochondria contact sites region and we clearly observed that it is increased at the ER-mitochondria interfaces, as shown by the RGB profile where TEX2 TEX2 co-peaked with the ER signal (**Fig. 3.4D**).

3.4 TEX2: a novel tether of ER-mitochondria MCSs

Prompted by the observations that TEX2 accumulates at ER-mitochondria contact sites interfaces, we asked whether TEX2 may also be functionally involved in ER-mitochondria contact sites formation, stability, and/or functions. To address all these questions, we depleted the TEX2 protein level in HeLa cells using an siRNA-mediated approach (knockdown validated by immunoblot; see Methods). After 72 hours of treatment with a specific siRNA targeting the TEX2 transcript, we observed a severe decrease in TEX2 signal by Western blot analysis (see Methods).

We next assessed the impact of TEX2 depletion on ER-mitochondria formation using the reporter system previously described (Cb5-TOM20). After siRNA treatment, cells were transfected with the plasmid carrying the Cb5-FKBP-mCherry TOM20-FRB-HA system and then treated with or without rapamycin. In control (MOCK) cells, rapamycin triggered the redistribution of the ER marker in regions closely associated with mitochondria, creating numerous puncta, consistent with what we have already observed. Surprisingly, in TEX2-KD cells, the number of observed Cb5 puncta is severely reduced compared to control cells, suggesting a huge decrease in the number of existing ER-mitochondria contact sites (**Fig. 3.5A-B**). Quantitative analysis revealed a marked reduction in the number of cells with MAM (***, $P < 0.001$). This finding pointed out that TEX2 is acting as a novel ER-mitochondria tethering molecule.

To further confirm that TEX2 is a tethering molecule for ER-mitochondria contact formation, we explored alternative strategies to measure those contacts. We decided to check whether the observed defect was not related to any possible detection defect in our system of reporters (lower amount of expression or lower recruitment efficiency extent, even though the expression of the reporters and rapamycin treatment remained consistent across conditions).

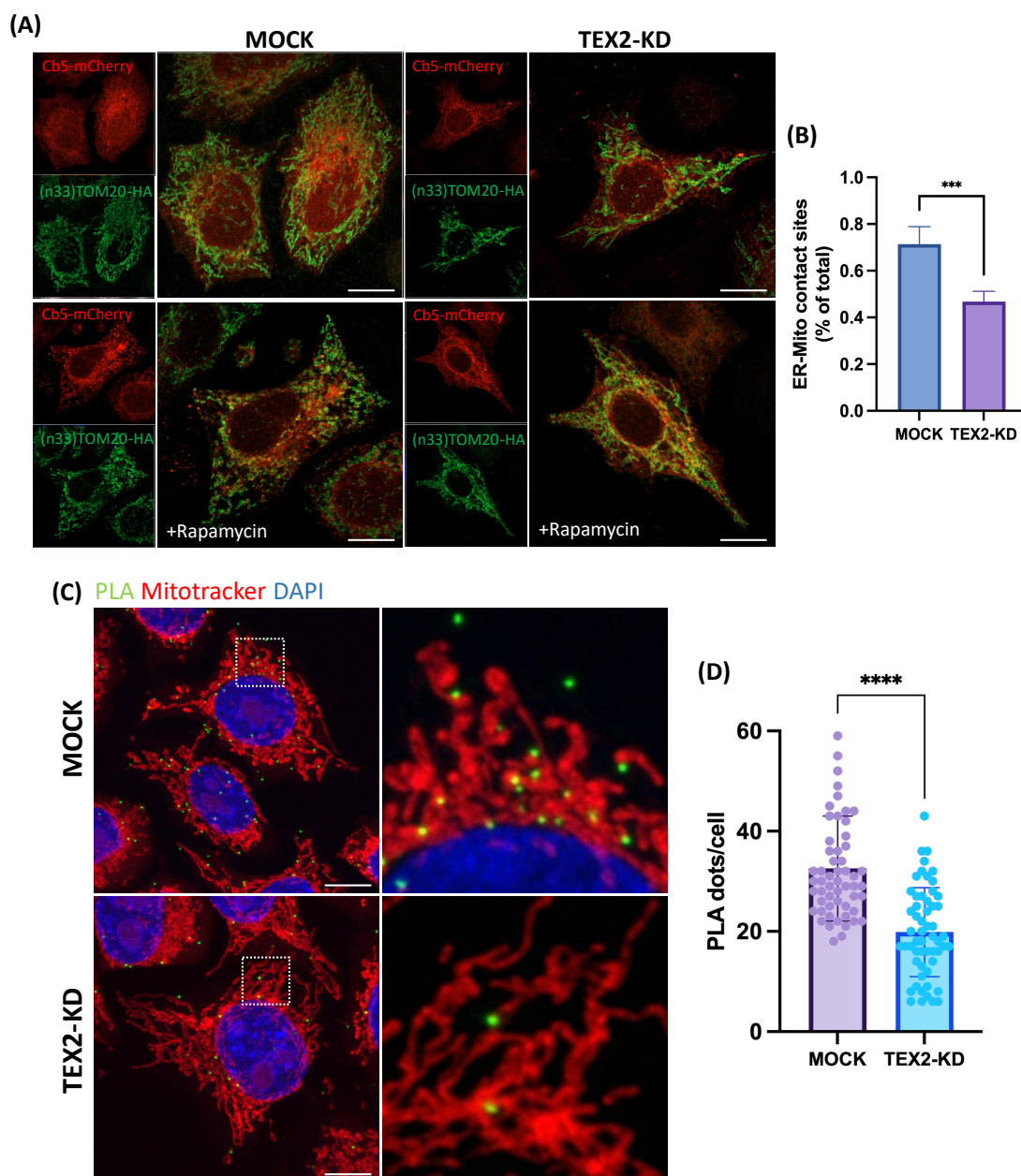


Figure 3.5 - TEX2 depletion reduces ER-mitochondria contact sites.

(A) Representative confocal images of HeLa cells expressing the rapamycin-inducible ER-mitochondria tethering system consisting of Cb5-mCherry-FKBP (ER anchor, red) and n33TOM20-HA-FRB (OMM anchor, blue). Cells were either control (MOCK) or subjected to TEX2 knockdown (TEX2-KD) by transfection with 40 nM siRNA using Oligofectamine (Thermo Fisher) for 72h. Cells were fixed with 4% paraformaldehyde and immunostained using goat anti-HA (1:200) and Alexa Fluor 488 secondary antibodies (1:400). Images were acquired with a Zeiss LSM 800 confocal microscope using a 63 $\times$ /1.4 NA oil objective. Scale bars, 10 μ m. This reporter system stabilizes pre-existing ER-mitochondria contacts upon

addition of 20nM rapamycin for 4 min. In control cells, rapamycin treatment caused a substantial redistribution of the ER signal along mitochondria, while TEX2-KD cells exhibited markedly weaker ER recruitment, indicating reduced ER-mitochondria coupling.

(B) Quantification of rapamycin-induced ER-mitochondria contacts, expressed as the percentage of cells displaying clear ER recruitment to mitochondria after treatment. Bars show mean $\pm$ S.D. from $n = 3$ independent experiments, totalling 60 cells per condition. Statistical significance was determined by an unpaired two-tailed t-test ($***P < 0.001$).

(C) Endogenous MAMs assessed by proximity ligation assay (PLA) in HeLa cells using the ORP8 (mouse, 1:200) and PTPIP51 (rabbit, 1:200) pair (in green) as an established ER-mitochondria contact marker. Before fixation, cells were incubated with MitoTracker Orange (Thermo Fisher, 1:10000, 30 min at 37 °C) to label mitochondria. PLA signals were overlaid DAPI contained in the Vectashield mounting medium (blue). Insets (right) show enlarged mitochondrial regions containing multiple PLA puncta in control cells, which were markedly reduced in TEX2-KD cells. Scale bars, 10 μ m.

(D) Quantification of PLA puncta per cell. Bars represent mean $\pm$ S.D. from $n = 3$ independent experiments, with = 50 cells analyzed per condition. Statistical significance was assessed using an unpaired two-tailed t-test ($****P < 0.0001$).

We performed a completely different experiment, based on the in-situ proximity ligation assay (PLA), using antibodies against the endogenous ER protein ORP8 and the OMM protein PTPIP51, a validated pair reports ER-mitochondria proximity (~tens of nm, REF, introduction) in control and TEX2-KD HeLa cells. In control cells, PLA yielded numerous puncta close to mitochondria (**Fig. 3.5B**). In TEX2-KD cells, PLA puncta were significantly reduced (spots per cell, normalized to area), indicating fewer endogenous MAMs. Imaging and analysis were performed in a blinded manner, with identical acquisition and threshold settings across conditions. These data confirm the hypothesis that TEX2 is acting as a tethering molecule at ER-mitochondria interfaces.

3.5 TEX2-KD impacts mitochondrial morphology

Our experiments demonstrated that TEX2 depletion reduces ER-mitochondria contacts. MAMs are very dynamic structures: ER tubules mark mitochondrial constriction sites, recruit and stabilize the DRP1 fission machinery via OMM adaptors (e.g., MFF/MID49–51/FUNDC1) (Friedman *et al.*, 2011; Otera *et al.*, 2010; Losón *et al.*, 2013; Wu *et al.*, 2016), and provide actin/myosin forces and lipid flux (PS→PE, cholesterol) that tune outer-membrane curvature and scission probability (Vance, 2014). When the MAM function is reduced, the balance typically shifts toward insufficient fission, yielding longer, hyper-connected mitochondria (de Brito & Scorrano, 2008; Sebastián *et al.*, 2012; Giacomello & Pellegrini, 2016). On this basis, we depleted TEX2 by siRNA in HeLa cells and visualized the mitochondrial network using TOM20 staining.

Compared to MOCK, TEX2-KD HeLa cells showed a clear shift toward elongated, highly connected mitochondria, with fewer isolated mitochondrial profiles (**Fig. 3.6A**). We measured the extension of mitochondria by analyzing single-cell morphological data derived from confocal images. We categorized mitochondria length into three main classes: first, from 0 to 1 μm , the second class, from 1 to 5 μm , and the third class, formed by the very long mitochondria, which measured more than 5 μm in length. Upon TEX2 depletion, we observed a significant decrease in the 0–1 μm class and a concomitant increase in the third class, which includes mitochondria longer than 5 μm , indicating an alteration in mitochondrial dynamics. This phenotype is compatible with both a reduced fission rate and an increased fusion rate (**Fig. 3.6B**).

To test whether the effect we have observed is specifically due to TEX2 depletion, we performed rescue experiments by expressing back a TEX2-GFP version that is resistant to siRNA activity. Reintroduction of TEX2 normalized mitochondrial morphology, restoring the prevalence of short- to medium-length objects and reducing the long-mitochondria fraction (**Fig. 3.6C and D**). The rescue supports a cell-autonomous role of TEX2 in maintaining proper mitochondrial dynamics and argues against off-target effects of siRNA.

(B) Distribution of mitochondrial length classes (0–1 μm , 1–5 μm , >5 μm) quantified using the Mitochondria Analyzer plugin in Fiji/ImageJ. Each bar represents the percentage of total mitochondria per class averaged from $n = 3$ independent experiments. In control (MOCK) HeLa cells, short mitochondria (0–1 μm) represented $\approx 54\%$ of the population, intermediate mitochondria (1–5 μm) $\approx 37\%$, and long mitochondria (> 5 μm) only $\approx 9\%$. Upon TEX2 knockdown, the proportion of long mitochondria increased nearly threefold to $\approx 25\%$, with a concomitant decrease of short mitochondria to $\approx 30\%$, while the intermediate fraction slightly rose to $\approx 45\%$. This shift toward longer mitochondria reflects impaired fission dynamics and increased network continuity in the absence of TEX2.

(C) Rescue experiment: HeLa cells were co-transfected with TEX2 siRNA (40 nM, 72 h) and a TEX2–GFP plasmid (1 μg DNA) for the final 16 h before fixation. Cells were immunostained for TOM20 (red) and imaged under identical confocal settings. Re-expression of TEX2–GFP (green) in TEX2–KD cells restored a fragmented mitochondrial morphology comparable to that of controls, confirming the specificity of the phenotype. Scale bars, 10 μm .

(D) Quantification of mean mitochondrial length under the indicated conditions, analyzed using the Mitochondria Analyzer plugin (Fiji/ImageJ). Data represent mean $\pm$ S.D. from $n = 3$ independent experiments (≥ 30 cells per condition). TEX2 depletion significantly increased mitochondrial length, while re-expression of TEX2–GFP restored normal morphology, supporting a model in which TEX2 contributes to maintaining mitochondrial fission and network homeostasis. Notably, even in MOCK cells, TEX2–GFP expression slightly increased mitochondrial fragmentation, consistent with the protein's role in promoting mitochondrial fission at ER–mitochondria contact sites.

3.5.1 TEX2-KD reduces mitochondrial fission/fusion hotspots

Considering the reduction in ER-mitochondrial contacts caused by TEX2 depletion and the close functional link between MAMs and mitochondrial remodeling, we then examined whether TEX2 might influence the local platforms where fission and fusion machinery gather. Recent studies describe distinct foci along mitochondrial tubules, called “mitochondrial nodes,” as sites where the fission GTPase DRP1 and the fusion protein MFN1 (or MFN2) briefly co-cluster (Abrisch *et al.*, 2020). These structures are usually visualized through mild co-expression of DRP1-mCherry and MFN1-GFP, with co-peak signals showing where the two fluorescent markers overlap on a mitochondrion; such puncta are viewed as dynamic hotspots for scission or fusion (Giacomello *et al.*, 2020) (**Fig. 3.7A**).

Using this assay, we first asked whether endogenous TEX2 is present at nodes. Immunofluorescence experiments revealed that TEX2 is enriched at a subset of DRP1/MFN1 co-positive puncta, yielding triple-positive spots on mitochondria. Line-scan analysis across individual foci showed coincident peaks for DRP1-mCherry and MFN1-GFP with a closely TEX2 peak, consistent with TEX2 being enriched at the ER side of the node-containing MAMs (**Fig. 3.7B**).

We then quantified the abundance of the nodes upon TEX2 depletion in HeLa cells. Compared to control, TEX2-KD cells displayed a significant reduction in DRP1/MFN1 co-positive nodes per cell, despite comparable overall expression levels of the reporters and intact gross mitochondrial labeling (TOM20). Because nodes represent assembly/stabilization points for opposing remodeling machineries (Abrisch *et al.*, 2020), their reduction reinforces the idea that TEX2 supports the formation and/or persistence of these dynamic platforms, in line with its role as an ER-mitochondria tether. Taken together with the elongated mitochondrial morphology observed in TEX2-KD cells, all these data indicate that TEX2 promotes the local organization of the fission–fusion machinery at MAMs.

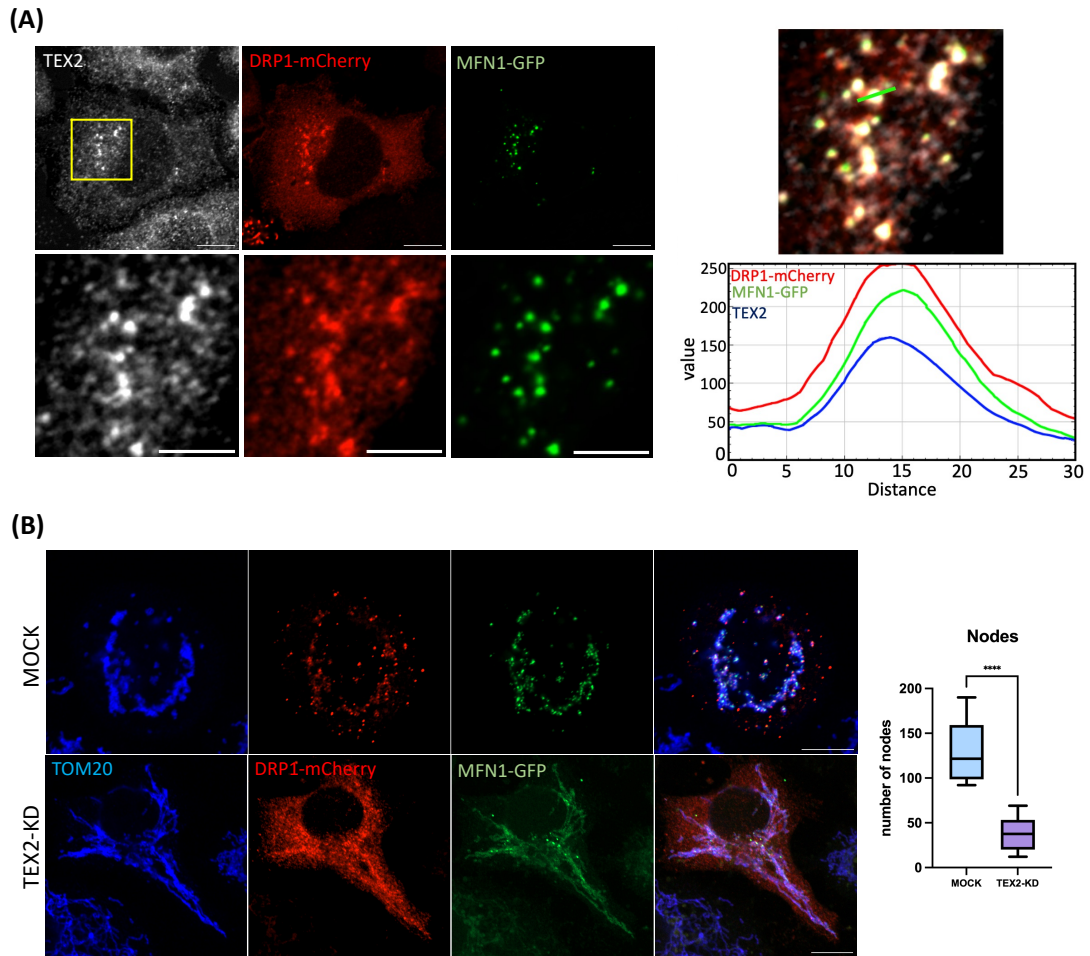


Figure 3.7 - TEX2-KD impairs mitochondrial nodes.

(A) Representative HeLa cell co-expressing DRP1–mCherry (red) and MFN1–GFP (green), immunostained for endogenous TEX2 (grayscale). Cells were fixed 16 h post-transfection, permeabilized with 0.05% saponin, and stained with rabbit anti-TEX2 (1:200) followed by Alexa Fluor 647-conjugated secondary antibody (1:400). Imaging was performed using a Zeiss LSM 800 confocal microscope equipped with a 63×/1.4 NA oil objective. The merged inset (right) shows an enlarged region of interest (ROI) corresponding to a mitochondrial “node,” where DRP1 and MFN1 co-cluster along a tubule. The corresponding line-scan profile (bottom right) displays overlapping DRP1–mCherry and MFN1–GFP fluorescence peaks, with a closely aligned TEX2 signal, indicating that TEX2 localizes at sites where fission and fusion machineries converge (see also Abrisch et al., 2020; Giacomello et al., 2020). Scale bar 10 μ m (cell) and 5 μ m (magnification).

(B) Representative images of HeLa control (MOCK) and TEX2-depleted (TEX2–KD) cells co-expressing DRP1–mCherry and MFN1–GFP, immunostained for TOM20 (blue, 1:400) to visualize the mitochondrial network. TEX2 silencing was achieved by transfection with 40 nM

siRNA using Oligofectamine (Thermo Fisher Scientific) and analyzed 72 h post-transfection. In control cells, DRP1 and MFN1 form multiple co-localizing foci (“nodes”) along mitochondria, while TEX2-KD cells show a marked reduction in both the number and intensity of these structures. The box plot (right) quantifies the number of DRP1–MFN1 co-localizing puncta per cell, defined as DRP1/MFN1 co-positive spots located on TOM20-positive tubules. Data represent mean $\pm$ S.D. from $n = 3$ independent experiments (≥ 25 cells per condition). TEX2 depletion significantly reduces the formation of mitochondrial nodes, suggesting that TEX2 contributes to organizing local ER-mitochondria platforms that coordinate DRP1- and MFN1-mediated remodeling events. Scale bar 10 μm .

3.5.2 TEX2-depleted cells exhibit mitochondrial fission impairment

Since TEX2 depletion resulted in reduced ER-mitochondria contacts and diminished node abundance, we tested whether the fission arm of mitochondrial dynamics was compromised. We first used the FCCP assay, a classic perturbation in which acute mitochondrial depolarization (1 μ M FCCP, 20–30 min) provokes rapid, DRP1-dependent fragmentation of the network (Smirnova *et al.*, 2001). In control (MOCK) cells, FCCP converted interconnected tubules into short fragments in the majority of cells. By contrast, TEX2-KD cells were significantly resistant to FCCP-induced fragmentation, retaining elongated or only partially fragmented networks. Quantitatively, the fraction of cells displaying a fully fragmented phenotype dropped markedly in TEX2-KD compared with MOCK (**Figure 3.8A**).

To directly visualize fission events, we performed live cell imaging in HeLa cells using DRP1-mCherry, along with a mitochondrial marker (mito-BFP, Friedman *et al.*, 2011). We scored fission when a DRP1 punctum formed on a pre-constricted mitochondrial segment and resolved into two daughter tubules. Across matched acquisition windows, TEX2-KD cells exhibited a strong reduction in fission frequency (events per cell per minute) relative to controls (**Figure 3.8B**), while overall DRP1-mCherry expression and mitochondrial labeling were comparable. Kymographs and time-lapse insets illustrate the loss of productive DRP1 events in TEX2-KD.

Prompted by this defect in DRP1 function, we examined the phosphorylation state of DRP1, since these post-translational modifications have been implied in the regulation of its fission activity (Chang *et al.*, 2007). In particular, phosphorylation occurring in the Ser616 (ERK/CDK sites) correlates to pro-fission competence, while phosphorylation of the Ser637 (PKA site) correlates to improper fission ability (Taguchi *et al.*, 2007; Cribbs *et al.*, 2007). Cell total extracts from control and TEX2-KD cells were probed using antibodies specific for the different phosphoSer of DRP1. Immunoblotting revealed similar total DRP1 levels between conditions but a shift in phosphorylation in TEX2-KD: reduced pSer616 and elevated pSer637 (Fig. C; ~40% decrease in pSer616 and ~5-fold increase in pSer637 relative to MOCK in this experiment), consistent with a DRP1 pool that is less fission-competent (**Figure 3.9C**).

Together, the FCCP resistance, the live fission assay, and the DRP1 phospho-signature support the conclusion: TEX2 promotes DRP1-mediated fission, likely by organizing ER-mitochondria interfaces where DRP1 is recruited and maintained in an active state.

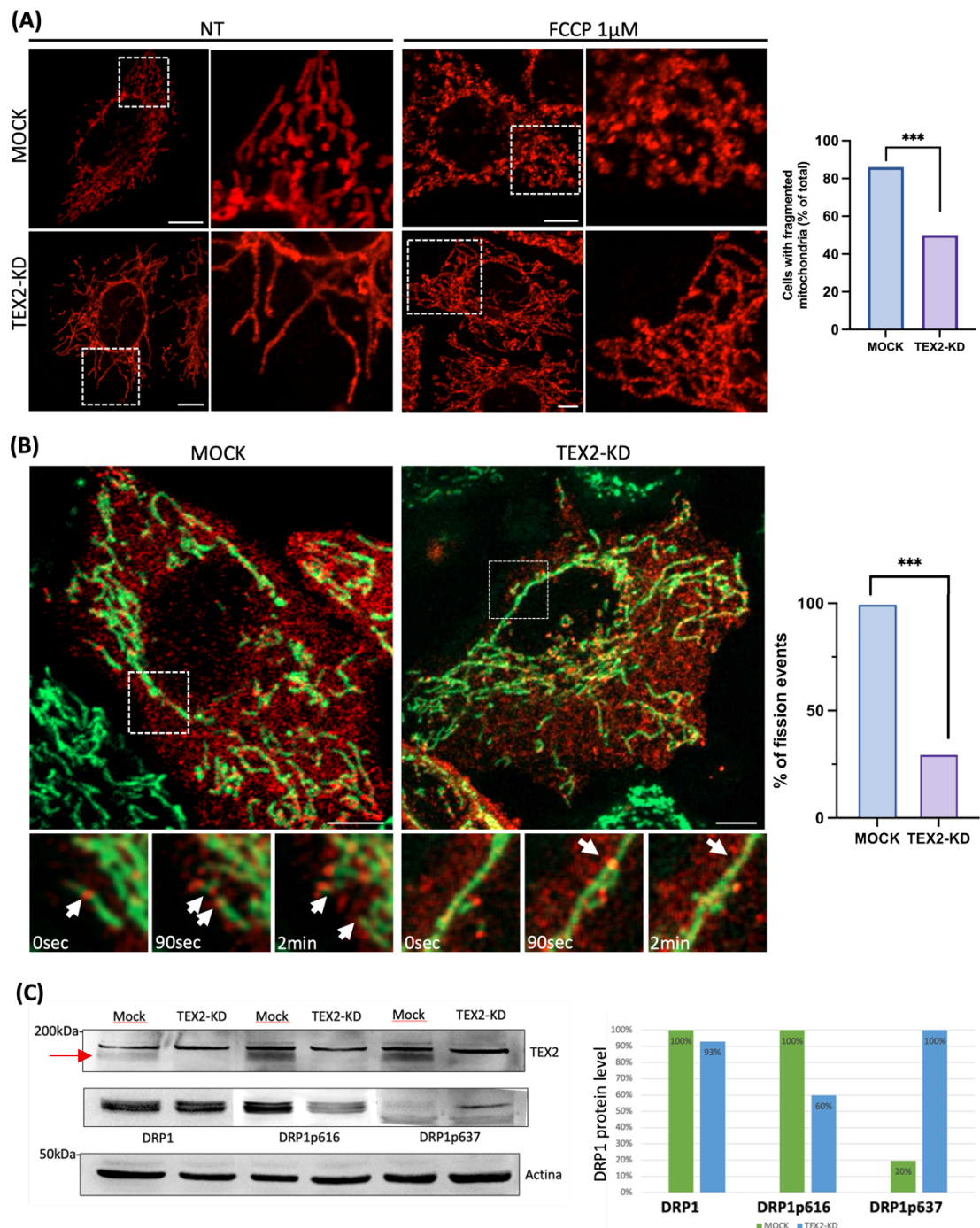


Figure 3.8 - TEX2 depletion impairs DRP1-dependent mitochondrial fission.

(A) HeLa cells, either control (MOCK) or TEX2-depleted (TEX2-KD), were immunostained for the mitochondrial outer membrane protein TOM20 (mouse, 1:400) to visualize mitochondrial morphology. TEX2 knockdown was achieved by transfection with 40 nM siRNA using Oligofectamine (Thermo Fisher Scientific) according to the manufacturer's protocol, and cells were analyzed 72 hours post-transfection. To assess mitochondrial fission capacity, cells were treated with 1 μ M FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) for 20–30 minutes at 37°C to acutely dissipate the mitochondrial membrane potential. FCCP treatment typically triggers a DRP1-dependent fragmentation of the mitochondrial network. In control cells, FCCP induced complete mitochondrial fragmentation, while in TEX2-KD cells, elongated mitochondria persisted, indicating defective fission. Insets show higher-magnification regions of the same cells. The bar graph (right) quantifies the percentage of cells displaying fragmented mitochondria after FCCP (mean $\pm$ S.D., **P < 0.001, n = 2 independent experiments, $\geq$ 100 cells per condition).

(B) HeLa cells were co-transfected with DRP1-mCherry (0.5 μ g DNA) and mito-BFP (0.5 μ g DNA) using Lipofectamine LTX Plus, and imaged 16 h post-transfection using a Zeiss LSM 800 confocal microscope with a 63 $\times$ /1.4 NA oil objective and on-stage incubation at 37 °C and 5 % CO₂. Time-lapse sequences (0, 90 s, 2 min) show DRP1-mCherry foci (red) transiently assembling on mito-BFP-labeled tubules (green) at productive fission sites (arrows). TEX2-KD cells display a strong reduction in DRP1 activity and completed fission events compared to MOCK. Quantification (right) shows the mean number of fission events per cell during 5 minutes of live acquisition.

(C) DRP1 phosphorylation state. Immunoblot analysis of total DRP1 and its phosphorylated forms at Ser616 and Ser637 in control and TEX2-KD HeLa cell lysates. Proteins (60 μ g per lane) were separated by 10 % SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with the following primary antibodies: anti-TEX2 (rabbit, 1:1000), anti-DRP1 (rabbit, 1:1000), anti-phospho-DRP1 Ser616 (rabbit, 1:1000), anti-phospho-DRP1 Ser637 (rabbit, 1:1000), and anti- β -actin (mouse, 1:8000) as a control. Secondary antibodies (1:400) were used, and signals were detected by chemiluminescence. Densitometric analysis (right) shows that total DRP1 levels remain unchanged, whereas phosphorylation at Ser616 (pro-fission site) decreased to ~60 % of control, and phosphorylation at Ser637 (anti-fission site) increased to ~120 % in TEX2-KD cells. These results indicate that TEX2 loss shifts the DRP1 phosphorylation balance toward an inactive state, impairing mitochondrial fission competence.

3.6 FUNDC1 recapitulate TEX2-KD phenotype

TEX2 is an ER-resident SMP-domain protein that we have found to be enriched at the ER-mitochondria contact sites interface, acting as a novel tethering molecule for this class of contacts. To further dissect the molecular mechanism underlying TEX2 activity, we next asked which OMM factors may function as a partner with TEX2 across the MAM to stabilize contacts and, potentially, coordinate fission. Our unbiased AP-MS identified FUNDC1 as one of the top interactors of TEX2.

FUNDC1 is an integral outer-mitochondrial membrane (OMM) protein. Its cytosolic N-terminus contains a canonical LC3-interacting region (LIR) that enables receptor-mediated mitophagy (Chen et al., 2016; Tang et al., 2023). The FUNDC1 function is post-translationally gated:

- inhibitory marks (e.g., phosphorylation at Ser13 by CK2 , REF; phosphorylation at Tyr18 by Src, REF) dampening LC3 binding (Chen et al., 2016; Tang et al., 2023.)
- activating marks (e.g., phosphorylation by ULK1 on Ser17, ref and PGAM5-dependent dephosphorylation of inhibitory sites), promoting LC3 engagement (Wu *et al.*, 2014).

FUNDC1 represents a valuable candidate to act as a molecular mitochondrial counterpart for TEX2 at the ER-mitochondria contact site. FUNDC1 depletion in HeLa cells perfectly mimicked TEX2 depletion at steady state (**Figure 3.10A**). FUNDC1-KD cells show a hyper-tubulated, elongated mitochondrial network (TOM20), which is a hallmark of impaired DRP1-dependent fission and weaker ER-mitochondria coupling, all of which are precisely the features we see when TEX2 is knocked down. Intriguingly, using the ER-mitochondria contact sites system of reports, we observed that FUNDC1 depletion resulted in a significant reduction of the percentage of cells exhibiting ER redistribution after rapamycin treatment compared to control cells (**Fig. 3.9B and C**). Since this method stabilizes existing contacts rather than forming new ones from scratch, the reduction in “recruitment-positive” cells truly indicates a deficiency in the presence of contact sites in the cells. In other words, reducing the OMM partner (FUNDC1) phenocopies the contact-site deficit observed when the ER partner (TEX2) is decreased, supporting the hypothesis that the two proteins may

operate within the same pathway to maintain MAMs and drive the organization of the fission machinery locally.

A mechanistic unsolved puzzle in the FUNDC1 field helps to explain why an additional ER protein similar to TEX2 might be needed. FUNDC1 has been reported to interact with the ER chaperone Calnexin at MAMs (Wu *et al.*, 2016). Yet, Calnexin is a type-I ER membrane protein with a large luminal lectin domain and a short cytosolic tail, whereas the binding-competent region of FUNDC1 is cytosolic. A direct, high-affinity FUNDC1–Calnexin interaction is therefore topologically complex: the likely binding surfaces (Calnexin luminal vs FUNDC1 cytosolic) are on opposite sides of the ER membrane. Our data add the missing piece of information : TEX2, an ER-resident protein with extensive cytosolic exposure, a PH-like/basic region, coiled-coils, and an SMP lipid-handling module, might serve as a cytosolic bridge for FUNDC1 to bind to the ER membranes. In this model, TEX2 co-resides with Calnexin in an ER microdomain at MAMs, while engaging FUNDC1 across the cytosolic gap, thereby stabilizing the platform for DRP1 assembly. The observed mitochondrial elongation upon either TEX2- or FUNDC1-KD fits with a shared failure to support productive DRP1 recruitment/activation at MAMs—importantly, this occurs even without a hypoxic challenge, extending prior studies that emphasized FUNDC1-dependent fission mainly under hypoxia.

Overall, the data collected so far (including the interaction between TEX2 and FUNDC1 in **Figure 3.9D** and the similarities between conditions in which the two proteins are individually depleted) support the hypothesis that TEX2 functions as a cytosolic ER adaptor connecting a Calnexin-containing ER microdomain to FUNDC1. This connection facilitates the formation of ER-mitochondria contact sites and contributes to DRP1-dependent fission events.

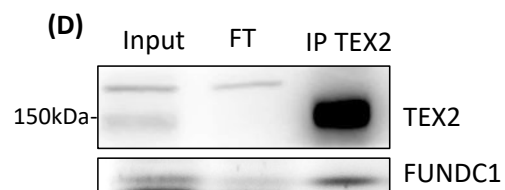
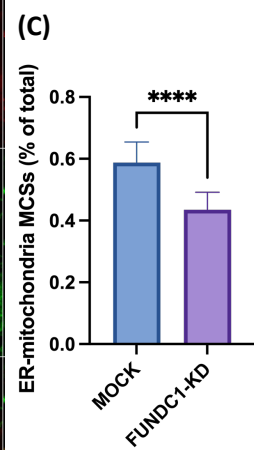
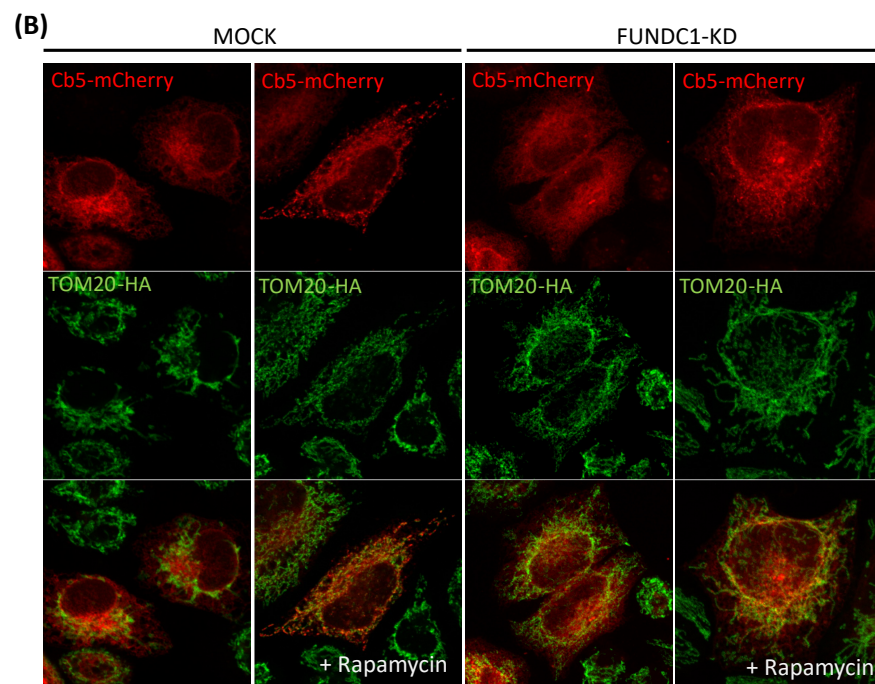
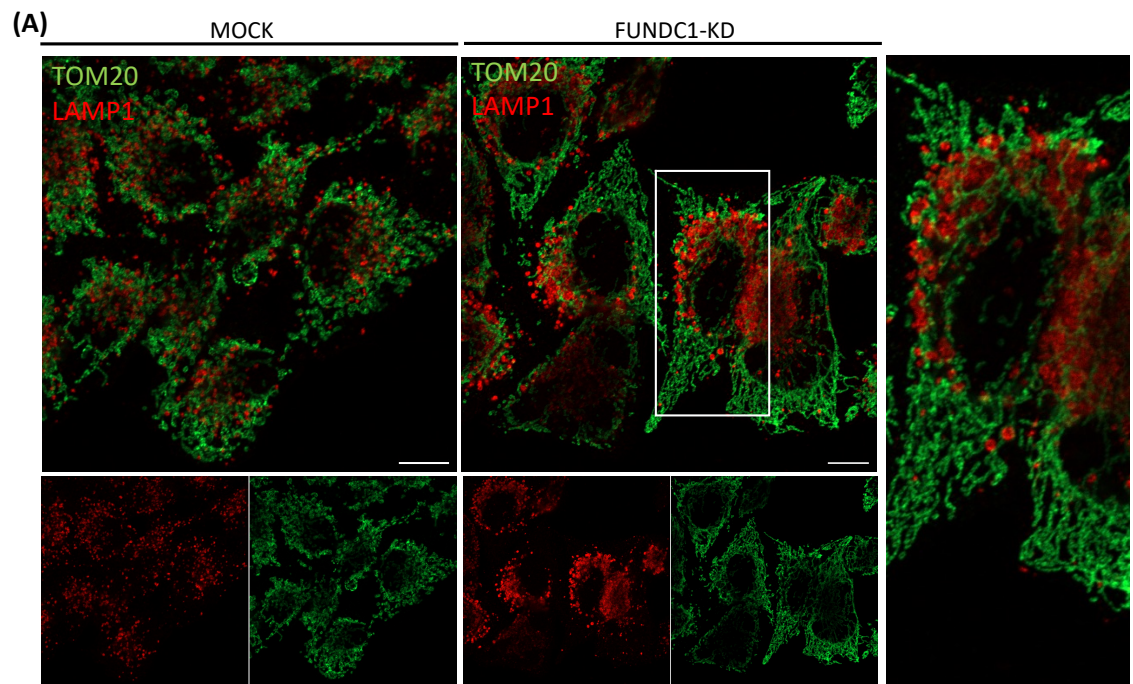


Figure 3.9 - FUNDC1 depletion phenocopies TEX2 loss-of-function at contact sites and alters lysosome positioning.

(A) Representative confocal images of HeLa cells control (MOCK) or transfected with control siRNA targeting FUNDC1 (FUNDC1-KD) and immunostained for TOM20 (mitochondria, green) and LAMP1 (lysosomes, red). FUNDC1 knockdown was achieved using 40nM siRNA for 72 hours, resulting in >80% protein reduction, as verified by immunoblotting (see Methods). FUNDC1-KD cells display elongated, interconnected mitochondria and a marked perinuclear clustering of LAMP1-positive vesicles, indicative of altered lysosome positioning (rightmost magnification).

(B) Rapamycin-inducible ER-mitochondria tethering assay. HeLa cells expressing ER-Cb5-mCherry-FKBP-OMM-TOM20-HA-FRB were treated with 100 nM rapamycin for 15 min to trigger acute FKBP-FRB dimerization and ER recruitment onto mitochondria. In control (MOCK) cells, robust relocalization of ER tubules onto the mitochondrial network was observed, whereas FUNDC1-KD cells exhibited a markedly attenuated recruitment, consistent with reduced ER-mitochondria contact formation.

(C) Quantification of the percentage of cells showing ER relocalization upon rapamycin addition (**mean $\pm$ S.D. ; n = cells from ≥ 3 independent experiments; **p < 0.0001; statistical test in Methods).

(D) Endogenous TEX2 co-immunoprecipitation assay. Whole-cell lysates from HeLa cells were subjected to IP using an anti-TEX2 antibody, followed by immunoblotting for FUNDC1. FUNDC1 was detected explicitly in the TEX2 immunoprecipitate, indicating a physical association between the two proteins. Input (60 μ g), flow-through (FT, 60 μ g), and immunoprecipitated (IP) fractions are shown.

3.7 TEX2's SMP domain is required to maintain mitochondrial morphology

Guided by the idea that SMP-domain proteins often couple membrane tethering with lipid handling at membrane contact sites (MCSs), we investigated whether the SMP of TEX2 is required for its pro-fission activity at ER-mitochondria contacts (MAMs). Several well-characterized tethers share this dual function. For instance, the extended synaptotagmins (E-Syts) bridge ER–plasma membrane contacts while transferring glycerophospholipids through their SMP barrels (Schauder et al., 2014; Saheki et al., 2016); VPS13 family members form lipidic bridges between multiple organelle pairs including ER-mitochondria (Kumar et al., 2018; Li et al., 2020); and PDZD8, an ER-resident SMP protein, functions as an ER–mitochondria tether in mammalian cells (Hirabayashi et al., 2017). Together, these examples illustrate how SMP-containing tethers integrate structural and metabolic coordination between organelles. Within this superfamily, the yeast homolog Nvj2p relocates upon ER stress. It has been mechanistically linked to non-vesicular ceramide transport to the Golgi, while engineered ER–Golgi tethers in mammalian cells are sufficient to accelerate ceramide transfer. These two findings demonstrate the feasibility and physiological importance of ceramide hand-off by SMP-containing factors (Liu et al., 2017; Jeyasimman et al., 2020).

MAMs are lipid-active hubs that integrate metabolic fluxes with mitochondrial dynamics. ER-mitochondria MCSs promote PS→PE conversion and regulate cholesterol/PA pathways (Vance, 2014). In addition, they mark mitochondrial constriction sites where ER tubules recruit the DRP1 machinery via OMM adaptors (MFF, MiD49/51, FUNDC1) to complete scission (Friedman et al., 2011; Otera et al., 2010; Losón et al., 2013). Lipid composition at these sites critically tunes curvature and protein assembly: cone-shaped lipids (PE, PA) facilitate constriction, while sphingolipids such as ceramide can modulate membrane organization and DRP1-dependent remodeling. Under stress, ceramide accumulation has been shown to associate with DRP1-dependent mitochondrial fragmentation and mitophagy (Sentelle et al., 2012) and to enhance fission in cardiomyocytes (Parra et al., 2008). In human trophoblast models and preeclamptic placentae, C16-ceramide increases

mitochondrial fission with BOK-mediated regulation of DRP1 and localization of fission events to ER/MAM regions (Ausman et al., 2018), supporting the view that ceramide-driven remodeling at MAMs can act as a rheostat linking fission to mitochondrial turnover. In our system, TEX2 is enriched at MAMs, and its depletion reduces ER-mitochondria contacts and impairs DRP1-dependent fission, resulting in elongated networks (Figures 3.5–3.9).

To test whether lipid-handling by TEX2's SMP is required for its function, we generated two double-mutant versions of TEX2 within conserved SMP motifs by analogy to catalysis-impairing substitutions defined in ESYT SMP barrels (see Materials and Methods). We then performed side-by-side rescue in MOCK and TEX2-KD cells. Re-expression of siRNA-resistant TEX2-GFP (wild type) fragmented mitochondria and restored mean length toward control values, whereas both SMP mutants failed to rescue: mitochondrial networks remained elongated in TEX2-KD and, importantly, did not hyper-fragment in MOCK cells (**Fig. 3.10A**). Quantitatively, the mean mitochondrial length decreased from ~8.0 μm in TEX2-KD NT to ~5.1 μm upon TEX2-GFP (wild type), while SMP mutants remained long (~7.7–8.3 μm) (**Fig. 3.10B**). These results argue that an intact SMP barrel is necessary for TEX2's morphological activity.

Mechanistically, we support a model in which TEX2 uses its SMP to remodel local lipid composition at MAMs to promote productive DRP1 constriction. Cone-shaped lipids such as PE and PA lower line tension and assist outer-membrane curvature during fission; neutral sphingolipids like ceramide can also reorganize membrane domains and influence curvature and protein recruitment (Reinisch & Prinz, 2021; Vance, 2014). Because our proteomics place TEX2 in the same pathway as FUNDC1, a MAM-enriched OMM factor able to scaffold DRP1 (Wu et al., 2016; Chen et al., 2016), we propose that TEX2's SMP maintains/feeds a MAM lipid environment that licenses FUNDC1/DRP1 activity. Consistently, TEX2 loss shifts DRP1 phosphorylation toward a less fission-competent state and reduces acute fission responses (FCCP, live DRP1 assay), phenotypes that wild-type TEX2, but not SMP mutants, counteract.

Notably, several studies have demonstrated non-vesicular ceramide transfer at ER interfaces. The best-characterized example is the ceramide transfer protein CERT,

which shuttles ceramide from the ER to the Golgi apparatus for sphingomyelin synthesis through its START domain and PH-domain-mediated tethering to PI4P-enriched Golgi membranes (Hanada et al., 2003; Kumagai et al., 2005). Similarly, FAPP2 and Nvj2p have been shown to mediate ceramide or glycosphingolipid transport between the ER and Golgi under stress conditions (D'Angelo et al., 2007; Liu et al., 2017). In addition, recent lipidomic and proteomic studies place key enzymes of sphingolipid metabolism, such as ceramide synthases and sphingomyelin synthase 1, at ER-mitochondria contact sites (MAMs) (Area-Gomez et al., 2012; Vance, 2014). However, direct evidence for ER-to-mitochondria ceramide transfer driving DRP1 activity remains incomplete, leaving open the possibility that MAM-localized LTPs, such as TEX2, could perform this function.

Our data therefore support the conservative conclusion that TEX2's SMP-dependent lipid handling—potentially including ceramide together with PA/PE—acts as a rheostat for fission probability at MAMs, without requiring us to assert a specific stress-induced ceramide surge at MERCSs.

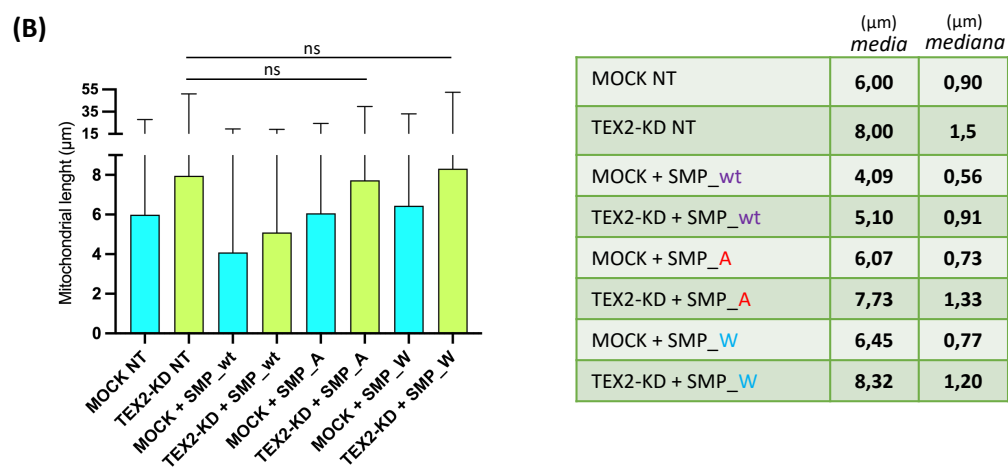
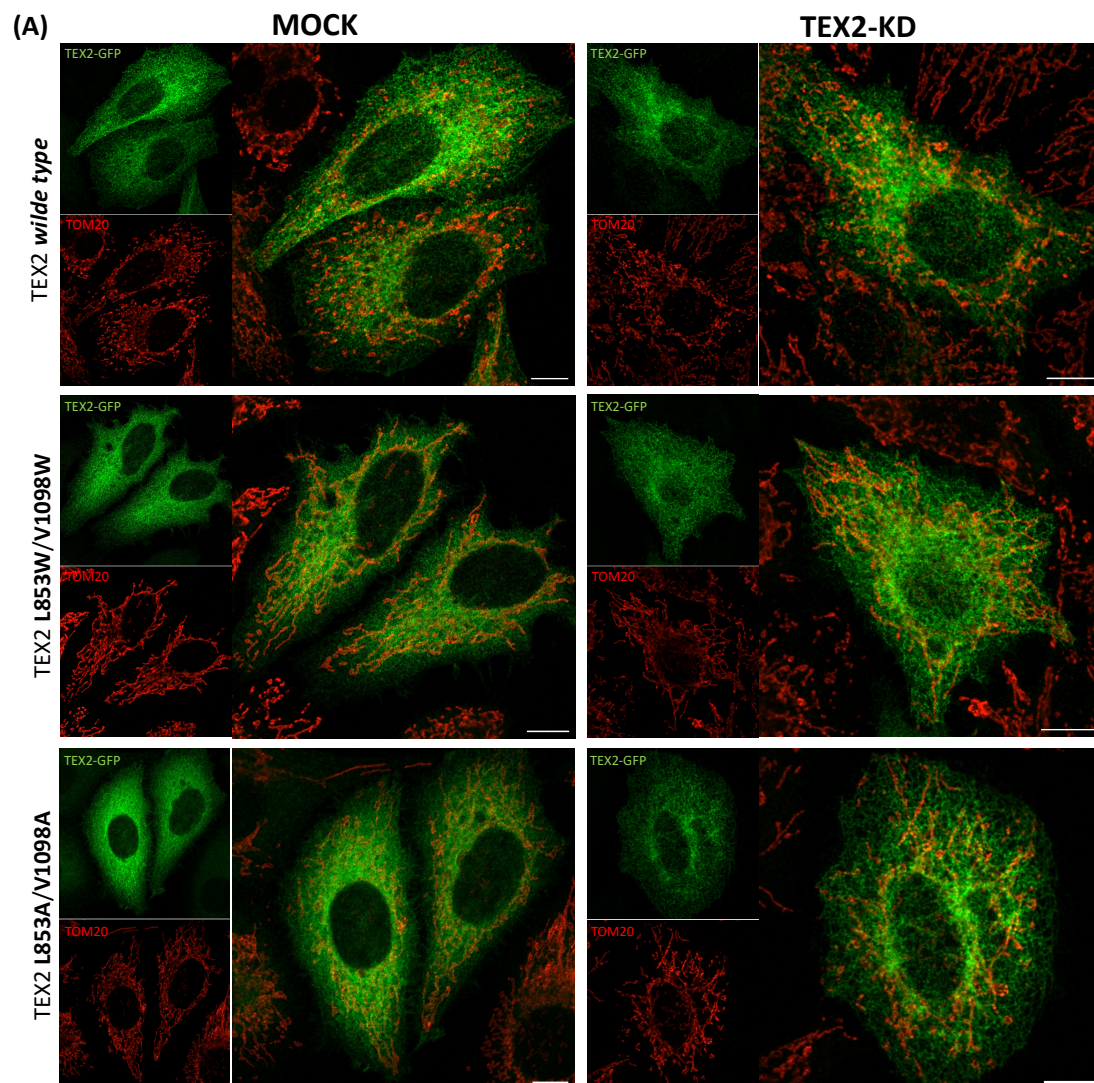


Figure 3.10 - TEX2 SMP mutants fail to rescue mitochondrial morphology

(A) Representative confocal images of HeLa cells stained for TOM20 (red) and expressing the indicated TEX2–GFP constructs (green). Cells were subjected to TEX2 siRNA knockdown for 72 h using Oligofectamine, followed by transfection of siRNA-resistant TEX2–GFP plasmids for 16 h using Lipofectamine LTX Plus reagent before fixation. Left panels show MOCK-transfected cells (control siRNA), and right panels show TEX2-KD cells. Top: expression of wild-type TEX2–GFP localizes to tubular ER structures and promotes mitochondrial fragmentation in MOCK cells, while restoring a fragmented network in TEX2-KD (rescue). Middle/bottom: the two SMP double mutants (as indicated in panels; see Materials and Methods for mutation details) exhibit comparable ER-like distribution but fail to induce mitochondrial fragmentation in MOCK and do not rescue the elongated phenotype in TEX2-KD. Scale bars, 10 μm .

(B) Quantification of mean mitochondrial length (μm per cell) under the indicated conditions (bar plot, mean $\pm$ SD; n = cells as in Methods). Mitochondrial morphology was analyzed from maximum-intensity projections using the Mitochondria Analyzer plugin (Fiji/ImageJ), which segments and computes morphometric parameters (length, branch count, aspect ratio) on thresholded TOM20-labeled networks. The accompanying table (right) summarizes averaged values and statistical significance. Wild-type TEX2–GFP significantly reduces mitochondrial length, indicating rescue of DRP1-dependent fission, whereas both SMP mutants remain comparable to non-transfected TEX2-KD cells (ns, not significant).

3.8 Consequences of disrupting ER-mitochondria coupling

3.8.1 Lipid homeostasis in TEX2-KD cells

Since the TEX2 SMP domain binds polar lipids, we explored whether specific lipid pools change upon TEX2 depletion. Du *et al.* demonstrated that TEX2-SMP binds to phosphatidic acid (PA) and binds strongly to ceramide, moderately to PE and PS, and weakly to PC, with a baseline level in the presence of cholesterol or in the absence of lipids (Du *et al.*, 2020). This profile ($PA > \text{ceramide} \gtrsim PS/PE > PC \gg \text{cholesterol}$) aligns with an SMP barrel that prefers cone-shaped lipids and neutral sphingolipids, which are precisely the species that shape membranes at MAMs and support fission.

To evaluate whether these candidate lipids redistribute in cells after TEX2 loss, we used both genetically encoded and exogenous reporters. PS readouts, Lactadherin-C2 (a cytosolic-leaflet PS sensor), and pulse-chased TopFluor-PS showed no relocalization in TEX2-KD compared to the control, suggesting either that redundant pathways buffer PS trafficking or that our probes mainly detect long-lived pools not immediately impacted by the contact defect (**Figure 3.11**). Conversely, the PA biosensor OPI1 displayed a clear shift: in TEX2-KD cells, the reporter accumulated on LAMP1-positive compartments, indicating PA enrichment in lysosomes/late endosomes. This observation suggests an additional role for TEX2 at the ER-lysosome interface and supported the hypothesis that when TEX2-dependent MAMs are disrupted, a portion of PA—usually consumed or directed at ER-mitochondria contacts—is re-routed to the endo-lysosomal system (for example, through DGK activity or impaired lipid transfer at MAMs, **Figure 3.11**).

Finally, the TopFluor-PE showed a loss of the specific, although weak mitochondrial-associated signal that appears in control cells (above an endosomal signal, **Figure 3.11**). Since mitochondrial PE is mainly produced by PS→PE decarboxylation in the inner membrane, with PS supplied from the ER at MAMs, a reduction in mitochondrial PE is exactly what you'd expect from decreased ER-mitochondrial PS transfer. Mechanistically, this aligns with the defect in the mitochondrial fission we have observed in TEX2-KD cells: PE promotes negative curvature and DRP1 constriction; reduced mitochondrial PE likely hinders fission, resulting in elongated networks.

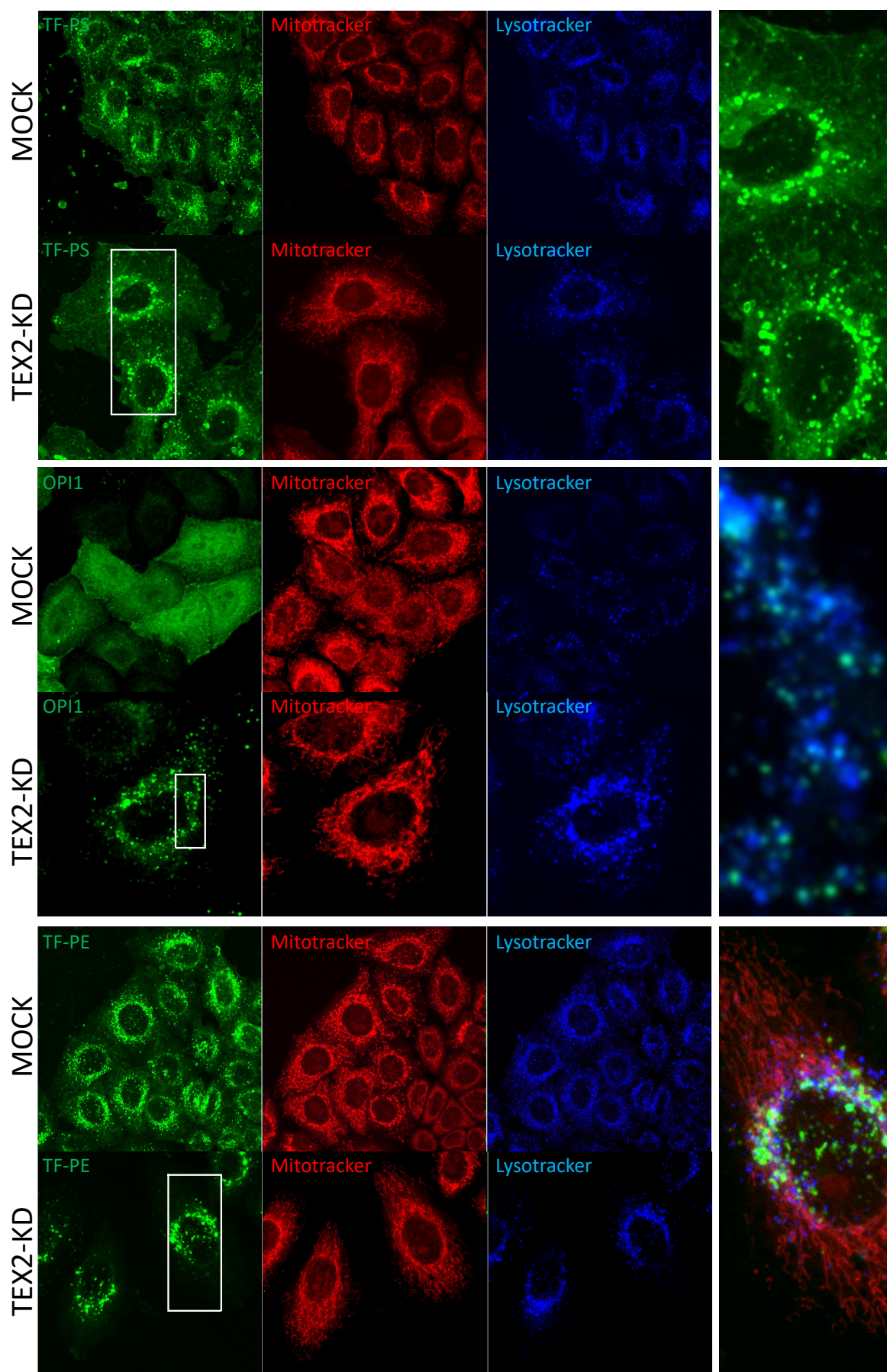


Figure 3.11 - TEX2 depletion alters PA and PE distribution.

Confocal images of control (MOCK) and TEX2-KD HeLa cells labeled with organelle markers (MitoTracker, red; LysoTracker, blue) and lipid probes (green). Dashed boxes indicate regions enlarged on the right. TopFluor-PS (TF-PS): in both MOCK and TEX2-KD, TF-PS shows a predominantly endomembrane pattern with puncta and internal membranes; no overt redistribution toward mitochondria or lysosomes is evident upon TEX2 knockdown. PA sensor (OPI1): in MOCK cells, the fluorescent PA reporter displays a relatively diffuse cytosol and endomembrane signal. In TEX2-KD, OPI1 accumulates in bright puncta that coincide with LysoTracker-positive structures, indicating PA enrichment on lysosomes when TEX2 is depleted. TopFluor-PE (TF-PE): in MOCK cells, TF-PE labels endomembranes, including a detectable mitochondrial-associated component (overlaps with MitoTracker). In TEX2-KD, this mitochondrial contribution is reduced, consistent with impaired delivery/retention of PE at mitochondria when ER-mitochondria contacts are weakened. All images were acquired and displayed using identical acquisition and display settings within each probe.

3.8.2 Impairment in lipid droplet (LD) biogenesis

Lipid droplets (LDs) do not form randomly in the ER; they nucleate and grow at specialized ER subdomains where neutral-lipid synthesis, phospholipid remodeling, and organelle exchange are spatially coordinated (Prinz et al., 2020; Joshi et al., 2018). A large body of evidences, exemplified by Giordano and colleagues, highlights ER-mitochondria contact sites as key players in this process (Ma et al., 2021; Guyard & Giordano, 2025). MAMs concentrate enzymes for TAG/sterol-ester production, channel PS→PE through the mitochondrial PSD, and return it to the ER, providing ATP/Ca²⁺ and fatty acids needed for LD expansion (Vance, 2014; Ma et al., 2021). In this perspective, efficient LD growth relies on continuous lipid and information flow between the ER and mitochondria.

We investigated how TEX2 depletion, which decreases ER-mitochondria coupling and disrupts lipid organization, impacts lipid droplets (LDs). Using Lipi-blue staining, we observed a significant reduction in LD size in TEX2-KD cells compared to MOCK controls (a leftward shift in size distribution and a lower average diameter, **Fig. 3.12A**). Although the LD number was not the main focus of this experiment, the reduction of the LD size is caused by impaired growth or expansion rather than a complete block in the nucleation process. This phenotype aligns with a MAM-centric model: when ER-mitochondria contact is weakened, local pools of important lipids and the enzymes that modify them are less efficiently accumulated at the site of LD formation, restricting its growth.

Our lipid-sensing probes further support a MAM-related mechanism. First, the OPI1 PA reporter shows a clear redistribution toward lysosomal and endolysosomal compartments in TEX2-KD, suggesting that phosphatidic acid (PA), a precursor that promotes LD budding and supplies DAG and TAG synthesis, becomes mispartitioned away from ER-mitochondria subdomains when TEX2 is absent. Second, TopFluor-PE labelling, which in controls marks endomembranes with a visible mitochondrial component, loses this mitochondrial-associated signal in TEX2-KD, consistent with decreased PS→PE flux through mitochondria or reduced return and retention of PE at ER-mitochondria regions. Together, these observations are perfectly in line with the

literature (Giordano *et al.*), according to which MAMs organize the PE and PA circuits necessary for LD growth: lower PE availability at the LD monolayer (important for curvature and surface tension) and mislocalized PA (crucial for budding and TAG production) would both be expected to produce smaller droplets.

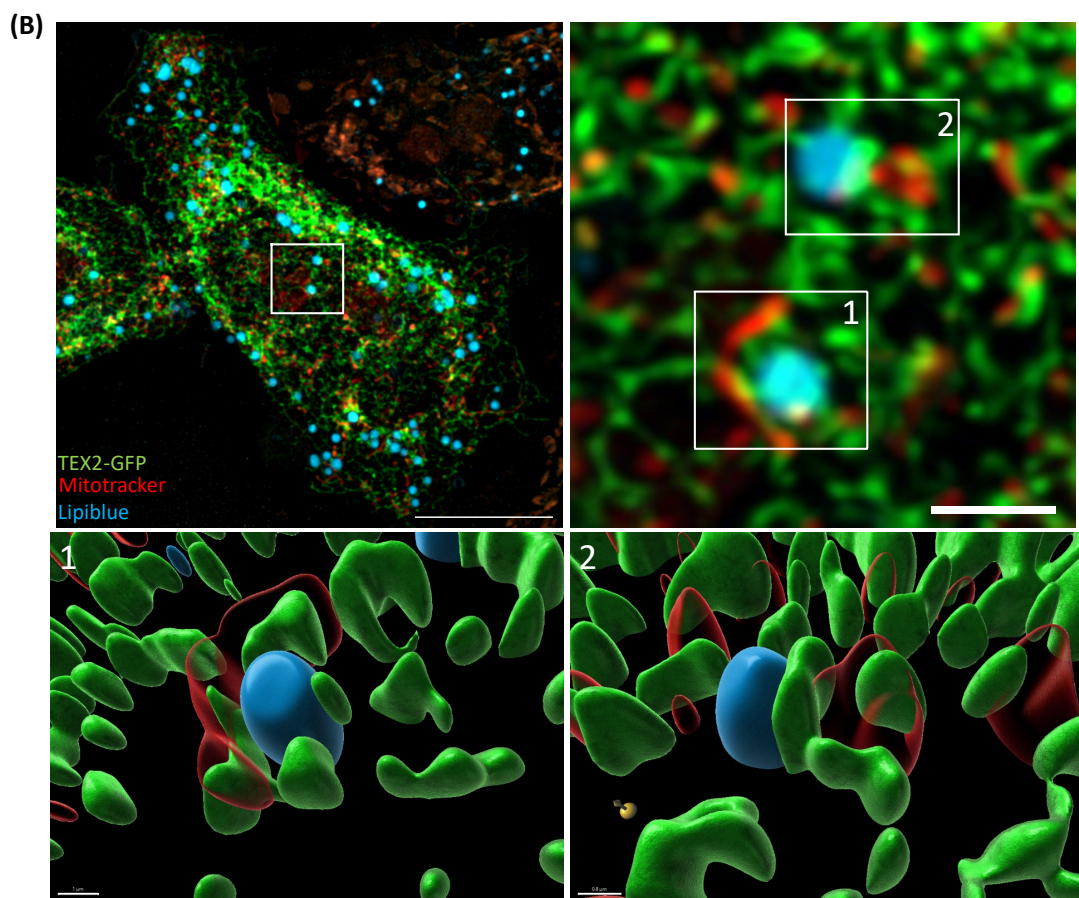
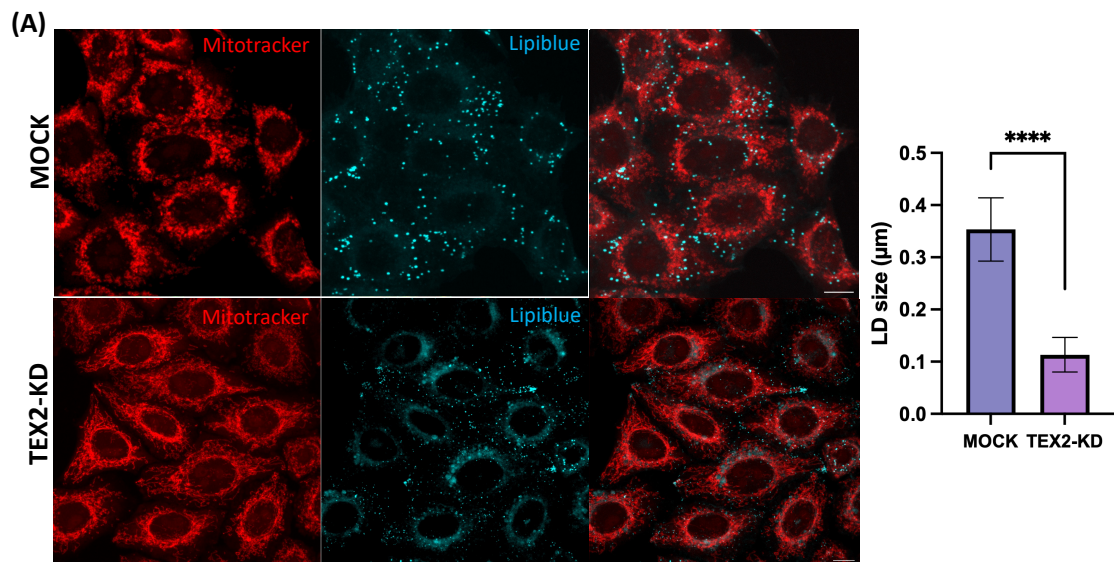


Figure 3.12 - TEX2 controls LD size and accumulates at LD–ER–mitochondria MCSs.

(A) Confocal images of control (MOCK) and TEX2-KD HeLa cells stained with MitoTracker (red) and the neutral-lipid dye Lipi-blue (cyan). Right, quantification of lipid droplet (LD) size (mean LD diameter per cell). Bars show mean $\pm$ S.D.; **** indicates $p < 0.0001$. TEX2 depletion yields significantly smaller LDs.

(B) Super-resolution imaging of HeLa cells expressing TEX2-GFP acquired on a ZEISS Elyra 7 Lattice SIM² system. TEX2-GFP (green) delineates ER strands that frequently run adjacent to mitochondria (MitoTracker, red) and LDs (Lipi-blue, cyan). A zoomed view (top right) highlights hotspots where TEX2-positive ER, mitochondria and LDs coincide. Bottom, 3D reconstructions in Imaris illustrate representative tri-partite junctions (1–2), with TEX2-positive ER (green), LDs (blue) and mitochondria (red). Scale bars 10 μ m and 1 μ m (magnification).

Then, we observed that TEX2 accumulates on the surface of LDs and is repeatedly enriched at tri-partite junctions where LDs, ER, and mitochondria meet (**Figure 3.12 B**). These structures are exactly localized where neutral-lipid synthesis, phospholipid remodelling, and metabolite exchange coordinatively occur, highlighting the functionally significant role of TEX2.

Consistent with a positive role at LD growth sites, TEX2-GFP overexpression resulted in visibly larger LDs compared to controls. Along with the TEX2-KD phenotype, these findings suggest that TEX2 is not only a scaffold that maintains ER-mitochondria proximity. We hypothesized that TEX2 supports MAMs organization, which is necessary for LD formation, and functions locally at LD–ER–mitochondria tri-partite contacts to regulate lipid availability and membrane mechanics, thereby promoting growth. These findings will be further investigated in future studies.

3.8.3 Increase in mitochondria-lysosome MCSs

In our TEX2 loss-of-function model, where MAMs are reduced, we consistently observe a fission-defective state—elongated mitochondria. Although these findings were initially observed in HeLa cells, we decided to analyse whether TEX2 depletion results in similar phenotypes in different cell types. Therefore, we used BJ-5ta fibroblasts, a non-transformed human cell line. Two main reasons drove this choice. First, fibroblasts allowed us to test whether the defects caused by TEX2 depletion are reproducible in a different cell type, strengthening the overall relevance of our results. Second, their larger, more spread-out cytoplasm compared to epithelial cell lines offers a clear advantage for imaging-based quantifications, as individual mitochondria and lysosomes are easier to resolve in space.

Using this system, we performed siRNA based experiments to reduce TEX2 protein level, similarly to what obtained in HeLa cells (see Methods for details). TEX2-KD fibroblasts displayed the same fission-defective mitochondrial morphology observed in HeLa KD cells (**Figure 3.13 A**). Importantly, we also observed a striking increase in mitochondria–lysosome contacts (MLCs), as revealed by the enhanced juxtaposition of TOM20- and LAMP1-positive structures (**Figure 3.13 B**). This increase is consistent with the work done by Krainc and colleagues, in which they demonstrated that lysosomes transiently dock onto mitochondria at sites of division (Wong *et al.*, 2018). Mechanistically, this process is regulated by the Rab7 cycle: the GAP TBC1D15, recruited to the OMM via Fis1, terminates Rab7 activity, thereby limiting the duration of mito–lyso interactions. When the fission machinery is dysfunctional, or when ER-derived cues that normally position and activate DRP1 are missing, Rab7 remains in its GTP-bound state for longer periods, and MLCs consequently increase in number or persistence (Wong *et al.*, 2018).

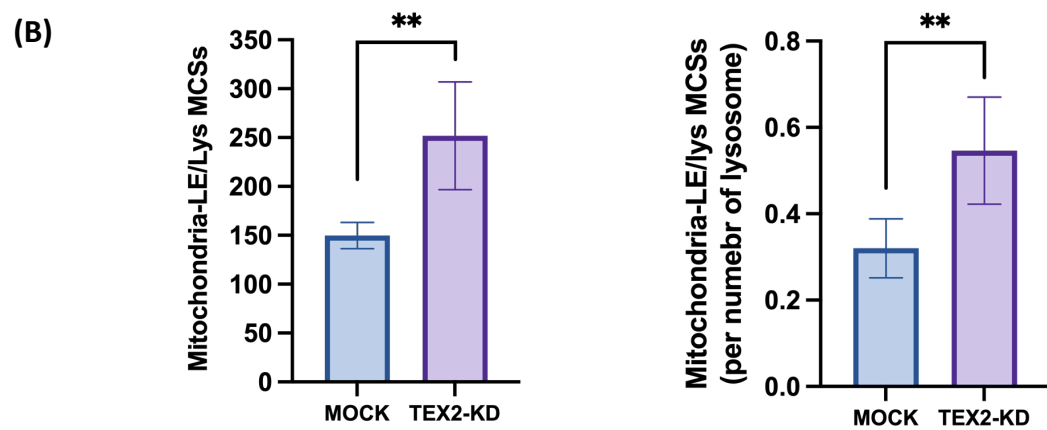
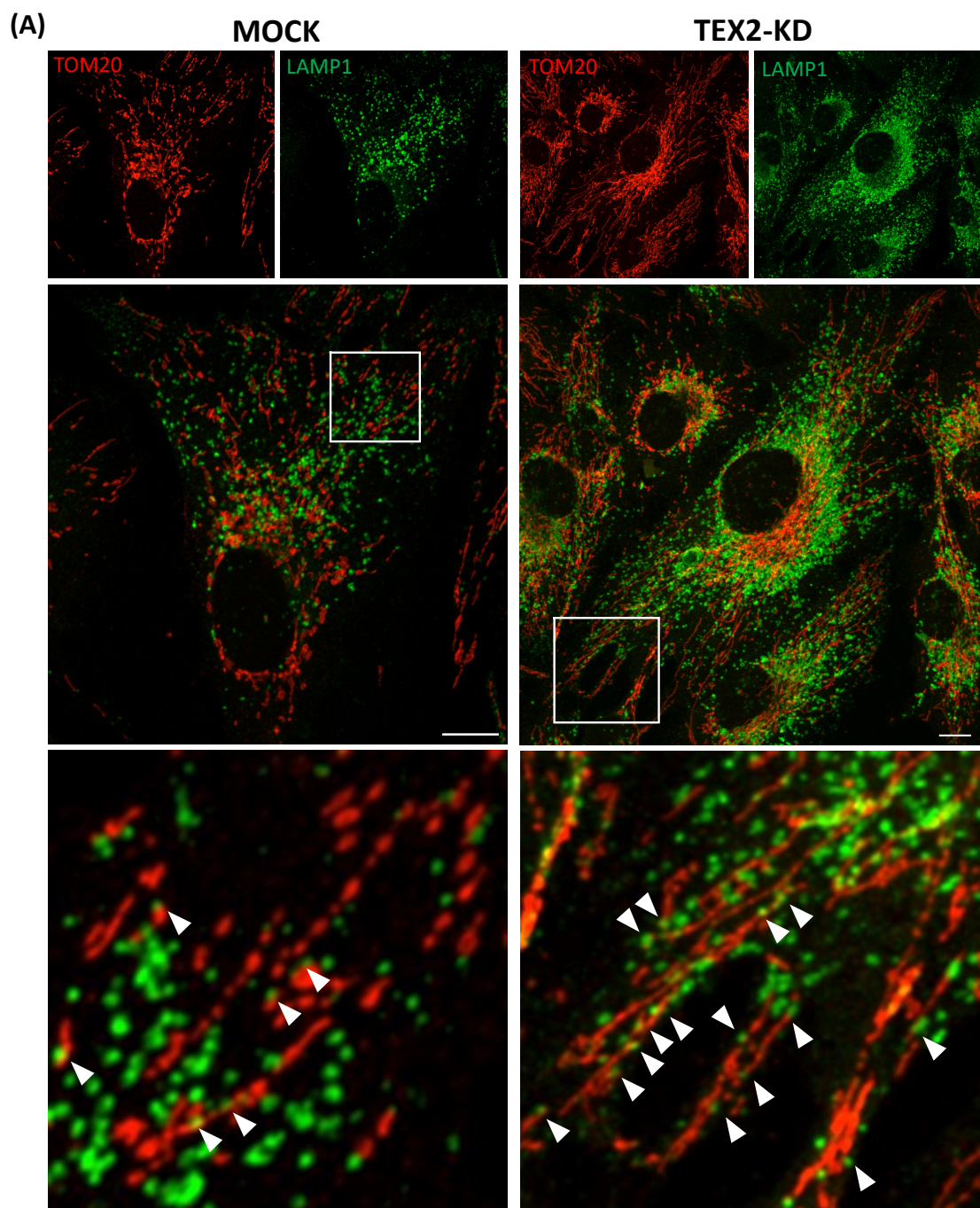


Figure 3.13. TEX2 depletion enhances mitochondria–lysosome contacts.

(A) Representative confocal images of human fibroblast-derived Bj-5ta cells, either control (MOCK) or transfected with TEX2-targeting siRNA (TEX2-KD) for 72 hours, immunostained for TOM20 (mitochondrial outer membrane marker, in red) and LAMP1 (lysosomal membrane marker, in green). Images show maximum-intensity projections of z-stacks acquired with a Zeiss LSM880 confocal microscope equipped with an Airyscan detector. Insets (dashed boxes) display higher magnification views of representative regions where mitochondria and lysosomes appear in close apposition (arrowheads), corresponding to mitochondria–lysosome membrane contact sites (MLCs). Compared to control cells, TEX2-KD cells show an increased number of TOM20–LAMP1 juxtaposition events, often at elongated mitochondrial tips, indicating stabilization of MLCs. Scale bars 10 μ m.

(B) Quantitative analysis of mitochondria–lysosome contacts in MOCK and TEX2-KD cells. Contacts were identified using an object-based colocalization approach in FIJI/ImageJ, with a distance threshold of 0 μ m between TOM20- and LAMP1-positive pixels: when the distance between the two objects is zero, we consider it as a contact between mitochondria and lysosome. Data are expressed both as the absolute number of MLCs per cell (left) and as the ratio of MLCs normalized to lysosome number (right). Values represent mean $\pm$ S.D. from 2 independent experiments (n = 30 cells per condition).

Our data in BJ-5ta fibroblasts support this interpretation. TEX2 depletion not only reduces MAMs and DRP1 activity, but also shifts the balance of the contact landscape, leading to a pronounced increase in MLCs. This suggests that when ER-mitochondria communication is compromised, lysosomes compensate by engaging more persistently with mitochondria.

Rather than being a specialized tether restricted to MAMs, TEX2 thus emerges as a more versatile organizer: its ER anchorage, lysosomal interactions, and cooperation with the OMM factor FUNDC1 place TEX2 at the crossroads of organelle crosstalk. We propose that TEX2 may locally coordinate multiple classes of contact at the same ER locus, creating microdomains where ER, mitochondria, and lysosomes converge.

3.9 TEX2 as a tether of ER-late endosome MCSs

Prompted by the observation that upon TEX2 depletion we measured an increase in mitochondria–lysosome contacts when ER-mitochondria contacts are lower, we next asked whether TEX2 actively functions as a tether at ER-lysosome junctions rather than simply being present there, for two reasons. First, in TEX2-KD cells, we observed a redistribution of contacts, characterized by a reduction in ER-mitochondria junctions and an increase in mitochondria–lysosome interfaces. This shift suggested that TEX2 may normally contribute to balancing organelle engagement, and that ER domains associated with TEX2 could possess an intrinsic ability to tether lysosomes. Second, previous studies had already identified TEX2 at ER-lysosome contacts through the lysosomal PI-phosphatase TMEM55B, suggesting that TEX2 may play a direct structural role in bridging the two membranes (Du *et al.*, 2020).

To test this, we employed a rapamycin-inducible stabilization assay, which traps pre-existing ER-lysosome contacts. In this system, FKBP is anchored to the ER (Cb5–mCherry–FKBP), and FRB is targeted to late endosomes (TMEM192–3×HA–FRB). In control cells, rapamycin treatment rapidly and robustly relocated ER membranes toward TMEM192-positive compartments, resulting in a high percentage of cells showing ER recruitment. By contrast, TEX2 knockdown markedly reduced this recruitment, demonstrating that endogenous TEX2 is required for efficient ER-lysosome tethering at steady state (**Fig. 3.14A**).

We then asked whether TMEM55B, known to interact with TEX2, acts as the lysosomal counter-receptor completing the tether. Unexpectedly, however, TMEM55B knockdown increased ER-lysosome recruitment in the reporter assay (**Fig. 3.14A**). This finding is completely unexpected and it seems apparently difficult to be reconciled with a model in which TMEM55B functions as the sole lysosomal anchor for TEX2. Several explanation can be hypothesised: one possibility is that TMEM55B is competing for the binding to several tethering molecules (TEX2, VAP proteins, MOSPDs), physically impeding to other lysosomal proteins to interact. When TMEM55B is depleted, more sites become available and larger ER-LE contact sites are formed. The other possibility is that while TEX2 is an important tethering molecule

for ER-LE contacts, TMEM55B is not the only recognized partner on LE7Ly surface. We can speculate longer on these findings, but additional experimental work is needed to address this specific issue.

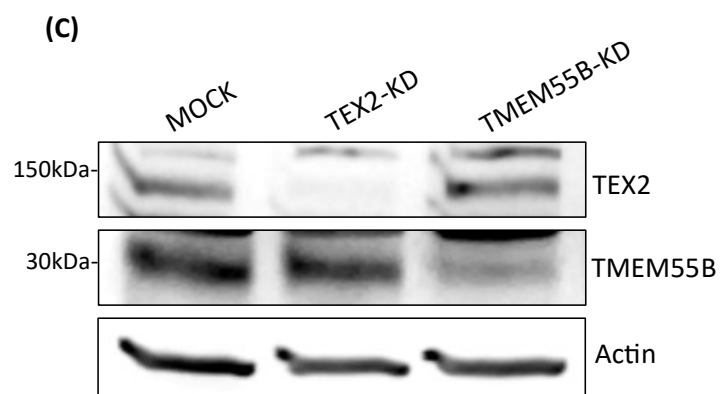
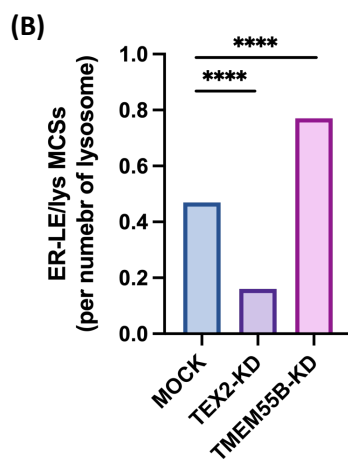
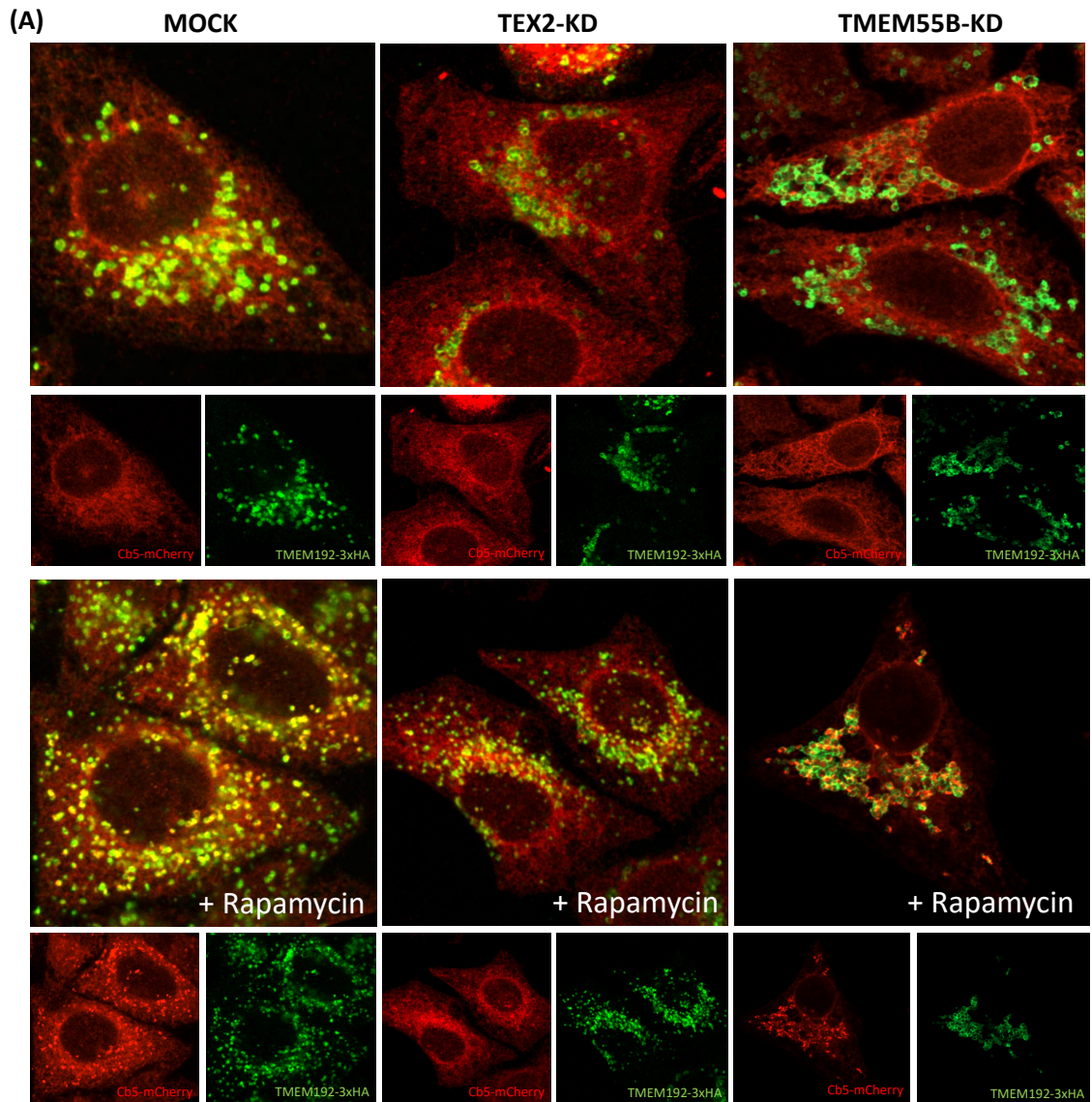


Figure 3.14 - TEX2 depletion impairs ER-lysosome tethering.

(A) Rapamycin assay for ER-lysosome contacts using Cb5 (ER, red)–mCherry–FKBP–TMEM192–3×HA–FRB (lysosomes, green) in HeLa cells. In this system, addition of 200 nM rapamycin for 4 min induces FKBP–FRB dimerization and visible relocalization of ER tubules onto lysosomal membranes. Representative confocal images show that under control conditions (MOCK), rapamycin triggers robust ER recruitment to lysosomes. In contrast, TEX2 knockdown (TEX2-KD) markedly reduces ER-lysosome tethering, whereas TMEM55B knockdown (TMEM55B-KD) enhances basal ER association with lysosomes. This data suggests that TEX2 acts as a tether at ER-LE/lys MCSs, while TMEM55B, although it has been described as an interacting partner of TEX2, doesn't function as a tethering molecule.

(B) Quantification of ER-lysosome contact formation, expressed as the fraction of lysosomes positive for ER signal following rapamycin treatment. Values are normalized to total lysosome number per cell (mean $\pm$ SEM from ≥ 3 independent experiments; ****p < 0.0001; unpaired two-tailed Student's t-test). TEX2-KD cells display a significant reduction in contact frequency, whereas TMEM55B-KD cells exhibit a corresponding increase, consistent with the phenotypes shown in (A).

(C) Immunoblot validation of knockdowns, showing efficient depletion of TEX2 and TMEM55B in HeLa cell lysates (60 μ g total protein per lane). Actin was used as a loading control.

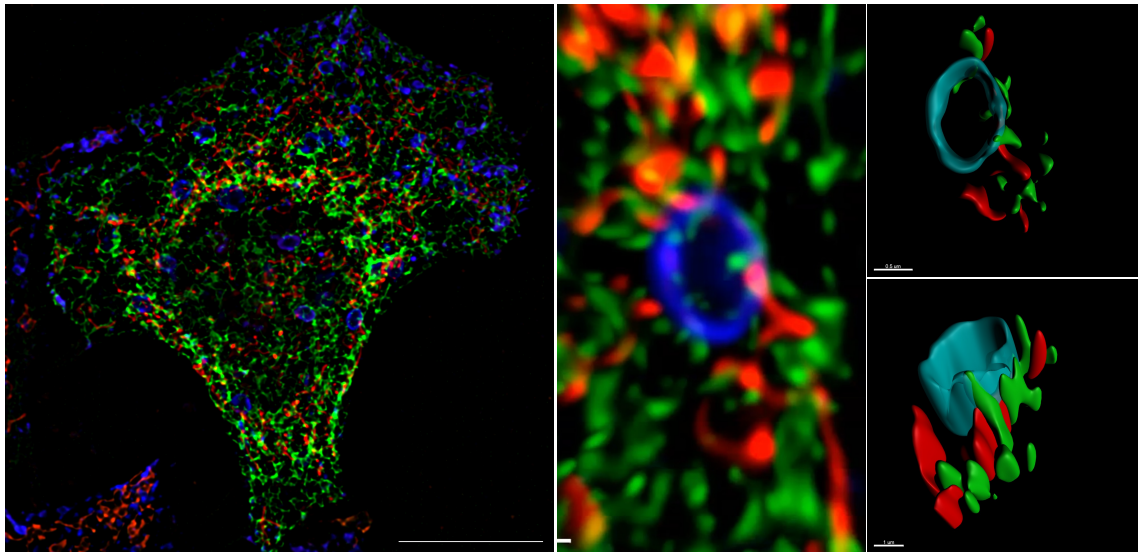
3.10 TEX2 as hub for ER-mitochondria-lysosome MCSs

We found that TEX2 supports ER-mitochondria coupling and, independently, ER-lysosome coupling. Due to this dual role, we asked whether TEX2 might also mark individual ER microdomains that simultaneously connect with mitochondria and lysosomes, thereby forming tripartite membrane contact sites. To visualize such junctions with sufficient spatial accuracy, we employed lattice structured-illumination super-resolution microscopy (Zeiss Elyra 7, Lattice SIM²), which we had previously validated in earlier chapters.

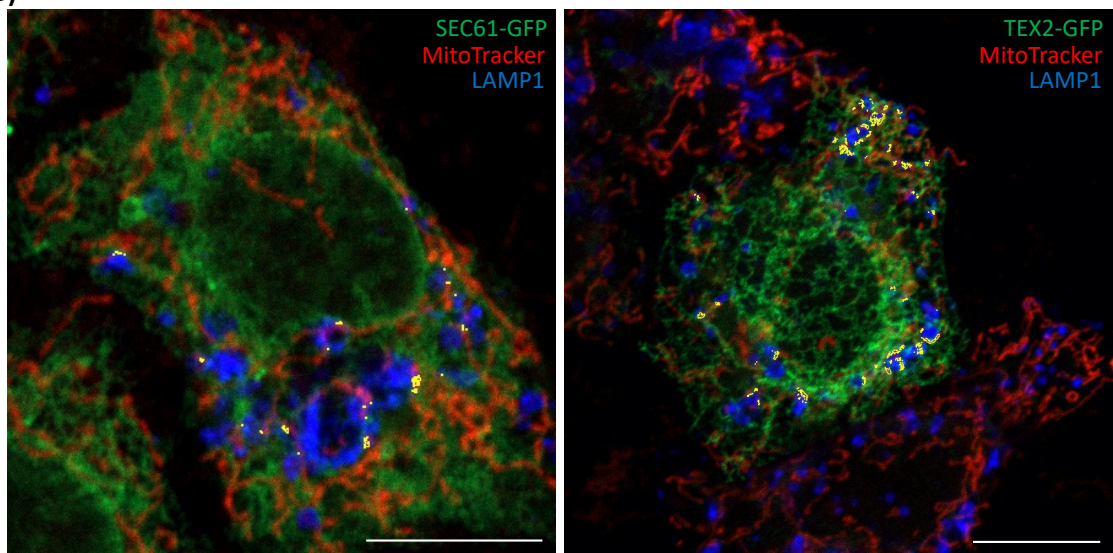
We expressed TEX2–GFP to label TEX2-positive ER microdomains and imaged mitochondria (using MitoTracker) and lysosomes (using LAMP1). Fixed-cell datasets were reconstructed in IMARIS with 3D rendering. We defined a tripartite contact as a TEX2–GFP punctum located at the interface between a mitochondrial segment and a LAMP1-positive vesicle. According to these criteria, TEX2 was consistently enriched at mito–lyso interfaces, and TEX2-positive ER strands often bridged the two organelles (**Fig. 3.15A**).

To assess whether TEX2 influences the formation of ER–mitochondria–lysosome junctions, we compared cells expressing TEX2–GFP with those expressing the general ER marker Sec61 β –GFP (**Fig. 3.15B**). Quantification was performed in three complementary ways: (i) the absolute number of tripartites per cell, (ii) the number of tripartites normalized to the total mitochondrial area (μm^2), which provides a measure of their frequency relative to the mitochondrial network, and (iii) the number of tripartites normalized to the total number of lysosomes, which reflects how often lysosomes participate in such junctions. This combined analysis allowed us to evaluate not only the overall abundance of tripartites, but also their relative likelihood when scaled to mitochondria or lysosomes (**Fig. 3.15C**).

(A)



(B)



(C)

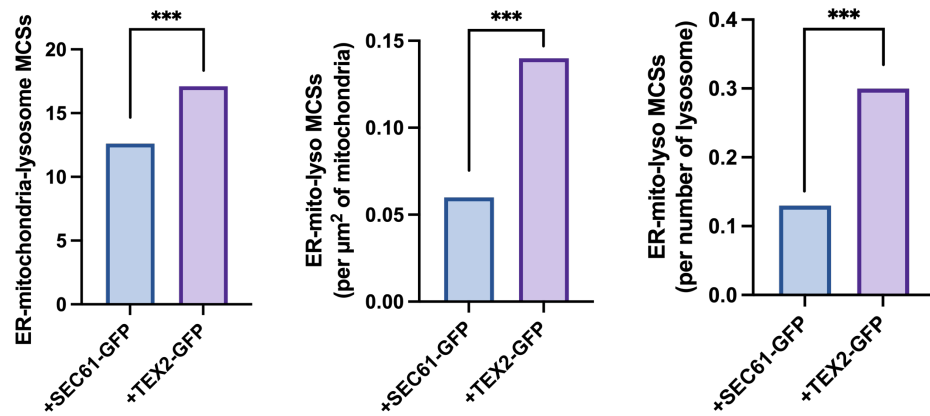


Figure 3.15 - TEX2 promotes the formation of ER–mitochondria–lysosome tripartite contacts.

(A) Lattice SIM super-resolution microscopy of HeLa cells expressing TEX2-GFP (green), stained with LAMP1 (blue) and MitoTracker (red), reveals the presence of tripartite ER–mitochondria–lysosome junctions (magnified views and 3D reconstructions generated with IMARIS are shown on the right).

(B) Representative images of cells overexpressing either the ER marker SEC61 β -GFP or TEX2-GFP, along with LAMP1 and MitoTracker. The yellow outlines indicate sites of tripartite interactions.

(C) Quantification of tripartite contacts shows a significant increase in TEX2-expressing cells compared to SEC61 β controls, normalized per cell, per mitochondrial area, and per lysosome number. These findings suggest that TEX2 not only localizes at tripartite junctions but also actively promotes their formation.

From these quantifications and normalization strategies, it became clear that the presence of TEX2 significantly increases the likelihood of forming ER-mitochondria-lysosome junctions. In absolute terms, cells expressing TEX2–GFP showed a higher number of tripartite contacts per cell compared to those expressing the general ER marker Sec61 β –GFP (**Fig. 3.15C**). When adjusted for mitochondrial area, TEX2 expression still resulted in a greater density of tripartites relative to the mitochondrial surface, indicating that TEX2 helps concentrate these structures locally rather than just reflecting differences in organelle abundance. Finally, when normalized to the number of lysosomes, TEX2-expressing cells again exhibited a significant increase, suggesting that individual lysosomes are more likely to engage simultaneously with both ER and mitochondria in the presence of TEX2 (**Fig. 3.15C**).

Taken together, these results suggest that TEX2 is not only a passive resident of tripartite junctions but rather an active organizer that promotes their formation. Its enrichment at ER subdomains appears to facilitate the coordinated recruitment of mitochondria and lysosomes to shared interaction sites, reinforcing the idea that TEX2 functions as a molecular hub for tripartite MCSs. Importantly, the stabilization of these junctions aligns with our live-cell observations, where TEX2-positive tripartites

frequently precede mitochondrial constrictions. Since lysosomes are increasingly recognized as markers of fission sites (Wong *et al.*, 2018), TEX2-mediated tripartites could serve as the structural platform where ER, mitochondria, and lysosomes converge to coordinate mitochondrial division. In this view, TEX2 not only defines the architecture of tripartite MCSs but also provides the spatial framework that positions fission machinery at the correct sites along the mitochondrial network.

Chapter 4 - Discussion

4.1 TEX2 is a hub for ER-mitochondria-lysosome MCSs

Understanding the molecular mechanisms that govern the formation and regulation of ER-mitochondria contact sites remains a central challenge in cell biology. Over the last decades, mitochondria-associated membranes (MAMs) have been described as multifunctional signalling and metabolic platforms that coordinate communication between the endoplasmic reticulum (ER) and mitochondria (Voeltz & Carvalho, 2024; Prinz *et al.*, 2020). These contact sites are not static structural junctions but dynamic regulatory hubs where lipid metabolism, calcium signalling, mitochondrial dynamics, and the initiation of autophagy converge (Prinz *et al.*, 2020, Rowland & Voeltz, 2012; Scorrano *et al.*, 2019).

An increasing number of molecular effectors have been identified to act at MAMs interface, defining the different functions occurring there. Among many functions regulated by this specific class of contacts, MAMs serve as privileged sites for calcium signalling (Csordás *et al.*, 2010; Rizzuto *et al.*, 2012). MAMs provide the structural and molecular framework that enables efficient Ca^{2+} transfer between the ER and mitochondria (Giacomello & Pellegrini, 2016). The IP_3R –GRP75–VDAC1 complex forms a physical bridge that allows direct Ca^{2+} flux from the ER lumen to the mitochondrial matrix, thereby coupling ER Ca^{2+} release to mitochondrial metabolism and ATP production (Csordás *et al.*, 2018). Additional tethers such as the VAPB–PTPIP51 complex and the SMP-domain-containing protein PDZD8 further modulate Ca^{2+} microdomains and the extent of physical coupling between the two organelles, integrating Ca^{2+} dynamics with mitochondrial function and cell survival (De Vos *et al.*, 2012; Hirabayashi *et al.*, 2017).

A key role of MAMs is the coordination of lipid synthesis and transfer from the ER (Rowland & Voeltz, 2012; Scorrano *et al.*, 2019; Prinz *et al.*, 2020; Reinisch & Prinz, 2021). Enzymes like phosphatidylserine synthase-1 and -2 (PSS1/2) and phosphatidylethanolamine N-methyltransferase (PEMT) drive key steps in the production of phosphatidylserine (PS) and phosphatidylethanolamine (PE), respectively (Kimura *et al.*, 2021; Vance, 2014). Meanwhile, lipid-transfer proteins such as ORP5 and ORP8 facilitate non-vesicular transfer of PS from the ER to mitochondria, supporting PE synthesis in the inner membrane (Vance, 2014; Monteiro-Cardoso *et al.*, 2022). MAMs are also involved in sterol metabolism through

STARD1, which moves cholesterol from the ER to mitochondria to aid steroid production and maintain mitochondrial membrane composition (Larsen *et al.*, 2020).

MAMs are also involved in the regulation of autophagy and mitophagy, acting as signaling platforms and membrane sources for the formation of autophagosomes. During stress or nutrient deprivation, MAMs recruit components of the ATG and PI3K–beclin–VPS34 complexes, including ATG5, ATG14, and BECLIN1, which nucleate the phagophore membrane and initiate autophagy (Hamasaki *et al.*, 2013; Gelmetti *et al.*, 2017).

Beyond their established role in calcium signaling, MAMs are recognized as active controllers of mitochondrial dynamics. In particular, one of the most unexpected functional roles of these contacts is their ability to promote mitochondrial fission events (Friedman *et al.*, 2011). At MAMs, ER tubules physically wrap around mitochondria, marking the sites of future division and defining the constriction geometry of the organelle (Friedman *et al.*, 2011). These ER segments locally recruit the fission GTPase DRP1 through its outer mitochondrial membrane receptors, MFF, FIS1, MID49, MID51, and FUNDC1, while also coordinating actin polymerization via ER-anchored nucleation factors such as INF2 and Spire1C (Korobova *et al.*, 2013; Chakrabarti *et al.*, 2018; Gatti *et al.*, 2025). The concerted action of these protein complexes and cytoskeletal elements generates the mechanical forces necessary to drive mitochondrial scission (Friedman *et al.*, 2011; Korobova *et al.*, 2013; Chakrabarti *et al.*, 2018; Gatti *et al.*, 2025; Kleele *et al.*, 2021). In parallel, mitofusins (MFN1/2), which mediate mitochondrial fusion, play also an additional role in tethering the two organelles: MFN2, in particular, forms homotypic or heterotypic interactions between ER and mitochondrial membranes, contributing to the maintenance and remodeling of MAMs architecture (de Brito & Scorrano, 2008; Naon *et al.*, 2016). The action of these tethering complexes together with cytoskeletal elements generates the mechanical and topological cues required for mitochondrial division (Friedman *et al.*, 2011; Korobova *et al.*, 2013; Chakrabarti *et al.*, 2018; Gatti *et al.*, 2025; Kleele *et al.*, 2021).

Lipids are key determinants of MAM function, in particular phosphatidylserine (PS), phosphatidylethanolamine (PE), cholesterol, and ceramide, which regulate membrane curvature, signaling, and protein recruitment (Vance, 2014; Liu *et al.*, 2017; MonteiroCardoso *et al.*, 2022; Reinisch & Prinz, 2021; Giacomello & Pellegrini,

2016). The PS synthesized at the ER by PSS1/2 and transferred to mitochondria through MAMs, is decarboxylated into phosphatidylethanolamine (PE) by mitochondrial PSD (Vance, 2014). This PS–PE pathway sustains mitochondrial membrane integrity and bioenergetics, and its disruption leads to defects in respiration and cristae morphology (Galmes *et al.*, 2016; Monteiro-Cardoso *et al.*, 2022). In addition, the negative charge of PS promotes the electrostatic recruitment of several fission and signaling proteins, including DRP1, INF2, and Orai1, at MAMs (Joshi *et al.*, 2018; Reinisch & Prinz, 2021). On the other hand, PE, due to its cone-shaped geometry, induces negative membrane curvature and thereby facilitates mitochondrial fission and fusion (Kleele *et al.*, 2021). Although biophysical and cell-based studies support this structural role, the precise molecular mechanism by which PE modulates membrane remodeling at MAMs remains unclear (Giacomello & Pellegrini, 2016; Vance, 2015). Cholesterol regulates membrane fluidity and permeability, influencing lipid raft formation and protein organization at MAMs (Area-Gomez & Schon, 2011). Its transfer from the ER to mitochondria by STARD1/3 and GRAMD1 proteins supports mitochondrial steroidogenesis and membrane potential, whereas excessive cholesterol accumulation at MAMs impairs respiration and autophagy (Area-Gomez & Schon, 2017; Larsen *et al.*, 2020).

Besides the recruitment of proteins and reorganization of cytoskeleton elements (Gatti *et al.*, 2025) contributing to the regulation of mitochondrial dynamics at MAMs, the lipid precise composition of MAMs required to support those events remains to be fully understood.

How are the MAMs regulated? If it is true that a lot of clues about their functions have been addressed, some of the tethering molecules and of the functional proteins able to act at their interface have been identified, the full picture of their regulation still awaits being achieved. Well-characterized lipid transfer proteins (LTPs) and tethering factors have been identified to operate at mitochondria-associated membranes (MAMs), where they coordinate lipid exchange, calcium signaling, and mitochondrial dynamics (Vance, 2014; Prinz *et al.*, 2020; Scorrano *et al.*, 2019). Among these, ORP5 and ORP8, two members of the oxysterol-binding protein-related (ORP) family initially characterized acting at ER–plasma membrane contact sites, have been shown to function at the MAM interface, where they mediate the non-vesicular transfer of PS

from the ER to mitochondria to sustain PS synthesis (Galmes *et al.*, 2016; Monteiro-Cardoso *et al.*, 2022).

A more recently characterized player acting at MAMs is PDZD8, an ER-resident protein that contains a synaptotagmin-like mitochondrial lipid-binding (SMP) domain and functions as both a tether and a lipid transfer protein (Hirabayashi *et al.*, 2017). The SMP domain of PDZD8 is homologous to SMP domain of Mmm1, the ER-resident subunit of the yeast ERMES complex, which bridges the ER and mitochondria to facilitate lipid exchange and signaling (Kornmann *et al.*, 2009; Hirabayashi *et al.*, 2017). Despite this structural similarity, PDZD8 was first identified at ER–late endosome (ER–LE) contact sites, where it regulates endosomal positioning and neurite outgrowth through an interaction with FYCO1, Rab7 and to form dynamic ER–endosome–mitochondria tripartite interfaces that coordinate lipid exchange and endosomal transport (Raiborg *et al.*, 2015; Elbaz-Alon *et al.*, 2020). Subsequent studies revealed that loss of PDZD8 protein severely affects the ER-mitochondria contacts, demonstrating its role as a tethering molecule for this class of contacts, and, consequently, resulting in the impairment of the Ca^{2+} transfer-dependent activities (Hirabayashi *et al.*, 2017; Wu *et al.*, 2018). Additional data have been provided reinforcing the hypothesis that PDZD8 exerts a crucial role in MAMs regulation, as it directly interacts with the outer mitochondrial membrane protein FKBP8, forming a tethering complex that regulates ER-mitochondria apposition (Nakamura *et al.*, 2025).

As a tethering molecule, PDZD8 not only influence the Ca^{2+} signaling started from the MAMs, but it also actively participates in the lipid transfer and remodeling of those contact sites (Hirabayashi *et al.*, 2017). It binds PS through its SMP domain (Shirane *et al.*, 2020)

All together, these findings reveal that PDZD8 is not confined to a single class of membrane contact sites, but dynamically localizes to both ER–late endosome and ER–mitochondria interfaces (Guillén-Samander *et al.*, 2019; Elbaz-Alon *et al.*, 2020). This dual distribution raises an intriguing question: does PDZD8 represent distinct functional pools specialized for different contact sites, or does it behave more like VAP proteins, whose site-specific activity is defined by the repertoire of partner proteins encountered at each interface? Such versatility would enable the cell to coordinate a

broader crosstalk between different organelle networks, fine-tuning local lipid flux and signaling in response to metabolic or stress conditions.

From a structural perspective, the SMP domain of PDZD8 plays a key role in this adaptability. PDZD8 demonstrates how SMP-domain-containing tethers can coordinate lipid transfer, Ca^{2+} regulation, and membrane remodeling across multiple organelle interfaces, adjusting their function to different physiological contexts (Reinisch & Prinz, 2021). However, the diversity of SMP-domain proteins is still only partially understood, and the molecular mechanisms linking lipid transfer to mitochondrial fission, autophagy, and MAM remodeling are still in early stages. Many SMP-domain proteins remain poorly characterized, and the mechanisms regulating MAM formation and remodeling, especially how lipid transfer is connected to mitochondrial fission and autophagy, are just beginning to be explored.

In this context, the TEX2 protein, a mammalian homolog of the yeast ceramide transporter Nvj2p, has recently attracted attention as a potential dual-function tether and lipid transfer factor acting at membrane contact sites.

We have studied TEX2, a novel ER-resident tethering and lipid transfer protein that contributes to the organization and function of MAMs. TEX2 features the general hallmarks of SMP-domain-containing lipid transfer proteins, including a predicted tubular lipid-binding domain (Liu *et al.*, 2017; Du *et al.*, 2023). However, TEX2 cannot be considered a canonical SMP protein. Structurally, the SMP domain of TEX2 diverges from other members of the TULIP superfamily by containing an extended C-terminal insertion of approximately 100 residues, absent in classical SMP proteins such as extended synaptotagmins (E-Syts) or PDZD8 (Giordano *et al.*, 2013; Guillén-Samander *et al.*, 2019). This additional segment may confer greater structural flexibility or modify the shape of the hydrophobic groove, potentially allowing the accommodation of bulkier and more hydrophobic lipids.

Importantly, the yeast homolog of TEX2, Nvj2p, has been shown to directly transfer ceramide between the ER and Golgi under stress conditions (Liu *et al.*, 2017). During ER stress or ceramide accumulation, Nvj2p relocates from the nucleus–vacuole junction to ER–Golgi contact sites, where it alleviates lipotoxicity by mediating the non-vesicular transport of ceramide (Liu *et al.*, 2017).

The human TEX2 homolog of Nvj2 can only partially compensate for the yeast protein in a genetic assay where the survival rate of yeast strains upon lipid stress was measured (Liu *et al.*, 2017). These observations, together with the extensive degree of conservation between the SMP domains of the yeast and mammalian homologues, support the hypothesis that TEX2 may possess a similar ability to bind and/or transport ceramide in mammalian cells.

What is the role of ceramide or other lipids in MAMs regulation and/or functions?

Multiple lines of evidence show that the local lipid composition of MAMs critically influences mitochondrial dynamics (Losón *et al.*, 2013; Nagashima *et al.*, 2020). Phosphatidic acid (PA), cardiolipin (CL), and ceramide have all been implicated in mitochondrial fission through distinct yet complementary mechanisms. Phosphatidic acid, a cone-shaped anionic lipid enriched at constriction sites, promotes membrane curvature and facilitates DRP1 oligomerization by directly interacting with its GTPase domain, thus enhancing mitochondrial division (Adachi *et al.*, 2016). Cardiolipin, a mitochondria-specific phospholipid, transiently redistributes from the inner to the outer membrane during fission events and directly binds to DRP1, stimulating its polymerization and GTPase activity (Bustillo-Zabalbeitia *et al.*, 2014; Macdonald *et al.*, 2014). Accumulation of ceramide at mitochondrial constriction sites promotes DRP1 recruitment and activation, likely by altering local membrane curvature and the biophysical properties of the outer mitochondrial membrane (Smith *et al.*, 2013; Ausman *et al.*, 2018; Fugio *et al.*, 2020). In light of these findings ceramide has emerged as a critical sphingolipid regulator of mitochondrial dynamics (Jamil and Cowart, 2023).

To further support the importance of ceramide content at outer mitochondria membrane, enzymes involved in ceramide metabolism, such as ceramide synthases (CerS) and neutral sphingomyelinases (nSMase), are enriched at ER–mitochondria contact sites, reinforcing the notion that this interface serves as a hub for sphingolipid synthesis and turnover (Vance, 2014; Jamil and Cowart, 2023). Studies have shown that CerS1 and its product, C18-ceramide, can induce mitophagy independently of Bak, Bax, or caspases (Sentelle *et al.*, 2012). Sentelle *et al.* found that C18-ceramide interacts directly with lipidated LC3 on the mitochondrial membrane, initiating mitophagy. Recently, it has been shown that PERMIT (Protein that Mediates ER–

Mitochondria Trafficking) functions as an essential mitochondrial trafficking chaperone that, during aging or injury-induced stress, facilitates the ER-to-mitochondria translocation of Ceramide Synthase 1 (CerS1), thereby triggering localized C18-ceramide production needed for mitophagy and mitochondrial quality control. (Oleinik *et al.*, 2025). This lipid acts as a crucial signal for the recruitment of LC3-positive autophagosomes to initiate mitophagy, serving as a lipid-mediated mechanism for mitochondrial quality control (Oleinik *et al.*, 2025). However, the precise mechanism by which ceramide is spatially organized or transferred to these sites remains to be elucidated.

Like its yeast homolog Nvj2p, TEX2 localizes primarily to ER tubules and binds ceramide through its SMP domain, as well as specific phosphoinositides via a pleckstrin homology (PH) domain (Liu *et al.*, 2017; Du *et al.*, 2023). In yeast, Nvj2p relocates from the nucleus–vacuole junction to ER–Golgi contacts under ceramide-induced stress, where it transfers ceramide to the Golgi to alleviate ER lipotoxicity (Liu *et al.*, 2017). We hypothesize that mammalian TEX2 may represent the mammalian missing link in ceramide regulation at the ER-mitochondria interface, acting as the lipid transfer protein responsible for delivering ceramide to MAMs. Through this function, TEX2 could establish a ceramide-enriched microenvironment that modulates mitochondrial dynamics and membrane remodelling.

Consistent with this hypothesis, our data demonstrate that TEX2 is a component of the ER-mitochondria interface. Immunofluorescence and subcellular fractionation analyses revealed that TEX2 is enriched at mitochondria-associated membranes, where it colocalizes with mitochondrial markers (**Fig 3.3**). Biochemical isolation of MAM fractions further confirmed the presence of TEX2 within the ER-mitochondria pool, together with canonical MAM proteins such as calnexin and VAPA (**Fig 3.4**). Functionally, loss of TEX2 profoundly affects ER-mitochondria association (**Fig 3.5**). In TEX2-depleted cells, both confocal and super-resolution imaging revealed a significant reduction in the number of MAMs, accompanied by a marked elongation of the mitochondrial network, thereby defining TEX2 as a novel tethering molecule of ER-mitochondria membrane contact sites (**Fig 3.6**)

MAMs act as platforms for mitochondrial division, where ER tubules mark constriction sites, facilitate DRP1 recruitment, and drive local lipid and actin dynamics

to drive fission (Friedman *et al.*, 2011; Korobova *et al.*, 2013; Chakrabarti *et al.*, 2018). The decrease in DRP1 phosphorylation at Ser616 and the increase in inhibitory phosphorylation at Ser637 in TEX2-depleted cells indicate that the absence of TEX2 impairs DRP1 activation, likely by disrupting the molecular or lipid environment needed for DRP1 assembly on mitochondria (**Fig 3.8**). This phenotype is similar to what is observed after decreasing the levels of other known MAM components as PDZD8, ORP5/8, or VAPB–PTPIP51, whose loss resulted in decreasing the efficiency of the mitochondrial fissioning events and contributed to a global change of the mitochondrial shape (Hirabayashi *et al.*, 2017; De Vos *et al.*, 2012; Filadi *et al.*, 2015).

To better understand how TEX2 operates and to identify its potential interaction network, we performed mass spectrometry analysis of TEX2 immunoprecipitates. Among the proteins enriched in TEX2 complexes, we detected the mitochondrial outer-membrane protein FUNDC1, a well-established regulator of mitophagy and mitochondrial dynamics at MAMs (Liu *et al.*, 2012; Wu *et al.*, 2016; Chen *et al.*, 2016; Kraus *et al.*, 2021) (**Fig 3.2**). The identification of FUNDC1 among TEX2 interactors suggests that the two proteins may functionally cooperate at ER-mitochondria interfaces. Although this approach does not directly define the localization of the TEX2 protein, the presence of a known MAM component within its interactome further confirm TEX2 localization and enrichment at ER-mitochondria contact sites.

FUNDC1 (FUN14 domain-containing protein 1) was first characterized in 2012 as a mitochondrial outer membrane protein involved in mitophagy (Liu *et al.*, 2012), and was later recognized as an important component of mitochondria-associated membranes (Wu *et al.*, 2016). Structurally, FUNDC1 contains an N-terminal cytosolic tail facing the cytoplasm and three transmembrane helices that insert into the outer mitochondrial membrane, positioning its short N-terminus to interact with cytosolic and ER-resident factors. It acts as a receptor for the autophagy protein LC3 through a conserved LIR (LC3-interacting region) motif, thereby mediating the selective removal of damaged mitochondria by mitophagy (Liu *et al.*, 2012; Chen *et al.*, 2016).

Beyond its role in mitophagy, FUNDC1 also regulates mitochondrial fission and MAM integrity. It interacts with the ER chaperone calnexin, forming a physical bridge between the two organelles that facilitates Ca²⁺ exchange and spatial coordination of fission events (Wu *et al.*, 2016). Additionally, FUNDC1 associates with the fission

GTPase DRP1, promoting its recruitment to the mitochondrial surface. These interactions are dynamically regulated by post-translational modifications: under basal conditions, FUNDC1 is phosphorylated at Ser13 by the kinase CK2 and at Tyr18 by Src, which prevents its binding to LC3 and inhibits mitophagy. During stress conditions such as hypoxia, ischemia, or mitochondrial depolarization, these inhibitory phosphorylations are removed by the mitochondrial phosphatase PGAM5, leading to dephosphorylation and activation of FUNDC1 (Liu *et al.*, 2012; Wu *et al.*, 2016; Chen *et al.*, 2016; Kraus *et al.*, 2021). Activated FUNDC1 thus functions as a signaling platform that coordinates mitochondrial fragmentation with the subsequent recruitment of the autophagic machinery. Through its simultaneous interactions with DRP1 and LC3, it couples mitochondrial division to quality control, ensuring that damaged fragments are selectively engulfed and degraded. The dual role of FUNDC1 in both fission and mitophagy positions it as a central hub at the ER-mitochondria interface, where it links membrane remodeling, metabolic signaling, and stress adaptation.

Given these properties, the identification of FUNDC1 as a TEX2 interactor provides a compelling explanation for TEX2 localization at MAMs. TEX2 may associate with FUNDC1 to access mitochondrial constriction sites or mitophagy initiation zones, where lipid transfer and membrane curvature remodeling are most dynamically regulated. This binding could integrate lipid-based mechanisms mediated by TEX2 with protein-driven pathways controlled by FUNDC1, establishing a composite tether that synchronizes membrane composition with mitochondrial fission and turnover. We still don't know whether the binding between the two proteins is direct or mediated by adaptor molecule/s, since we have observed this interaction by immunoprecipitation experiments (**Fig 3.9**). Additional studies are needed to discriminate between the two models for the two molecules to interact, so we cannot currently rule out any of them.

This context raises a key question: does FUNDC1 contribute to the maintenance of ER-mitochondria contacts at the steady state, or is its function restricted to stress-induced remodelling? To address those questions, we analyzed the impact of FUNDC1 depletion in our cell model system upon control conditions, without imposing any stress condition.

Surprisingly, at the steady state, FUNDC1 depletion phenocopies TEX2 knockdown, resulting in reducing the ER-mitochondria contact sites. In addition, elongated mitochondria were observed in FUNDC1-KO cells, indicating that FUNDC1 functions constitutively to maintain MAM integrity. Our findings provide the first evidence that FUNDC1 participates in MAM organization independently from stress (**Fig 3.9**)

Notably, the interaction between TEX2 and FUNDC1 was confirmed by co-immunoprecipitation. It has been proposed that calnexin is the ER partner of FUNDC1, although the molecular adaptor mediating their physical interaction has not yet been identified so far (Wu *et al.*, 2016). TEX2 emerges as an attractive candidate for bridging FUNDC1 and the ER membrane at the ER-mitochondria interface. Given its localization at ER-mitochondria contact sites and its structural features compatible with both tethering and lipid transfer, TEX2 might function as an adaptor protein between FUNDC1 and calnexin, stabilizing ER-mitochondria contacts and coordinating local membrane remodelling. Although our mass spectrometry analysis did not detect a direct TEX2-calnexin interaction, future targeted experiments will be necessary to confirm this hypothesis.

In addition to its role in shaping ER-mitochondria contacts, TEX2 appears to influence autophagy pathways more broadly. Consistent with the findings by Du *et al.* (2023), TEX2 depletion was associated with altered lysosomal digestive capacity and autophagic flux, suggesting that TEX2 may contribute to the regulation of membrane turnover and organelle quality control. This observation is particularly relevant in light of TEX2's interaction with FUNDC1, a well-established mitophagy receptor. FUNDC1 regulates mitochondrial quality control under stress by coupling DRP1-dependent fission with LC3-mediated autophagosome recruitment (Liu *et al.*, 2012; Chen *et al.*, 2016; Wu *et al.*, 2016). It is therefore possible that the defects in mitochondrial division observed after TEX2 knockdown partially stem from impaired mitophagy, in addition to the loss of physical ER-mitochondria coupling.

Impaired autophagy or mitophagy results in the accumulation of hyperfused mitochondria, as the inability to remove dysfunctional fragments leads to compensatory elongation and network interconnection (Gomes *et al.*, 2011; Rambold *et al.*, 2011; Mao *et al.*, 2013). A similar mechanism may partially contribute to the elongated phenotype observed in TEX2-depleted cells, where reduced contact

formation and defective mitochondrial turnover may cooperate to stabilize oversized organelles. In this scenario, TEX2 and FUNDC1 might function within the same regulatory axis, linking lipid transfer and contact site maintenance to mitochondrial quality control efficiency.

An intriguing, alternative possibility is that the relationship between MAM integrity and mitophagy is bidirectional. A primary defect in mitophagy might alter MAM formation by affecting the lipid composition or biophysical properties of the outer mitochondrial membrane, thereby resulting in changes in the affinity properties of tethering factors for the organellar surface. This feedback mechanism may represent the connection between structural and degradative pathways in maintaining mitochondrial homeostasis. Further experiments will be performed to clarify this point (**Fig 4.1**).

In summary, identifying FUNDC1 as a TEX2 interactor creates a functional link between lipid metabolism, mitophagy and mitochondrial dynamics, connecting a lipid transfer component with the core fission machinery. FUNDC1 acts as a mitochondrial receptor for DRP1, facilitating its recruitment to the outer membrane, whose lipid composition strongly influences DRP1 binding and activation (Wu *et al.*, 2016; Kraus *et al.*, 2021). In this context, TEX2 is ideally positioned to modulate the lipid landscape through its SMP domain at ER-mitochondria contact sites, thereby controlling the efficiency of FUNDC1–DRP1 complex formation.

Based on its homology with the yeast ceramide transporter Nvj2p, we propose that TEX2 mediates ceramide delivery at MAMs, generating lipid nanodomains that stabilize FUNDC1 and facilitate DRP1 recruitment and activation. In this model, TEX2 acts upstream as a lipid provider and structural tether, whereas FUNDC1 functions as the effector translating these lipid cues into mechanical constriction and fission signaling. Supporting this view, TEX2 depletion leads to reduced DRP1 phosphorylation at Ser616 and increased inhibitory phosphorylation at Ser637, consistent with a failure to activate the fission machinery (**Fig 4.1**). Moreover, loss of TEX2 compromises MAM integrity and impairs DRP1-dependent fission, while re-expression of wild-type TEX2, but not SMP-domain mutants, rescues both MAM formation and mitochondrial morphology. These findings demonstrate that the lipid transfer activity of TEX2 is essential for its function and support a model in which

TEX2 serves as a lipid-dependent scaffold ensuring the proper alignment and activation of FUNDC1–DRP1 complexes at MAMs, thereby coupling ceramide flux to mitochondrial fission efficiency even under basal conditions. This integrated model suggests a dual role for TEX2: as an ER-mitochondria tether that maintains MAM structure, and as a lipid transfer protein that creates ceramide-enriched nanodomains necessary for effective mitochondrial fission. Future studies involving MAM lipidomics, in vitro ceramide transfer assays, and live imaging of DRP1 recruitment will help clarify how this lipid–protein interaction governs the timing and efficiency of mitochondrial fission.

Beyond ceramide, our results suggest that TEX2 also influences additional lipid pathways at MAMs. In particular, fluorescent lipid probes revealed a reduction of mitochondrial PE levels (TopFluor-PE) and an accumulation of phosphatidic acid (PA) at lysosomes (Opi1 sensor) upon TEX2 depletion (**Fig 3.11**). Since PE synthesis in mitochondria depends on the import of its ER precursor phosphatidylserine (PS), this observation may reflect a defect in PS transfer or conversion at MAMs, consistent with the reported ability of TEX2 to bind PS in addition to ceramide (Du *et al.*, 2023). However, it remains unclear whether the decreased PE signal results directly from a loss of TEX2-mediated lipid transfer or indirectly from reduced ER-mitochondria contacts. Further experiments, such as targeted lipidomics and in vitro reconstitution assays, will be required to discriminate between these possibilities. In either case, the data indicate that TEX2 plays an important role in maintaining the local lipid composition of MAMs, a prerequisite for the curvature and membrane plasticity required for DRP1-driven fission.

The increased PA signal detected at lysosomes is particularly intriguing, as PA is a cone-shaped lipid that regulates membrane curvature and dynamics at multiple contact sites (Adachi *et al.*, 2016). Its accumulation might indicate a rerouting of lipid fluxes toward the ER-lysosome interface when TEX2 is absent, potentially linking defective MAM organization to altered mitochondria–lysosome communication. This observation prompted us to investigate whether the loss of TEX2 also affects mitochondria–lysosome contacts (MLCs), which coordinate the late stages of mitochondrial fission and turnover (Wong *et al.*, 2018; Peng *et al.*, 2020).

We found that TEX2 loss increases mitochondria–lysosome contacts (MLCs), a phenotype consistently observed in models with defective mitochondrial fission (**Fig 3.13**) (Wong *et al.*, 2018; Kleele *et al.*, 2021). MLCs are transient structures regulated by the lysosomal GTPase Rab7 and its GAP TBC1D15, which coordinates the untethering of lysosomes from mitochondria after DRP1-driven scission (Peng *et al.*, 2020). When fission efficiency is reduced, lysosomes can remain attached to mitochondrial tips, leading to MLC buildup. This raises the question of whether the MLC accumulation observed upon TEX2 depletion represents an independent phenotype or rather a secondary effect due to the loss of ER-mitochondria coupling.

One possibility is that reduced ER-mitochondria contact frequency delays DRP1-mediated constriction, allowing lysosomes to remain tethered to mitochondria for longer periods as part of a compensatory quality-control response. In this view, the expansion of MLCs would arise indirectly from impaired fission, consistent with the role of lysosomes in the terminal stages of mitochondrial division and mitophagy (Peng *et al.*, 2020). Alternatively, TEX2 might directly influence the establishment of MLCs, independent of its role at MAMs: TEX2 has been detected at ER-lysosome interfaces (Du *et al.*, 2023), and its depletion could alter the lipid composition or dynamics of lysosomal membranes, thereby indirectly promoting prolonged interaction between lysosomes and mitochondria. Although additional work is needed to determine whether MLC accumulation is a direct or indirect outcome of TEX2 loss, our data indicate that TEX2 functions at the interface of multiple contact types.

TEX2 also localizes to ER–late endosome/lysosome interfaces, where it interacts with the late endosomal phosphatase TMEM55B (Du *et al.*, 2023). TMEM55B is a PI(4,5)P₂ 4-phosphatase that converts phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂] into phosphatidylinositol-5-phosphate [PI(5)P], thereby regulating endosomal membrane identity, maturation, and autophagy (Puertollano R *et al.*, 2024 Araki M *et al.*, 2021; Takemasu S *et al.*, 2019). It also contributes to endosome–lysosome trafficking and cholesterol export, linking phosphoinositide metabolism to lysosomal homeostasis (Medina MW *et al.*, 2014).

Our data extend these observations by showing that TEX2 functions as a tether at ER-lysosome interfaces, suggesting a broader role for TEX2 in coordinating inter-organelle communication. However, TMEM55B knockdown does not mimic TEX2

depletion, as it does not produce the significant changes in organelle coupling and morphology seen when TEX2 is depleted (**Fig 3.14**). This discrepancy suggests that TEX2-mediated ER-lysosome tethering requires additional, yet unidentified partners that stabilize the complex and possibly confer specificity to the contact interface.

Given that TEX2 depletion leads to both reduced MAM formation and increased mitochondria–lysosome contacts, it is tempting to speculate that TEX2 coordinates lipid flux and structural communication between these interfaces. By dynamically partitioning between ER-mitochondria and ER-lysosome contact sites, TEX2 may act as a central node that synchronizes lipid exchange and organelle remodeling across interconnected contact networks. Identifying the molecular components that cooperate with TEX2 at ER-lysosome contacts will be essential to fully understand how this coordination is achieved.

Together, these findings allow us to propose a working model where TEX2 and FUNDC1 form a functional ER-mitochondria tethering complex that coordinates lipid transfer, mitochondrial division, and quality control (**Fig 4.1**). By binding and possibly transferring ceramide and other sphingolipids at contact sites, TEX2 may influence the local lipid landscape and membrane curvature, thereby facilitating FUNDC1-dependent recruitment of DRP1 and promoting mitochondrial fission. At the same time, TEX2 activity at ER-lysosome interfaces could enable the exchange of lipids or signaling molecules, supporting mitochondrial turnover or stress response. Without TEX2, reduced ceramide transfer and disrupted MAM structure would impair FUNDC1 signaling, poorly activate DRP1, and prolong mitochondria–lysosome contacts. This model identifies the TEX2–FUNDC1 pathway as a key component of a tripartite communication system linking lipid metabolism with mitochondrial dynamics. Notably, TEX2 shares conceptual similarities with PDZD8, another SMP-domain tether that operates at multiple contact types (ER–mitochondria, ER–endosome, and ER–lysosome), suggesting that a subset of SMP-domain proteins acts as multi-interface organizers that dynamically coordinate organelle communication depending on cellular state and metabolic demand.

Beyond their cellular roles, alterations in the architecture or regulation of MAMs have been increasingly linked to human disease. Defects in key tethering proteins compromise mitochondrial dynamics, bioenergetics, and quality control, resulting in a

broad spectrum of pathologies. Mutations in MFN2, a core component of the mitochondrial fusion machinery and ER–mitochondria tether, cause Charcot–Marie–Tooth disease type 2A, a peripheral neuropathy characterized by axonal degeneration (Zaman *et al.*, 2022). Similarly, loss or mutation of VAPB or its binding partner PTPIP51 disrupts ER-mitochondria coupling and contributes to amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (Paillusson *et al.*, 2016). Dysfunction of PDZD8, a multi-contact SMP-domain protein, has been implicated in neurodevelopmental and psychiatric disorders, consistent with its role in calcium homeostasis and synaptic activity (Janikiewicz *et al.*, 2018; Truong *et al.*, 2025). Moreover, FUNDC1 has attracted increasing attention for its roles in mitophagy and mitochondrial quality control. Alterations in FUNDC1 signaling have been linked to cardiac ischemia-reperfusion injury, metabolic syndrome, and tumor progression (Chen *et al.*, 2016; Tan *et al.*, 2022; Bai *et al.*, 2023). Moreover, genetic variants in FUNDC1 have recently been associated with a Kabuki-like congenital syndrome, highlighting the pathological consequences of impaired mitophagy and ER-mitochondria communication in human development (Khan *et al.*, 2023, Clin Genet).

Beyond advancing our fundamental understanding of membrane contact sites, these insights could provide a framework for targeting these sites as a therapeutic approach. Adjusting lipid flux or restoring proper tethering at MAMs might improve mitochondrial dysfunction and cellular stress responses disease. By clarifying the role of TEX2 within this complex network, our study contributes to understanding how the coordination among the ER, mitochondria, and lysosomes sustains cellular homeostasis and opens possibilities for future research on MAMs as diagnostic and therapeutic targets in mitochondrial medicine.

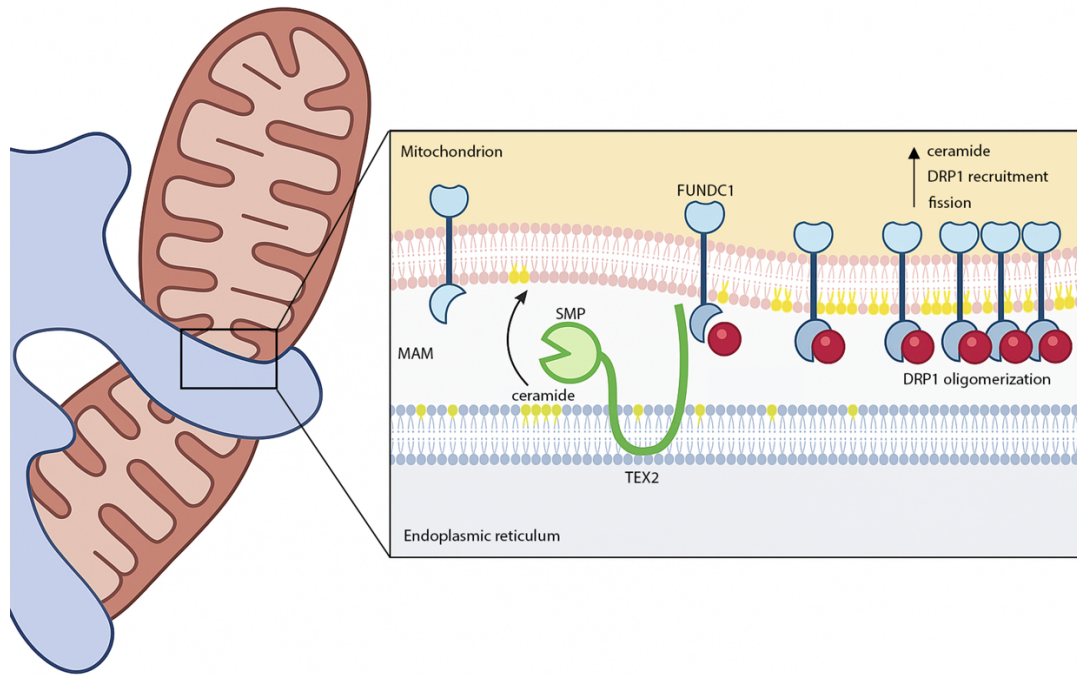


Figure 4.1- TEX2-mediated lipid transfer at ER-mitochondria contact sites regulates DRP1-dependent mitochondrial fission.

Schematic representation of the proposed model for TEX2 function at mitochondria-associated membranes (MAMs). TEX2, an ER-resident SMP-domain-containing tether, mediates the non-vesicular transfer of ceramide from the endoplasmic reticulum to the mitochondrial outer membrane. Through its SMP domain, TEX2 shuttles ceramide molecules across the MAM interface, contributing to the local enrichment of cone-shaped lipids that promote negative membrane curvature. This lipid remodeling facilitates the recruitment of the mitochondrial receptor FUNDC1 and enhances DRP1 oligomerization at the constriction sites, ultimately promoting mitochondrial fission. In this model, TEX2 serves as a lipid-transfer hub that couples ER lipid metabolism with mitochondrial division, linking the structural and metabolic functions of MAMs. The ceramide-dependent modulation of curvature may thus represent a mechanistic step through which ER-mitochondria contacts coordinate membrane remodeling and organelle dynamics.

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