ARTICLE

Overexpressed yeast Bax inhibitor (Bxi1p/Ybh3p) is a calcium channel in *E. coli*

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ABSTRACT

Human Bax Inhibitor-1 (HsBI-1/TMBIM6) is the founding member of the evolutionary conserved TMBIM superfamily of proteins that share sequence homology within the transmembrane Bax inhibitor-containing motif (TMBIM). Mechanistically, BI-1/TMBIM6 and all the other mammalian TMBIM proteins appear to be involved in the maintenance of calcium homeostasis, and the crystal structure of a bacterial TMBIM protein, BsYetJ, suggests that the protein is a pH-sensitive calcium leak. The budding yeast, *Saccharomyces cerevisiae*, has a single TMBIM family member (YNL305C) named Bxi1p/Ybh3p. To determine the function Bxi1p/Ybh3p, we overexpressed Bxi1p-GFP in *E. coli* to interrogate its putative calcium channel function. We show that bacterial cells expressing Bxi1p-GFP are more permeable to calcium than controls. Our data suggests that yeast Bax inhibitor (Bxi1p) is a calcium channel in *E. coli*, lending support to our proposal that Bxi1p is a *bona fide* member of the TMBIM family of proteins. Finally, parallel experiments also revealed that the human Bax Inhibitor-1 (HsBI1/TMBIM6) is also a calcium channel in bacteria.

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INTRODUCTION

Human Bax Inhibitor-1 (HsBI-1/TMBIM6) is the founding member of the evolutionary conserved TMBIM superfamily of proteins that share sequence homology within the transmembrane Bax inhibitor-containing motif (TMBIM) (Xu & Reed, 1998; Reimers et al., 2006; Carrara et al., 2012; Rojas-Rivera & Hetz, 2015; Gamboa-Tuz et al., 2018; Lebeaupin et al., 2020). The human genome encodes six members of the superfamily (TMBIM1-6) that are homologous to other BI-1 like proteins in vertebrates, plants, yeast, bacteria and viruses. TMBIM1-3 are localized to the Golgi apparatus, TMBIM4-6 are found in the endoplasmic reticulum, and TMBIM5 is a mitochondrial protein (Lisak et al., 2015).

KEYWORDS

Bax Inhibitor, Bxi1p, Ybh3p, calcium channel, TMBIM6, BI-1

Phenotypically, BI-1/TMBIM6 is an ER-localized, anti-apoptotic protein that was first identified in a screen for human proteins that could inhibit Bax-mediated cell death in yeast (Xu & Reed, 1998). Mammalian cells stably overexpressing BI-1/TMBIM6 are protected against ER-stress induced apoptosis (Chae et al., 2004; Bailly-Maitre et al., 2006). Moreover, mice lacking BI-1/TMBIM6 are more sensitive to stroke-induced cerebral damage and tunicamycin-induced kidney toxicity (Chae et al., 2004). Clinically, BI-1 is known to be overexpressed in breast cancer, glioma, lung, and prostate carcinoma (van 't Veer et al., 2002; Schmits et al., 2002; Grzmil et al., 2003, 2006; Lu et al., 2015). Strikingly, downregulation of BI-1 in prostate cancer cells by RNAi leads to cell death (Grzmil et al., 2003). The other mammalian TMBIM proteins are also cytoprotective against different triggers known to induce cell death (Rojas-Rivera & Hetz, 2015).

Mechanistically, BI-1/TMBIM6 and all the other mammalian TMBIM proteins appear to be involved in the maintenance of calcium homeostasis (Lisak et al., 2015; Liu, 2017). Knocking out TMBIM6 in hepatocytes leads to higher Ca^{2+} content in the ER of hepatocytes (Chae et al., 2004), while overexpressing the gene leads to reduced ER Ca^{2+} content (Westphalen et al., 2005). Similarly, stably overexpressing each HA-tagged TMBIM1-6 protein family member in HT22 cells reduced ER Ca^{2+} content, and all the TMBIM proteins except TMBIM5 also reduced the basal concentration of calcium in the cytosol (Lisak et al., 2015). TMBIM6 regulates Ca^{2+} flux in a pH-dependent manner (Ahn et al., 2009, 2010; Kiviluoto et al., 2013).

Structurally, the role of the TMBIM family of proteins in calcium homeostasis has been confirmed by the solution of the crystal structure of a bacterial TMBIM protein, BsYetJ, from Bacillus subtilis that suggests that the protein is a pH-sensitive calcium leak (Chang et al., 2014; Guo et al., 2019). It has a seven-transmembrane-helix fold structure that has either a closed or an open channel conformation depending upon the pH of its environment. It also has a di-aspartyl pH sensor in its C-terminal pore domain (Asp171-Asp195) that corresponds to two aspartate residues in BI-1/TMBIM6 (Asp188-Asp213) (Chang et al., 2014; Guo et al., 2019). Biochemical characterization of BsYetJ proteoliposomes at various pHs revealed that the pH-sensitive calcium-leak activity is intrinsic to the protein (Chang et al., 2014). Finally, reconstitution of BsYetJ in membrane vesicles with a lipid composition similar to that of the ER suggests that the charged residues E49 and R205 work together as a major gate, regulating calcium conductance in these ER-like lipid vesicles (Lan et al., 2023).

The budding yeast, *Saccharomyces cerevisiae*, has a single TMBIM family member (YNL305C) named Bxi1p/Ybh3p (Chae et al., 2003; Cebulski et al., 2011; Büttner et al., 2011). The protein is homologous to the mammalian TMBIM1-6 family members and contains the conserved di-aspartyl pH sensor in its C-terminal pore domain (Asp255-Asp278) that has been identified as the latch responsible for opening and closing the calcium leak. In a previous study, we showed that Bxi1p-GFP is localized to the ER, and that mutant yeast cells deleted of *BXI1* are more susceptible to a range of pharmacological and environmental triggers that induce cell death, especially pharmacological triggers associated with the unfolded protein response (Cebulski et al., 2011). This pro-survival function for Bxi1p in a genetic screen for cell death associated genes in yeast and when cells are treated with acetic acid has been confirmed by two other laboratories (Teng et al., 2011; Mentel et al., 2023) and is consistent with the anti-apoptotic function associated with the TMBIM superfamily. However, a subsequent paper from the Madeo Laboratory at the University of Graz, published days after our original publication, suggested that the protein encoded by the ORF – which they called Ybh3p for yeast BH3-only protein – is a pro-apoptotic member of the BH3-only family of proteins that translocates from the vacuole to the mitochondria to trigger BH3-domain dependent apoptosis (Büttner et al., 2011).

How do we resolve this discrepancy in the reported putative functions of Bxi1p/Ybh3p? First, it is notable that the inclusion of Bxi1p/Ybh3p in the BH3-only family of proteins has been criticized by those who have proposed that the BH3-domain-like sequence in this yeast ORF is not a bona fide BH3 domain (Aouacheria et al., 2013). These critics point out that the candidate yeast BH3 sequence is somewhat truncated as it is located at the C-terminus of the Ybh3p protein, and it overlaps one of six transmembrane segments, two unprecedented features among any other known BH3-containing proteins (Aouacheria et al., 2013). Finally, there is data that suggests that Bxi1p/Ybh3p is not required for the action of Bcl-2 family proteins on yeast cell viability suggesting that it does not play a role in Bcl-2 mediated cell death (Mentel et al., 2023).

Nonetheless, to begin to experimentally resolve the apparent discrepancy between our data that suggests that Bxi1p/Ybh3p is an anti-apoptotic member of the TMBIM superfamily, and the data that proposed instead that Bxi1p/Ybh3p is a pro-apoptotic member of the BH3-only family of proteins, we overexpressed Bxi1p-GFP in *E. coli* to interrogate its putative calcium channel function. We show that bacterial cells expressing Bxi1p-GFP are more permeable to calcium than controls. Our data suggests that yeast Bax inhibitor (Bxi1p) is a calcium channel in *E. coli*, lending support to our proposal that Bxi1p is a *bona fide* member of the TMBIM family of proteins. Finally, parallel experiments also revealed that the human Bax Inhibitor-1 (HsBI-1/TMBIM6) is also a calcium channel in bacteria.

MATERIALS AND METHODS

Bacterial Strain, Plasmids, and Growth Conditions: All experiments were done with DH5α *E. coli* cells obtained from New England Biolabs. Bacterial plasmids overexpressing yBxi1p-EGFP and the hTMBIM6-EGFP fusion were constructed and verified by VectorBuilder. The vector ID for plasmid, pBAD-EGFP(ns):4XGS:{yBXI1}, which overexpresses wildtype yBxi1p-EGFP in media containing arabinose is VB160522-1016qch; and the vector ID for plasmid, pBAD-EGFP(ns):4XGS:{hTMBIM6}, which overexpresses hTMBIM6-EGFP is VB170103-1020maq. These vector IDs can be used to retrieve detailed information and plasmid maps from vectorbuilder.com. Cells were transformed and cultured using standard bacterial protocols and media (Ausubel et al., 2002). Unless noted otherwise, all drugs and reagents were purchased from SIGMA-Aldrich.

Bxi1p-GFP Induction Assays: DH5α *E. coli* cells transformed with a bacterial plasmid overexpressing either yBxi1p-EGFP or hTMBIM6-EGFP were inoculated in 20mL of LB containing 100µg/mL ampicillin and allowed to grow to an OD_{600} of 0.6 at 37°C. Expression of the Bxi1p-EGFP fusion protein was then initiated by the addition of 200µL of 20% arabinose. Expression levels of the yBxi1p-EGFP or hTMBIM6-EGFP fusion proteins were then visualized, 1, 3, 6, 12, 18, and 24 hours after induction with a Zeiss LSM700 confocal microscope and quantified with an Accuri C6 Flow Cytometer. For all measurements with the Accuri C6 Flow Cytometer, bacterial cells were first diluted in 10mL water to an OD_{600} of 0.003.

Calcium Permeability Assays: DH5α *E. coli* cells transformed with a bacterial plasmid overexpressing either yBxi1p-EGFP or

hTMBIM6-EGFP were inoculated in 20mL of LB containing 100µg/mL ampicillin and cultured overnight at 37°C in a floor shaker set to 250 RPM. The following day, 1mL of cells were re-inoculated into 10mLs of fresh LB amp and allowed to grow to an OD_{600} of 0.60. Both induced (with 2% arabinose) and uninduced cultures then were incubated at 37°C, 250 RPM for six hours. Next, the cells were spun down at 3,000g for 10mins, washed twice with 10mL Buffer A (50mM Tris pH 7.5; 100mM KCl; 1mM $MgCl_2$), and resuspended in 20mL Buffer B (120mM Tris pH 8.0; 0.2mM EDTA) where they were incubated at 37°C, 250 RPM for ten minutes. The cells were then spun down at 3,000g for 10 mins and washed twice with 10mL of buffer A. Finally, the cells were re-suspended in 2.5mL Buffer A containing 10 µM Fura-2AM and incubated at 37°C, 250 RPM for two hours.

Following Fura-2AM incubation, cells were pelleted at 3,000g for 10mins and washed twice with 10mL aliquots of buffer A. Following the washes, the cells were re-suspended in 4mL Buffer A, and plated in 250 µL aliquots in a 96-well black Costar plate. Cells were excited at the standard wavelength for Fura-2AM (510 nm) and measured for wavelength emission of the bound (340nm) and unbound (380 nm) state using a Biotek Cytation 3 Cell Imaging Reader. Emissions were assessed every two minutes for six minutes. Following the initial six minutes, 5 µL of 500 mM $CaCl_2$ was added and run at the same conditions for an additional 10 minutes. Calcium concentration was represented by the ratio of R_{340}/R_{380} of Fura-2 as has been typically done in previous studies (Hudson et al., 1998; Luo et al., 2019). All experiments were done in triplicate. Statistical significance was determined with the Student's t-test, using Graph Pad Prism 6. By default, one asterisk is $p<0.05$; two asterisks is $p<0.01$; three asterisks is $p<0.001$; and four asterisks is $p<0.0001$.

RESULTS AND DISCUSSION

To determine if yeast Bax inhibitor (Bxi1p) has calcium channel function in *E. coli*, we overexpressed a Bxi1p-EGFP fusion protein in bacterial cells using the arabinose-inducible araBAD promoter (Guzman et al., 1995). A similar experiment had been done with the *Bacillus subtilis* homolog, BsYetJ, in bacteria (Chang et al., 2014). Expression levels of the Bxi1p-EGFP fusion protein were visualized 1, 3, 6, 12, 18, and 24 hours after induction on a Zeiss LSM700 confocal microscope and were quantified with an Accuri C6 Flow Cytometer to measure GFP fluorescence. As shown in Figures 1A and 1B, bacterial cells grown in media containing arabinose for six hours showed significant induction ($p<0.0001$) of the Bxi1p-EGFP fusion protein. This time point was chosen for all further experiments. We then loaded these DH5α cells with Fura-2AM, a membrane-permeable, fluorescent calcium indicator that has been used to determine the intracellular calcium dynamics of bacterial cells (Gangola & Rosen, 1987; Chang et al., 2014). Upon addition of external calcium, intracellular calcium concentration as indicated by the R_{340}/R_{380} ratio of Fura-2 increased more rapidly in bacterial cells induced with arabinose as compared with uninduced controls (Figure 1C).

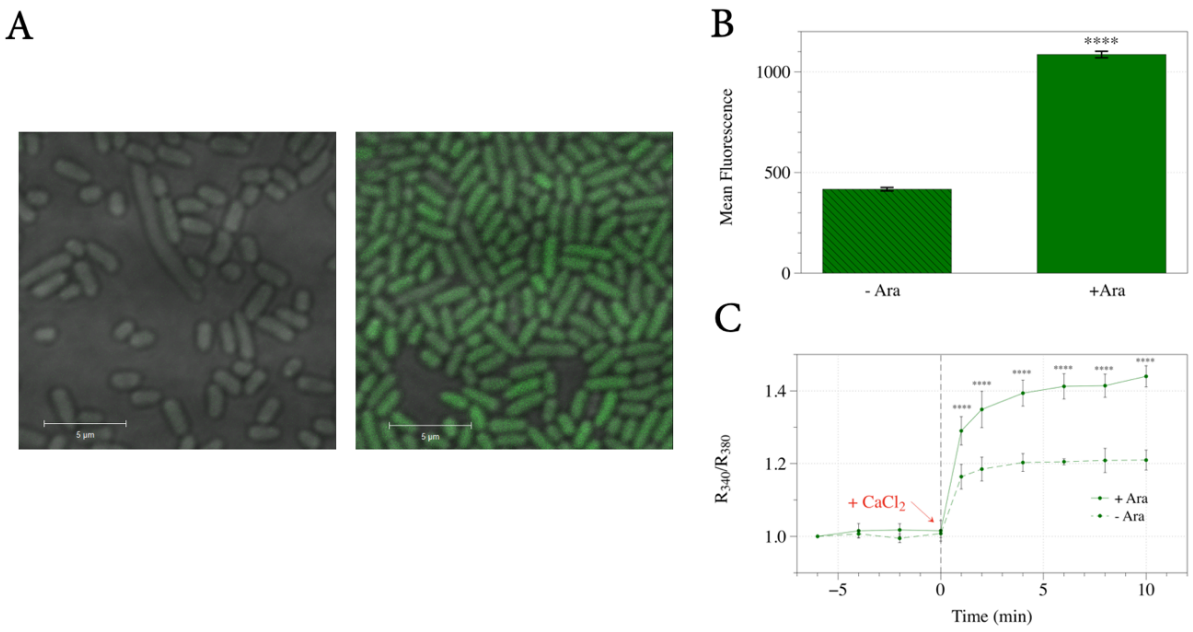

Figure 1: Yeast Bax Inhibitor (Bxi1p/Ybh3p) is a Calcium Channel in *E. coli*. (A) A yeast Bax inhibitor, Bxi1p-EGFP fusion protein was expressed in bacterial cells using the arabinose-inducible araBAD promoter and was visualized with a Zeiss LSM700 confocal microscope. (B) Bacterial cells grown in LB media containing arabinose for six hours showed significant induction ($p<0.0001$) of the Bxi1p-EGFP fusion protein as measured by an Accuri C6 Flow Cytometer. (C) We then loaded these DH5α cells with Fura-2AM, a membrane-permeable, fluorescent calcium indicator that has been used to determine the intracellular calcium dynamics of bacterial cells. Upon addition of external calcium, intracellular calcium concentration as indicated by the R_{340}/R_{380} ratio of Fura-2 increased more rapidly in bacterial cells induced with arabinose as compared with uninduced controls ($p<0.0001$).

This data suggests that Bxi1p-EGFP is a functional TMBIM superfamily member with channel activity that can increase the permeability of bacterial cells to calcium. It also suggests that Bxi1p-EGFP is not a member of the BH3-only family of proteins, which for the most part are known to be either globular or membrane bound with a C-terminal membrane anchor, and not transmembrane proteins (Glab, Mbogo & Puthalakath, 2017). We are still not sure how to reconcile this observation that Bxi1p is a protein with channel function with the published data that suggested that Bxi1p/Ybh3p is a protein that translocates from

the vacuole to the mitochondria to trigger cell death in a manner reminiscent of the BH3-only family of proteins (Büttner et al., 2011).

We repeated our experiments with the human Bax inhibitor (HsBI-1/TMBIM6) by overexpressing a HsBI1-EGFP fusion protein in bacterial cells, again using the arabinose-inducible araBAD promoter (Guzman et al., 1995). As shown in Figure 2A, bacterial cells grown in media containing arabinose for six hours showed significant induction ($p<0.05$) of the HsBI1-EGFP fusion protein. Once again, we loaded these DH5α cells with Fura-2AM, a membrane-permeable, fluorescent calcium indicator. Upon addition of external calcium, intracellular calcium concentration as indicated by the R_{340}/R_{380} ratio of Fura-2 increased more rapidly in bacterial cells induced with arabinose as compared with uninduced controls, though the difference between cells containing HsBI1-GFP and control cells was not as significant at the difference seen with bacterial cells overexpressing Bxi1p-GFP (Figure 2B). This data suggests that HsBI-1/TMBIM6 like Bxi1p/Ybh3p also has channel activity that can increase the permeability of bacterial cells to calcium.

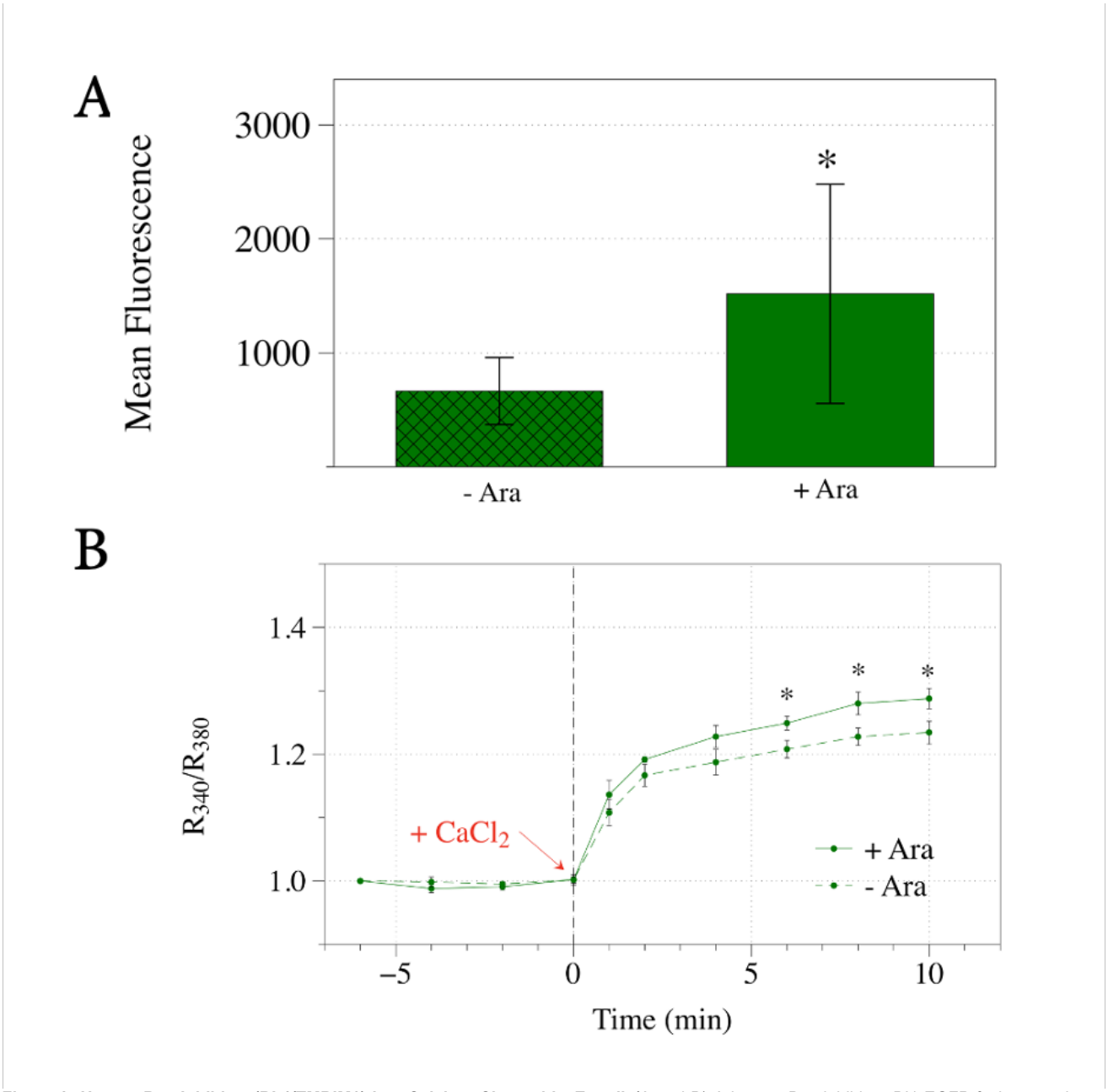

Figure 2: Human Bax Inhibitor (BI-1/TMBIM6) is a Calcium Channel in *E. coli*. (A and B) A human Bax inhibitor, BI1-EGFP fusion protein was expressed in bacterial cells using the arabinose-inducible araBAD promoter. Bacterial cells grown in LB media containing arabinose for six hours showed significant induction ($p<0.05$) of the BI1-EGFP fusion protein as measured by an Accuri C6 Flow Cytometer. (C) We then loaded these DH5α cells with Fura-2AM, a membrane-permeable, fluorescent calcium indicator that has been used to determine the intracellular calcium dynamics of bacterial cells. Upon addition of external calcium, intracellular calcium concentration as indicated by the R_{340}/R_{380} ratio of Fura-2 increased more rapidly in bacterial cells induced with arabinose as compared with uninduced controls ($p<0.05$).

In sum, our results suggest that both Bxi1p/Ybh3p and HsBI-1/TMBIM6 have calcium channel activity when expressed in bacterial cells. Interestingly, in mammalian cells, HsBI-1/TMBIM6 is known to interact with the inositol trisphosphate receptor (IP_3R) to regulate ER calcium homeostasis (Rojas-Rivera & Hetz, 2015) and to regulate local calcium transfer between the ER and the lysosome (Kim et al., 2021). It also controls the movement of calcium from the ER to the

mitochondria (Zhou et al., 2023). It will be interesting to determine if the yeast homolog, Bxi1p, is also involved in calcium transfer between these organelles in budding yeast.

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