

# ER–Mitochondria contact sites: A new regulator of cellular calcium flux comes into play

Michiel Krols,<sup>1,2</sup> Geert Bultynck,<sup>3,4</sup> and Sophie Janssens<sup>5,6</sup>

<sup>1</sup>Peripheral Neuropathy Group, VIB Department of Molecular Genetics, University of Antwerp, 2610 Antwerpen, Belgium

<sup>2</sup>Neurogenetics Laboratory, Institute Born-Bunge, 2610 Antwerpen, Belgium

<sup>3</sup>Laboratory of Molecular and Cellular Signaling, Department of Cellular and Molecular Medicine and <sup>4</sup>Leuven Kanker Instituut, KU Leuven, 3000 Leuven, Belgium

<sup>5</sup>Immunoregulation and Mucosal Immunology Unit, Inflammation Research Center, VIB-UGent, BE-9030 Ghent, Belgium

<sup>6</sup>Department of Internal Medicine, Universiteit Gent, BE-9030 Ghent, Belgium

Endoplasmic reticulum (ER)–mitochondria membrane contacts are hotspots for calcium signaling. In this issue, Raturi et al. (2016. *J. Cell Biol.* <http://dx.doi.org/10.1083/jcb.201512077>) show that the thioredoxin TMX1 inhibits the calcium pump SERCA2b at ER–mitochondria contact sites, thereby affecting ER–mitochondrial calcium transfer and mitochondrial bioenergetics.

Ca<sup>2+</sup> flux from the ER to mitochondria is a major determinant of several mitochondrial processes. Basal Ca<sup>2+</sup> oscillations drive mitochondrial metabolism for the production of ATP and mitochondrial substrates used in anabolic processes (Cárdenas et al., 2010; Fig. 1). In contrast, mitochondrial Ca<sup>2+</sup> overload can lead to mitochondrial permeability transition pore opening and subsequent cell death (Rizzuto et al., 2012). Dysregulation of Ca<sup>2+</sup> fluxes is involved in several human disorders (Rizzuto et al., 2012; Marchi et al., 2014; Krols et al., 2016), and its tight regulation is therefore crucial. This regulation is achieved by local modulation of Ca<sup>2+</sup> transport systems, the tightness of the association between ER and mitochondrial membranes and the ER Ca<sup>2+</sup> load. The ER Ca<sup>2+</sup> load is determined by ER Ca<sup>2+</sup> uptake systems (sarco-ER Ca<sup>2+</sup> transport ATPases [SERCAs] with the ubiquitously expressed SERCA2b as the housekeeping isoform) and intraluminal Ca<sup>2+</sup>-binding proteins like calreticulin, calnexin (CNX), and GRP78/BiP, which maintain high Ca<sup>2+</sup> levels in the ER. Activation of the intracellular Ca<sup>2+</sup> release channels inositol 1,4,5-trisphosphate receptors (IP<sub>3</sub>Rs) on the ER membrane leads to stimulation-induced Ca<sup>2+</sup> release from the ER. At membrane contact sites between the ER and mitochondria (Fig. 1), where the ER and mitochondrial membranes are held in close proximity through protein tethers (Krols et al., 2016), specialized ER microdomains known as the mitochondria-associated membranes (MAM) are enriched in IP<sub>3</sub>Rs. IP<sub>3</sub>Rs are physically connected to voltage-dependent anion channels type 1 (VDAC1) in the outer mitochondrial membrane via the chaperone Grp75 (Szabadkai et al., 2006; Fig. 1). As a consequence, the local Ca<sup>2+</sup> concentration rises to higher levels within these microdomains than in the cytosol, creating Ca<sup>2+</sup> hotspots needed to overcome the low-affinity Ca<sup>2+</sup> uptake properties of the mitochondrial Ca<sup>2+</sup> uniporter complex (Rizzuto et al., 2012).

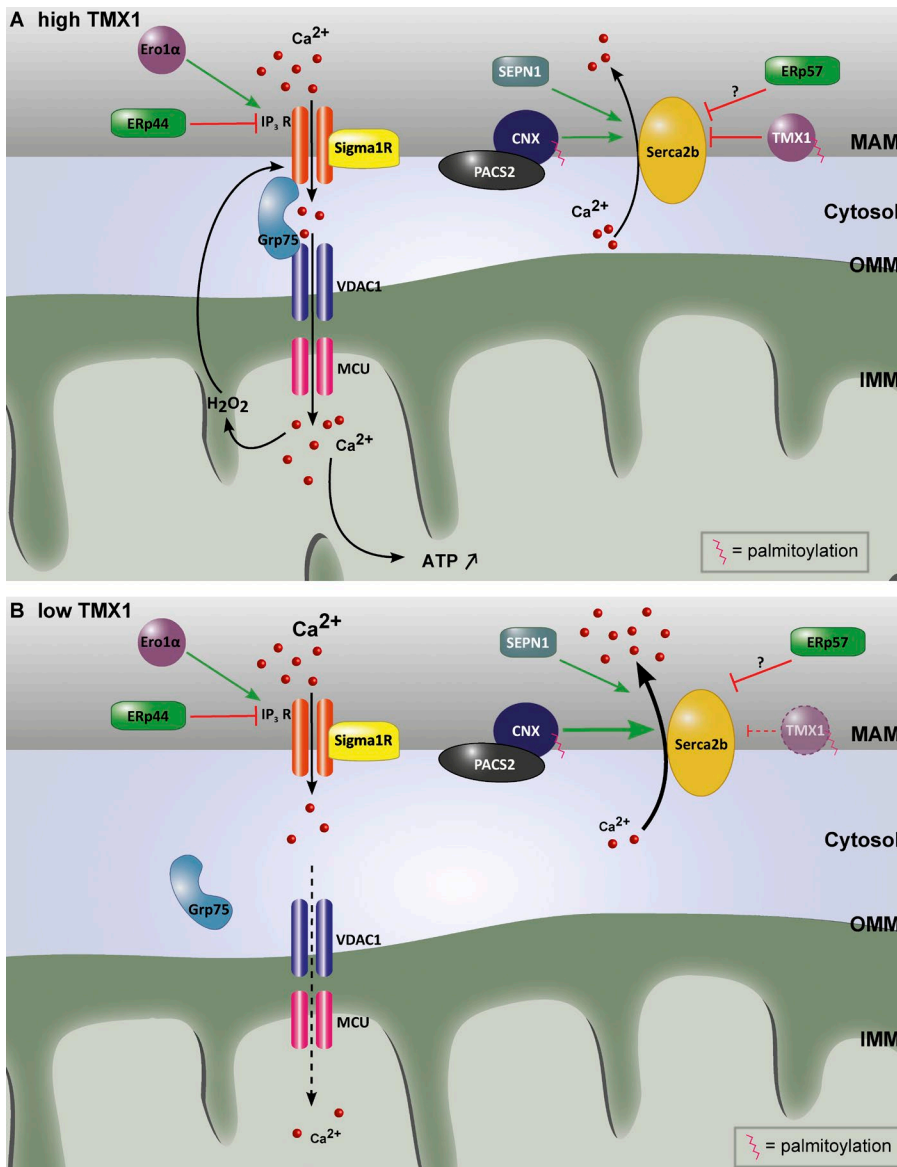
The activity of Ca<sup>2+</sup>-transport systems like SERCA and IP<sub>3</sub>Rs is controlled by several interactors. The oxidoreductases Ero1 $\alpha$  and ERp44 directly interact with IP<sub>3</sub>Rs and modulate its Ca<sup>2+</sup>-flux properties in a redox-sensitive manner (Higo et al., 2005; Anelli et al., 2012; Fig. 1). In addition, the Sigma1 receptor, which is enriched at MAMs, is released from the Ca<sup>2+</sup>-dependent chaperone BiP/GRP78 and promotes prolonged ER calcium release through the stabilization of IP<sub>3</sub>R3 when ER Ca<sup>2+</sup> content is low (Hayashi and Su, 2007; Fig. 1). SERCA2b activity is modulated by CNX, which is targeted to the MAM in a palmitoylation and phosphofurin acidic cluster sorting protein 2–dependent manner (Roderick et al., 2000; Lynes et al., 2012, 2013). Similar to IP<sub>3</sub>Rs, SERCA2b is sensitive to the redox state of the ER lumen. SERCA2b harbors cysteines in its fourth luminal loop (L4), and oxidation of these cysteines by ERp57 inhibits SERCA2b activity (Li and Camacho, 2004; Fig. 1), though this conclusion has been challenged (Appenzeller-Herzog and Simmen, 2016). Moreover, the redox-sensitive protein SEPN1 was recently shown to bind to the SERCA2b L4 cysteines and to enhance its ER Ca<sup>2+</sup> uptake activity, which protects cells against the reactive oxygen species (ROS) produced during oxidative protein folding (Marino et al., 2015; Fig. 1) In this issue, Raturi et al. describe thioredoxin-related transmembrane protein 1 (TMX1) as a novel SERCA2b-inhibiting protein at ER–mitochondria membrane contact sites, thereby providing new insights into SERCA2b regulation (Raturi et al., 2016; Fig. 1).

TMX1 belongs to the family of protein disulfide isomerases and consists of an ER luminal domain that harbors a CXXC reductase active site, a single transmembrane domain, and a cytosolic stretch that contains a palmitoylation motif, required for targeting and/or retention of TMX1 at the MAM (Lynes et al., 2012). Raturi et al. (2016) show that loss of TMX1 in cultured cells increases ER Ca<sup>2+</sup> uptake and enhances cytosolic Ca<sup>2+</sup> clearance. Interestingly, the impact of TMX1 on SERCA2b was partially antagonized by the SERCA2b regulator CNX (Roderick et al., 2000; Lynes et al., 2013). Consequently, TMX1–SERCA2b complexes were more prominent in CNX-deficient cells, and vice versa. Yet, it remains unclear how each of these proteins modulates SERCA2b activity and

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Correspondence to Sophie Janssens: [sophie.janssens@irc.vib-ugent.be](mailto:sophie.janssens@irc.vib-ugent.be)





**Figure 1. The impact of TMX1 on  $\text{Ca}^{2+}$  signaling at the ER-mitochondria interface.** The physical linkage of the ER-located IP<sub>3</sub>R channels and outer mitochondrial membrane-located VDAC1 channels establish “quasi-synaptic” transfer of  $\text{Ca}^{2+}$  from the ER to the mitochondria, where  $\text{Ca}^{2+}$  is imported into the mitochondrial matrix via the mitochondrial  $\text{Ca}^{2+}$  uniporter (MCU) complex. Mitochondrial  $\text{Ca}^{2+}$  transients are essential to stimulate ATP synthesis through the electron transport chain, whereas mitochondrial  $\text{Ca}^{2+}$  overload triggers apoptosis. Release of  $\text{Ca}^{2+}$  from the ER toward mitochondria is controlled by different factors, including redox-sensitive chaperones within the ER lumen, such as Ero1 $\alpha$ , ERp44, GRP78/BiP, and Sigma1 receptor. Mitochondrial  $\text{Ca}^{2+}$  transients are able to trigger  $\text{H}_2\text{O}_2$  release from mitochondrial cristae, stimulating ER  $\text{Ca}^{2+}$ -release events. In most cells, ER  $\text{Ca}^{2+}$  uptake is mediated by SERCA2b. The activity of SERCA2b is tightly regulated, and ER  $\text{Ca}^{2+}$  loading activity affects ER-mitochondrial  $\text{Ca}^{2+}$  flux. CNX is targeted to the MAM by palmitoylation and phosphofurin acidic cluster sorting protein 2 (PACS2) and stimulates SERCA2b activity. Similarly, SEPN1 positively modulates ER  $\text{Ca}^{2+}$  uptake through SERCA2b. Raturi et al. (2016) add TMX1 to the SERCA2b regulatory network: TMX1 is targeted to ER-mitochondria contact sites through palmitoylation. TMX1-SERCA2b complex formation is enhanced by ROS and is antagonized by CNX. TMX1 binds SERCA2b, causing its inhibition. (A) In conditions of high TMX1 expression, TMX1 causes strong SERCA2b inhibition. This leads to an augmented  $\text{Ca}^{2+}$  flux to the mitochondria after IP<sub>3</sub>-dependent stimulation of the cells. (B) In condition of low TMX1 expression, e.g., in cancer cells, ER  $\text{Ca}^{2+}$  uptake rates are increased and ER-mitochondria contacts are decreased. The increase in SERCA2b activity together with the increase in overall distance between ER and mitochondria, which may impact IP<sub>3</sub>R-GRP75-VDAC1 complex formation, limits ER-mitochondrial  $\text{Ca}^{2+}$  transfer and mitochondrial TCA cycling. This mechanism could contribute to the Warburg effect in cancer cells.

how they influence each other. At the functional level, TMX1 was proposed to inhibit SERCA2b based on the fact that loss of TMX1 led to an increased ER  $\text{Ca}^{2+}$  uptake rate and a corresponding increase in ER  $\text{Ca}^{2+}$ -store content, measured with genetically encoded ER-targeted  $\text{Ca}^{2+}$ -sensitive biosensors. However, further work is needed to assess TMX1's effect on SERCA2b activity, by using more direct SERCA2b activity assays like ATPase measurements in microsomes of SERCA2b-overexpressing cells expressing or lacking TMX1, or liposome-based systems using purified SERCA2b and TMX1, if feasible. Alternatively, changes in ER  $\text{Ca}^{2+}$ -leak pathways and/or intraluminal  $\text{Ca}^{2+}$ -buffering proteins could partially account for the observed TMX1 effects. Strikingly, cells lacking TMX1 displayed a reduced increase in cytosolic  $[\text{Ca}^{2+}]$  and “relative” drop in ER  $\text{Ca}^{2+}$  levels in response to extracellular agonists, despite a higher ER  $\text{Ca}^{2+}$  store content. This was explained by the enhanced ability of the ER to retain  $\text{Ca}^{2+}$  through the activation of SERCA2b. A challenging aspect of this model is the difference of several orders of magnitude in the kinetics of  $\text{Ca}^{2+}$  transport between fast  $\text{Ca}^{2+}$  channels like IP<sub>3</sub>R and slow  $\text{Ca}^{2+}$  pumps like SERCA. It is possible that this imbalance is compensated

for by a high local concentration of SERCA2b pumps over IP<sub>3</sub>R channels, e.g., in ER-mitochondria microdomains, or by very short opening times of IP<sub>3</sub>Rs.

As both TMX1 and CNX are enriched at the MAM and both their interactions with SERCA2b depend on their MAM-targeting palmitoylation motif (Lynes et al., 2013; Raturi et al., 2016), Raturi et al. (2016) hypothesized that TMX1 locally regulates SERCA2b, thereby impacting ER-mitochondrial  $\text{Ca}^{2+}$  flux. Indeed, cells overexpressing wild-type TMX1 showed an augmented flux of  $\text{Ca}^{2+}$  to the mitochondria after IP<sub>3</sub>-dependent stimulation of the cells (Fig. 1 A), whereas cells with lowered TMX1 levels displayed a diminished mitochondrial  $\text{Ca}^{2+}$  flux (Fig. 1 B). Though the modulation of ER-mitochondrial  $\text{Ca}^{2+}$  flux by TMX1 could be related to its effects on SERCA2b activity, other factors are likely involved as well. Indeed, Raturi et al. (2016) demonstrated that the ER and mitochondria in TMX1-deficient cells show a lower degree of tight association, which is a well-established determinant of ER-mitochondrial  $\text{Ca}^{2+}$  transfer (Csordás et al., 2006). How TMX1 affects ER-mitochondrial coupling is currently unclear. TMX1 may be involved in a protein complex tethering both

organelles or may impact the expression of ER–mitochondria tethering proteins. An intriguing alternative could be that it is the ER–mitochondria membrane association itself that is sensitive to ER Ca<sup>2+</sup> levels or local [Ca<sup>2+</sup>] in the ER–mitochondria interspace. This may negatively impact the formation of IP<sub>3</sub>R–GRP75–VDAC1 complexes, which are responsible for efficient ER–mitochondrial Ca<sup>2+</sup> transfer (Fig. 1 B). Loss of TMX1 leads to increased ER Ca<sup>2+</sup> content, thereby increasing the likelihood for proapoptotic Ca<sup>2+</sup> transfers into the mitochondria under certain conditions (Rizzuto et al., 2012). Therefore, it is possible that a decrease in the overall tight ER–mitochondria contacts constitutes a compensatory mechanism that is needed for cell survival in the absence of TMX1.

The augmented Ca<sup>2+</sup> flux in cells overexpressing TMX1 was abolished upon expression of the palmitoylation-deficient or thioredoxin mutants of TMX1, which fail to interact with SERCA2b at the MAM (Raturi et al., 2016). Consistent with the essential role of the thioredoxin motif of TMX1 for SERCA2b binding, the interaction of TMX1 with SERCA2b was sensitive to the ER lumen redox state (Raturi et al., 2016). Chemically induced hyperoxidation promoted TMX1 targeting to the MAM and the TMX1–SERCA2b association (Raturi et al., 2016), whereas reducing conditions had the opposite effects. Hence, it is possible that TMX1, like SEPNI, interacts with the L4 cysteines in SERCA2b. However, the individual contributions of the SERCA2b interaction partners, including SEPNI and CNX, and their interplay at steady-state or in varying redox-state conditions remain unclear.

Raturi et al. (2016) further underscore the emerging concept that Ca<sup>2+</sup> signaling and the luminal redox state of the ER are intertwined, especially at the MAMs. At the ER luminal side, ER Ca<sup>2+</sup> release and uptake mechanisms are directly modulated by redox-sensitive chaperones and oxidoreductases. In contrast, ER Ca<sup>2+</sup> levels regulate the activity of BiP, CNX, and calreticulin, proteins involved in oxidative protein folding. In addition, a close interconnection exists between Ca<sup>2+</sup> and ROS at the ER–mitochondrial junction. Using an H<sub>2</sub>O<sub>2</sub> sensor targeted to this interface, H<sub>2</sub>O<sub>2</sub> has been shown to be transferred from the mitochondrial intermembrane space to the ER–mitochondrial interface (Booth et al., 2016). H<sub>2</sub>O<sub>2</sub> release is stimulated by ER Ca<sup>2+</sup> release, and, in turn, these H<sub>2</sub>O<sub>2</sub> transients are able to modulate ER Ca<sup>2+</sup> discharge, as blocking H<sub>2</sub>O<sub>2</sub> release abolished normal Ca<sup>2+</sup> oscillations (Booth et al., 2016; Fig. 1 A). It is currently unclear how mitochondria-derived H<sub>2</sub>O<sub>2</sub> influences ER Ca<sup>2+</sup> release. Although new proteins and mechanisms regulating Ca<sup>2+</sup> release emerge, the redox-dependent regulation of Ca<sup>2+</sup> channels is not fully understood. Ca<sup>2+</sup> transfer to the mitochondrial matrix via ER–mitochondria contact sites is important for ATP production (Cárdenas et al., 2010), so ROS-sensitive proteins at the MAM could serve as sensors for ER protein folding capacity that use Ca<sup>2+</sup> to communicate the local ATP requirement to the juxtaposed mitochondria. As oxidative protein folding within the ER is a major consumer of mitochondria-derived ATP, such communication may be pivotal for normal cell proliferation.

Several proteins that regulate Ca<sup>2+</sup> and ROS signaling at the MAMs are implicated in diseases, such as cancer (Marchi et al., 2014) and neurodegeneration (Krols et al., 2016), emphasizing the need for further exploration of the mechanisms controlling Ca<sup>2+</sup> and ROS signaling. Raturi et al. (2016) demonstrate that TMX1 protein levels influence tumor growth. In cell culture, reduced TMX1 protein levels correlated with a higher rate of cell death and a reduced multiplication rate. These findings

are in agreement with a recent study showing the dependence of cancer cells to constitutive ER–mitochondrial Ca<sup>2+</sup> transfer (Cárdenas et al., 2016). Nevertheless, the conclusions of both studies diverge at the *in vivo* level. Whereas transplanting TMX1-depleted HeLa cells in mice led to increased tumor growth (Raturi et al., 2016), pharmacological inhibition of IP<sub>3</sub>R (through the injection of the drug Xestospongin B in the tumor) resulted in a significant reduction of tumor size (Cárdenas et al., 2016). A possible explanation for this discrepancy relates to the different mechanisms by which ER–mitochondrial Ca<sup>2+</sup> transfer is impaired. Indeed, a strong inhibition of IP<sub>3</sub>R channels by injection of the drug Xestospongin B directly impacts ER–mitochondrial Ca<sup>2+</sup> signaling, likely leading to a prominent and severe inhibition in ER–mitochondrial Ca<sup>2+</sup> fluxes. Because tumor cells depend on low-level Ca<sup>2+</sup> for their metabolic requirements, loss of ER–mitochondrial Ca<sup>2+</sup> transfer results in impaired tumor growth and reduced viability (Cárdenas et al., 2016). In contrast, partial depletion of TMX1 could indirectly impact ER–mitochondrial Ca<sup>2+</sup> signaling by activating SERCA2b, leading to a moderate reduction of Ca<sup>2+</sup> flux toward the mitochondria. This Ca<sup>2+</sup> flux could promote tumor growth *in vivo* by contributing to the Warburg effect (the switch to aerobic glycolysis in tumor cells) and possibly by preventing the occurrence of excessive, proapoptotic mitochondrial Ca<sup>2+</sup> signals, although *in vitro* data argue for increased cell death in the absence of TMX1 (Raturi et al., 2016). As the full range of TMX1 functions is unknown and the contribution of Ca<sup>2+</sup> transfer to the enhanced tumor growth upon TMX1 depletion was not studied, other mechanisms might be involved in promoting tumorigenesis upon TMX1 depletion. These seemingly opposite effects of lack of TMX1 *in vitro* (with increased cell death and decreased proliferation) and *in vivo* (with increased tumor growth) ought to be investigated further.

Overall, it has become clear that ER–mitochondrial Ca<sup>2+</sup> transfer plays a central role in oncogenesis and in the response of cancer cells to chemotherapy (Marchi et al., 2014). Other tumor suppressors, including p53, have been proposed to influence contact site formation and Ca<sup>2+</sup> transfer at the MAMs (Bittremieux et al., 2016). FATE1 was identified as an ER–mitochondria uncoupler, whereby it diminishes Ca<sup>2+</sup> flux and promotes chemotherapy resistance in adrenocortical carcinoma cells (Doghman-Bouguerra et al., 2016). Hence, advancing our knowledge on the composition, function, and regulation of these contact sites and on how all these different players cooperate to control interorganelle communication will be of utmost importance in the search for new treatments for diseases such as cancer, diabetes, and neurodegenerative disorders (Marchi et al., 2014; Krols et al., 2016).

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