HYPO-OSMOLARITY AND GLUCOSE STARVATION LOCALIZES SLM1 TO MITOCHONDRIA- A NOVEL STRESS RESPONSE

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ABSTRACT

Synthetic Lethal with Mss4 1 (Slm1) is a key protein found in the crossroad of the Membrane Compartment of Can1 (MCC) and the Membrane Compartment of TORC2 (MCT). The two compartments on the plasma membrane of budding yeast (*Saccharomyces cerevisiae*) are responsible for APC transporter regulation and sphingolipid synthesis respectively, altogether maintaining the homeostasis of the plasma membrane. By altering its localization between the MCC and the MCT, Slm1 serves as a signal that communicates the stress condition. And yet, neither the MCC, the MCT, nor Slm1 itself had been reported in the context of mitochondria or metabolism. In this study, we report Slm1 localizes to mitochondria under the combination of glucose starvation and hypo-osmotic stress. We also identified the first 173 amino acids to be critical for such localization. These findings give access to the study of Slm1's role in mitochondrial function, and a stress response pathway that has never been studied before—a pathway that relays stress signals to the regulation of cell metabolism.

INTRODUCTION
Role of Plasma Membrane Compartments in Yeast Stress Response

In unicellular organisms, any change in their environment such as ion concentration, osmolarity, nutrition, and energy source, can cause stresses on their structural integrity and metabolism. In cells stressed by osmotic pressure one of the first structures to be impacted is the plasma membrane which controls the permeability of molecules in and out of the cell. *Saccharomyces cerevisiae*, a unicellular eukaryote, has evolved dedicated pathways to respond to environmental stresses, including those that protect the plasma membrane.

The plasma membrane of yeast has been reported to contain five non-overlapping membrane compartments or rafts—the Membrane Compartment of the arginine permease Can1 (MCC), the Membrane Compartment of the sterol transporters Ltc3/4 (MCL), the Membrane Compartment of the H+ ATPase Pma1 (MCP), the Membrane Compartment of the kinase TORC2 (MCT), and the Membrane Compartment of the cell wall stress sensor Wsc1 (MCW). These membrane compartments are local regions of the plasma membrane that is composed of distinct lipids as well as a specific collection of proteins whose functions are somewhat inter-related. While some of the rafts are beyond the discussion of this study, MCC and MCT, both play roles in the stress response pathways of yeast and host many proteins with regulatory functions (for review, refer to Athanasopoulos et al., 2019) and are the focus of this study.

Structure and proteins of MCC

MCC, also known as the eisosome, is present as grooves on the plasma membrane of yeast folding inwards toward the cytoplasm (Strádalová et al., 2009). The lipid composition of eisosomes is unique to that of the plasma membrane, abundant in ergosterol as well as sphingolipids (Grossmann et al., 2007). In addition, the hosted proteins likely contribute to a thicker membrane in eisosomes compared to surrounding plasma membrane (Bharat et al., 2018). Together, these characteristics contribute to the lipid raft nature of eisosomes.

MCC hosts two BAR domain-containing proteins Pil1 and Lsp1 (Walter et al., 2006). The half-tubular shape of these proteins results in the signature shape of the eisosomes-- a groove approximately 50 nm in depth and 300 nm in length (Strádalová et al., 2009). There are roughly 50 to 100 eisosomes on each cell membrane (Deng et al., 2009). Due to their number and size, eisosomes usually appear as dots dispersed evenly on the plasma membrane under fluorescent microscopy, as shown in Figure 1.

In addition to Pil1 and Lsp1, eisosomes also host many other proteins. Nce102 is a protein whose function remained unknown until very recently. It was reported that Nce102 moves from eisosomes to the membrane of the vacuole under gradual glucose limitation, modulating the morphology and function of the vacuole (for detailed info, refer to Vaskovicova et al., 2020). The depletion of Nce102 at the eisosome causes the number of
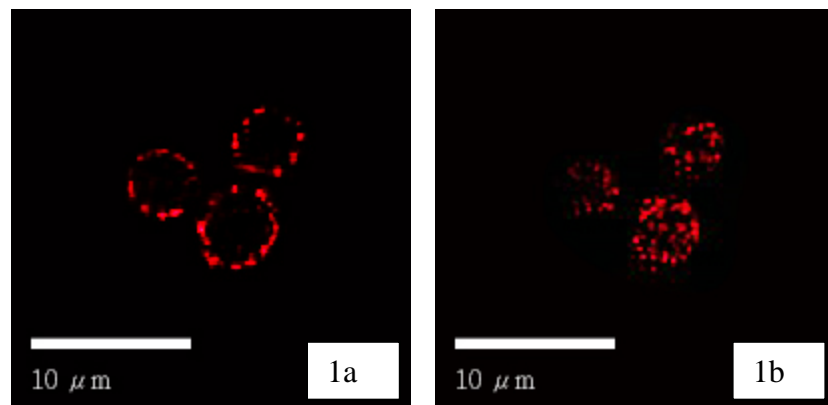
Figure 1. Typical Eisosomes of a Yeast Cell. Eisosomes are marked with RFP-tagged Pil1 (an eisosomal protein) for imaging under normal physiological condition. Figure 1a images the cross section of three cells. Figure 1b images the surface of the same cells. As demonstrated on the figure, eisosomes locate on the surface of the cells.

eisosomes to decrease significantly (Athanasopoulos et al., 2015), and a loss of eisosome's furrow shape (Strádalová et al., 2009). The amino acid-polyamine-organocation (APC) transporters are nutrient transporters that utilize the proton gradient across the plasma membrane for import (Keener and Babst, 2013). Eisosomes are also thought to host APC transporters to regulate the transporters (Moharir et al., 2018) and protect them from ubiquitination (Appadurai et al., 2020). A set of soluble proteins that localize to eisosomes under normal growth conditions are Slm1 and Slm2, paralogues that arose from whole genome duplication (Byrne et al., 2005). They have been reported to move away from eisosomes as a stress response signal (Berchtold et al., 2012).

Role of MCC (eiososomes) and MCT in stress response

Like its mammalian counterpart, the caveolae (Douglas et al., 2014), eisosomes sense stresses on plasma membrane (Berchtold et al., 2012). It has been observed that eisosomes flatten when the plasma membrane experiences various stresses, including high tension (Kabeche et al., 2015), loss of proton gradient across the membrane (Moharir et al., 2018), hypo-osmotic shock and heat shock (Appadurai et al., 2020). It has been previously hypothesized that the flattening of eisosomes compensates for the expansion of the plasma membrane under those conditions (Kabeche at al., 2015), but later studies showed that the surface area provided by the flattening of the eisosomes is not enough to account for the expansion in the surface area of the plasma membrane (Appadurai, et al., 2020). It has been proposed that eisosomes sense certain plasma membrane stresses, flatten in response, and regulate the metabolism of the cell downstream.

One consequence to the flattening of the eisosomes is the release, and endocytosis of the APC transporters, resulting in their degradation (Appadurai, et al., 2020) (Figure 2a). This consequence makes physiological sense because under stresses such as loss of proton gradient and hypo-osmolarity, the ability to import nutrients outside the cell or the nutrients themselves are usually lost. So, maintaining APC transporters during these conditions would be a waste of energy and material, and against the survival needs of the cell.

Another consequence to the flattening of eisosomes is the re-localization of protein Synthetic Lethal with Mss4 (Slm1) 1 and 2, working as signals for metabolic regulation. The two proteins are found in eisosomes under normal growth condition (Kamble et al., 2011). When stress is applied on the plasma membrane by either the inhibition of sphingolipid synthesis or mechanical tension, Slm1 and 2 leave eisosomes and are redistributed. Their affinity to the Target of Rapamycin Complex 2 (TORC2) is increased, localizing them to the Membrane Compartment of TORC2 (MCT), another membrane domain on the plasma membrane of yeast cells. The association also activates the TORC2 kinase (Berchtold et al., 2012).

The activity of TORC2 was reported to upregulate sphingolipid synthesis in part by the activation of the kinase Ypk2, which promotes ceramide formation (Aronova et al., 2008). TORC2 also activate YPk1. Ypk1/2 phosphorylate Orm1 and Orm2, two inhibitors for sphingolipid synthesis at the endoplasmic reticulum (ER), and inactivate Orm1/2 (Roelants et al., 2011). As a result, by activating TORC2 at the MCT, the re-localization of Slm1/2 ultimately up-regulate the synthesis of sphingolipids, helping to relieve the membrane tension (Berchtold et al., 2012) (Figure 2b). On the other hand, a decrease in plasma membrane tension due to hyper-
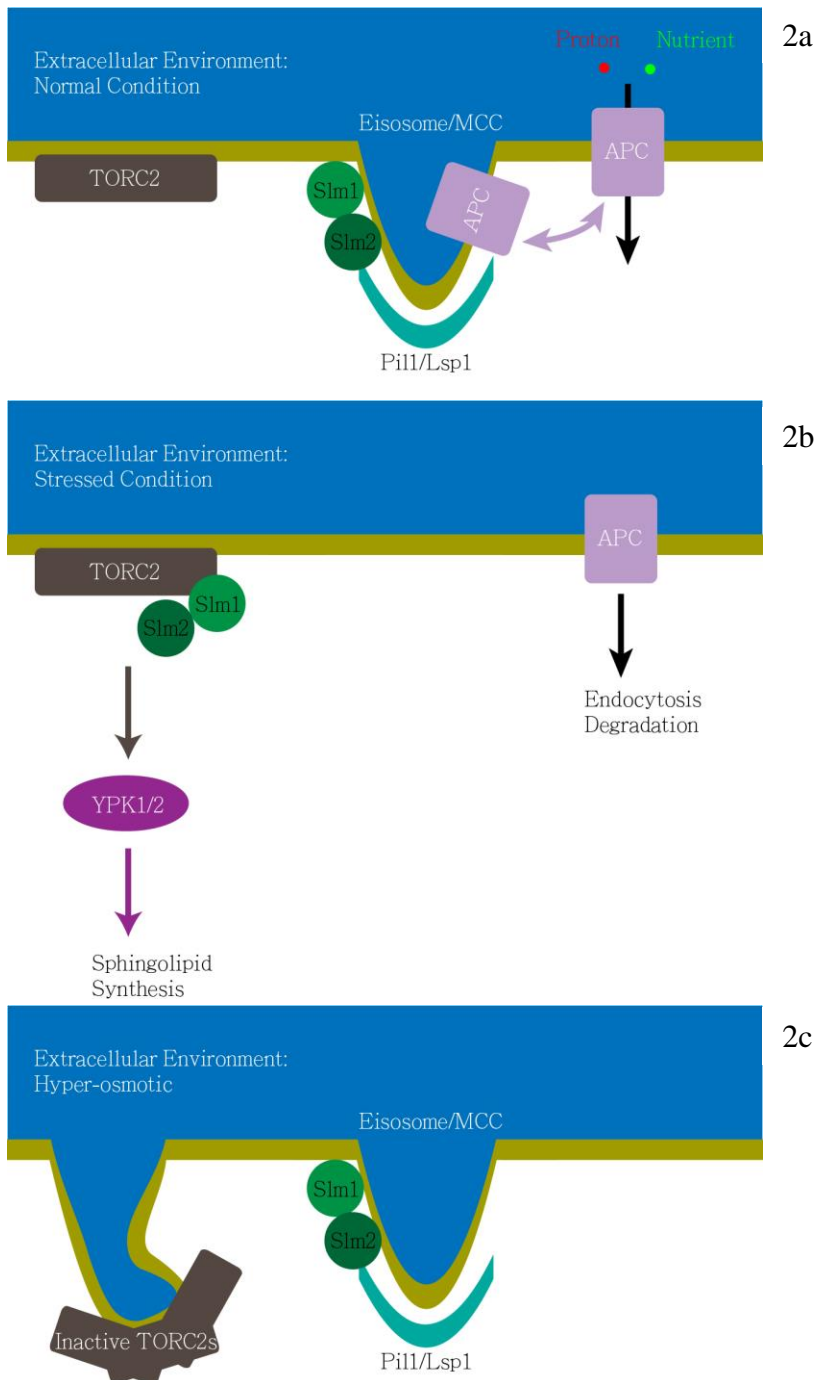
Figure 2. Pre-existing Models for Yeast Stress Response on MCC and MCT. (a) Under normal growth condition, MCCs (eisosomes) exist on the plasma membrane, and host Pil1, Lsp1, Slm1/2, APC transporters, and other proteins. (b) Under stress condition, eisosomes flatten, releasing APC transporters for endocytosis. Slm1/2 re-localize to TORC2, and trigger synthesis of sphingolipid downstream as a response. (c) Under hyper-osmotic condition, the plasma membrane invaginates, sequestering and inactivating TORC2s briefly.

osmolarity causes phosphatidylinositol-4,5-bisphosphate (PIP$_2$), a phospholipid in the plasma membrane, to phase separate, forming plasma membrane invaginations. The invaginations sequester and inactivate TORC2, which then restore the inhibition of sphingolipid synthesis (Riggi et al., 2018) (Figure 2c). Altogether, these two mechanisms are involved in maintaining the homeostasis of the plasma membrane.

Structure and role of Slm1 in Stress response via MCC and MCT

Amongst all the stress response pathways described so far, Slm1 is a key member that relays the signal from the stress-sensing eisosomes/MCC to the responding TORC2/MCT. The localization of Slm1 is thus important to be understood. Slm1's localization can be explained by its domain structure (Figure 3). Slm1 contains a Bin/amphiphysin/Rvs (BAR) domain, followed by a Pleckstrin Homology (PH) domain. The BAR domain is found in other eisosomal proteins

and recognizes the curved membrane of these furrow-like plasma membrane invaginations (Olivera-Couto et al., 2011). The PH domain binds to PIP2 of the plasma membrane and together with the BAR domain localizes Slm1 to eisosomes. The PH domain of Slm1 is also required for the binding to Avo2 and Bit61, two components of TORC2 (Fadri et al., 2005).

While the PH and BAR domains are relatively well-understood, the N-terminus of Slm1 is poorly studied, and yet of great interest. The first 173 amino acids in the N-terminus of Slm1 contains nine out of the total 24 phosphorylation sites (Albuquerque et al., 2008; Lanz et al., 2021; Swaney et al., 2013), as well as the only known ubiquitination site at K143 (Swaney et al., 2013).

In addition, the sequence retrieved from the Saccharomyces Genome Database (SGD) demonstrates several poly-glutamine and poly-asparagine sites. Poly-glutamine and poly-asparagine are known to drive protein phase separation. Protein phase separation is a novel discovery in cell biology, where proteins form their own phase, similar to the phasing between oil and water. Typical protein phase separation structures consist of more than one protein, allowing the proteins to interact in a non-specific manner (for review, refer to Boeynaems et al., 2018). Although the poly-glutamine and poly-asparagine sites on Slm1 are yet to be proven for functions related to protein phase separation, they offer a potential mechanism by which Slm1 function could be regulated. Together, the phosphorylation sites, the ubiquitination sits, and the potential site of protein phase separation suggest a potentially highly regulated region in Slm1 with an unknown function.
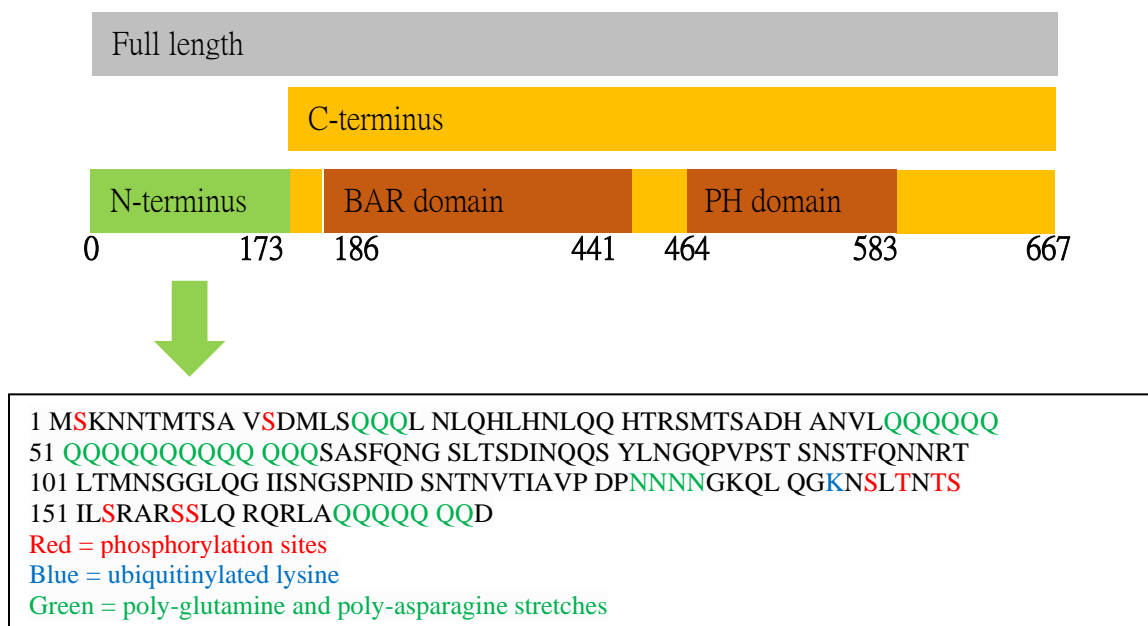
Figure 3. Structure of Slm1. Domains and regions of Slm1 discussed in this paper are demonstrated as colored bars. Numbers below the bars indicate numbers of the amino acids in the protein. The N-terminus contains many phosphorylation sites, a ubiquitination site, and multiple possible sites of protein phase separation. The amino acid sequence of the N-terminus (retrieved from the Saccharomyces Genome Database) is provided below for reference. The rest of the protein Slm1 contains the well-studied BAR domain and PH domain and is labelled as the C-terminus for contrast.

In this study, we study the first 173 amino acids of Slm1, using one of the most common stress yeasts encounter in the wild, water. We found that under water stress, Slm1 leaves

eisosomes and re-localizes to mitochondria in yeast cells, suggesting an additional and yet undiscribed regulatory pathway for yeast to respond to environmental stress.

METHODS
Media, Yeast Strains, and Plasmids
Media used for the growth of yeast strains are based on YPD (yeast extract, peptone), or synthetic dextrose (SD) medium (yeast nitrogen base, 2% glucose) supplemented with amino acids and nutrients (in mg/l: p-aminobenzoic acid [7.5], Ala [75], Arg [75], Asp [75], Asn [75], Cys [75], Glu [75], Gln [75], His [75], Ile [75], Leu [376], Lys [75], Met [75], Phe [75], Pro [75], Ser [75], Thr [75], Trp [75], Tyr [75], Val [75], adenine [20], inositol [75], and uracil [75]).

Homologous recombination was used in all cases of plasmid construction, deletion, and genomic tagging, as described previously (Longtine et al., 1998). In the case of strain construction, KANMX cassette, which creates resistance to antibiotic G418, was used for deletion and genomic tagging of genes. All genetic modifications of strains were selected with media containing antibiotic G418 and confirmed by genomic PCR. Plasmids were each constructed using vectors containing LEU2, TRP1, or URA3 gene, which provided the host strain with the ability to synthesize leucine (Andreadis et al., 1984), tryptophan (Miozzari et al., 1978), or uracil (Lacroute, 1968), respectively. Strains created by transformation of a plasmid or plasmids were confirmed and maintained by growing in SD medium lacking the corresponding nutrient or nutrients. The selection for strains and plasmids was maintained in all experiments to ensure the presence of proper genomic modification or plasmids. Details for all media, strains, and plasmids used in this study are listed in Table 1.

Organelle Markers
In most cases, organelles were tagged with an RFP-tagged protein that is signature to the organelle. Kar2 was used to tag the endoplasmic reticulum (Normington et al., 1989). Atg8 was used to tag the autophagosome (Lang et al., 1998). Pil1 was used to tag eisosomes (Walter et al., 2006). A signaling peptide was used to tag the mitochondria. The tags were cloned into a plasmid, unless stated otherwise. The plasmid was then transformed into the strain of interest, where a version of GFP-tagged Slm1 is co-transformed to mark the location of Slm1.

Treatments
Yeast cells were grown in the SD medium to exponential phase with and optical density (OD) of ~0.6 before any treatment. All treatments were done in the normal growth temperature of yeast, 30˚C.

Water treatment was performed by changing the medium from SD medium to pure water, followed by incubation at 30˚C for 5 minutes. The treatment lasted for 2, or 10 minutes in experiments in Figure 11, which specifically tested for the timing of mitochondrial fragmentation caused by water. The cells were then spun down for microscopy. Water treatment could lead to a combination of many stresses, including but not limited to hypo-osmolarity, glucose starvation, ion depletion, and amino acid starvation.

When performing glucose starvation, cells were also kept in SD medium to OD~0.6. The treatment was then performed by changing the SD medium to the starvation SD medium (refer to Table 1) for desired treatment time. In starvation SD medium, 2% glucose is replaced by 2% sorbitol. It has been reported that yeast cannot metabolize sorbitol without an extensive, seven-day period of adaptation to generate spontaneous mutation (Tanaka et al., 2020). As a result, our short substitution to sorbitol maintains the osmolarity while starving the cells for glucose.

Hypo-osmotic treatment was performed by keeping the cells in hyper-osmotic SD medium (refer to Table 1) until an OD of roughly 0.6 was reached, allowing the cells to adapt to the hyper-osmotic condition. The hyper-osmotic SD medium contained 1 molar sorbitol in addition to other pre-existing ingredients, to induce hyper-osmolarity without giving extra source of usable nutrient. The total culture time in each trail was less than a day, and cells were started freshly from stock in each trail to limit the effect of spontaneous mutation mentioned previously

Yeast Strains			
Name	Descriptive Name	Description	Reference or Source
SEY6210	Wild Type	MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL	Robinson et al., 1988
LGY31	Slm1-GFP, Mito-RFP	SEY6210, SLM1-GFP TRP1, PIL1-mCherry KANMX6	Appadurai et al., 2020
AMY48	Slm1 KO	SEY6210, SLM1 KANMX6	This study
AMY71	Slm2 KO	SEY6210, SLM2 KANMX6	This study
Plasmids			
Name	Descriptive Name	Description	Reference or Source
pLG13	Mito-RFP	LEU2 (pRS415) P(CUP1)-FUR4-GFP	This study
pYL1	NSlm1-GFP	URA3 (pRS416) P(CUP1)-SLM1(1-173)-GFP	This study
pYL3	Slm1-GFP	URA3 (pRS416) P(CUP1)-SLM1-GFP	This study
pYL4	CSlm1-GFP	URA3 (pRS416) P(CUP1)-SLM1(174-end)-GFP	This study
Kar2 KM-RFP	Kar2-RFP	TRP1 (pRS424) GAP2-KmRFP	Nakanishi et al., 2007
pWB38	RFP-Atg8	LEU2 (pRS415) P(VPS21)-mCherry-ATG8	This study
Media			
Name	Component		
YPD	yeast extract, peptone		
SD	yeast nitrogen base, 2% glucose, complete nutrient supplement*		
Hyper-osmotic SD	SD, 1M sorbitol		
Starvation SD	SD, -2% glucose, +2% sorbitol		

Table 1. Yeast Strains, Plasmids, and Media Used in This Study. Note: Details about the complete nutrient supplement used in SD medium is mentioned in the Media, Yeast Strains, and Plasmids section of Methods.

(Tanaka et al., 2020). The cells were then switched into regular SD medium. This treatment maintained a stable glucose level while applying hypo-osmotic stress, since the cells had taken the hyper-osmotic condition as normal before being treated.

In hypo-osmotic and glucose starvation combination stress, cells were kept in hyper osmotic SD medium until reaching OD~0.6 to adapt to the hyper-osmotic condition. The cells were then treated by switching the media to starvation SD medium. In this treatment, hyper-osmotic environment was replaced by standard osmolarity to induce hypo-osmotic stress, and 2% glucose was replaced by the unusable 2% sorbitol to induce starvation. This treatment allowed the combination of hypo-osmotic stress and glucose starvation to be applied at the same time without inducing other stresses that are present in a water stress.

Microscopy

All microscopy in this paper was performed using an Olympus fluorescent microscope, enhanced with the OLYMPUS cellSens Dimension software. Pictures were deconvolved using the Constrained Iterative software for better resolution. Some pictures were adjusted using the Adobe Photoshop software for demonstration of better contrast and coloring. The medium used in microscopy is always based on the SD medium, for that YPD seems to yield a higher background.

Mitochondrial Fragmentation Quantification and Data Analysis

Mitochondrial fragmentation was quantified by counting mitochondria in 30 cells from each group in the experiment, 10 cells per one of the three pictures. All cells were picked randomly and counted without knowing its corresponding experimental group. Each mitochondrial count was defined as a segment between two ends, two junctions to other mitochondria segment, or one end and one junction, as demonstrated in Figure 4. Only mitochondria on the surface of the cells were counted as representatives because counting mitochondria in the whole cell requires 3D reconstruction of the cell, which is impractical for manual analysis at large quantity. The resulting raw data is presented as the violin plots in Figures 7, 8, and 9.

The median number of mitochondria pieces in each group was calculated from the raw data. The median of a treatment group was then divided by the median of its corresponding control group that is without such treatment to yield a ratio of increased mitochondrial fragmentation. According to the raw data presented in the violin plots in Figures 7, 8, and 9, the mitochondria number per cell under normal growth condition is usually 3 to 5, and the population follows a somewhat centralized, though not necessarily Gaussian, distribution. In other words, a shift in median by 1 can easily cause the mitochondrial fragmentation ratio to fluctuate by 0.2 to 0.3, despite resulting from the error in random sampling. As a result, ratios that deviate from 1 by ~0.3 were considered insignificant, although advanced statistics might suggest a different value. Fortunately, the ratios resulting from the experiment can be categorized into two distinct groups, one with ratios within 1 ±0.3, the other with ratios much greater than 1.5. This allowed us to easily assign ratios that are within 0.3 rage to 1 to be insignificant increase or decrease, and ratios that are more than 0.5 away from 1 to be significant.
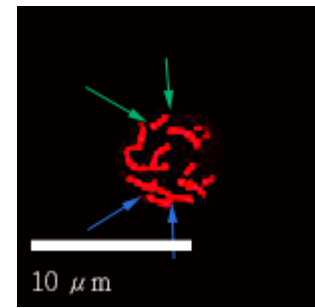
Figure 4. An Example of Mitochondria Count. The image shows the mitochondrial network of a yeast cell (SEY6210 + pYL3 + pLG13). The mitochondria are marked with RFP, resulting in the red signal. The green arrows indicate a mitochondrial count defined by two ends. The blue arrows indicate a mitochondrial count defined by one end and one junction.
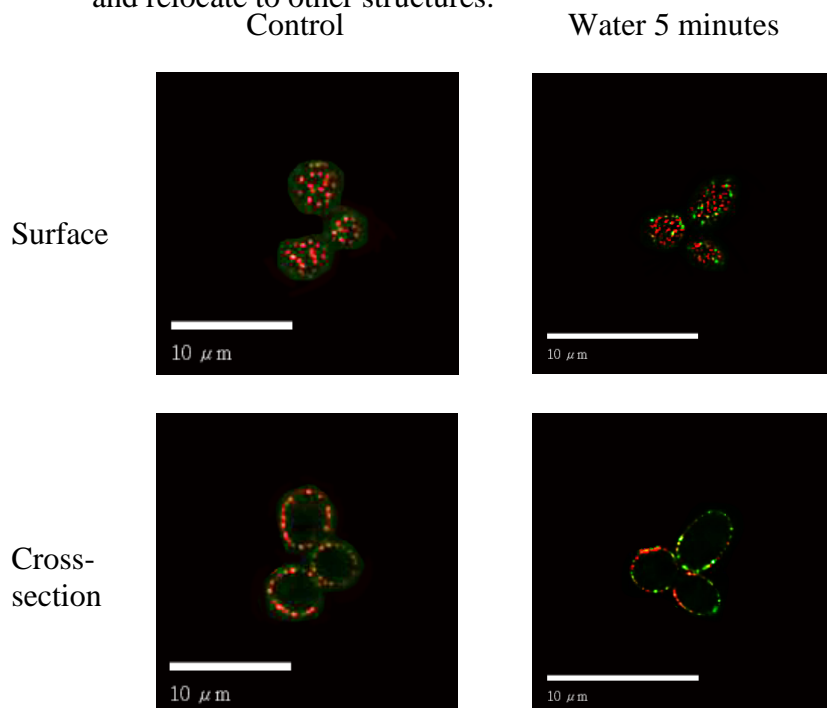
<div align="center">RESULTS</div>

Slm1 Co-localizes to Mitochondria Under Water Stress

Water is the most common stress yeasts in the wild would encounter. Rain can easily wash yeasts from its original dwelling that is rich in nutrient and proper ion concentration. This induces a combination of stresses to the yeast cells, including plasma membrane tension resulting from hypo-osmolarity, starvation for glucose and amino acids, as well as loss of proton gradient, which can cause the inability to utilize APC transporters, as previously discussed in the introduction.

To understand how Slm1 could potentially respond to the water stress, we genetically tagged full-length Slm1 with GFP on its C-terminus in a plasmid (pYL3), and transformed the wild type strain (SEY6210) with the plasmid (SEY6210 + pYL3), allowing it to express GFP-tagged Slm1. Another plasmid was co-transformed into the wild type strain (SEY6210), in which a RFP marker for endoplasmic reticulum (ER), autophagosome, or mitochondria was included. The markers of ER and autophagosome were Kar2-RFP, and RFP-Atg8, respectively, which are RFP-tagged proteins iconic to the organelles. Mitochondria were marked by RFP-tagged signaling peptide that targets RFP to the matrix. ER and autophagosome were chosen as candidates of Slm1 re-localization, for commonly being sources or recipients of protein trafficking from or to the plasma membrane, whereas mitochondria were chosen because water stress induces a starvation of energy sources, which potentially requires tuning in the metabolism of the cell. Eisosome were tagged with Pil1-RFP, and served as the control, for being the locations where Slm1 reside under normal growth condition, as described in the introduction.
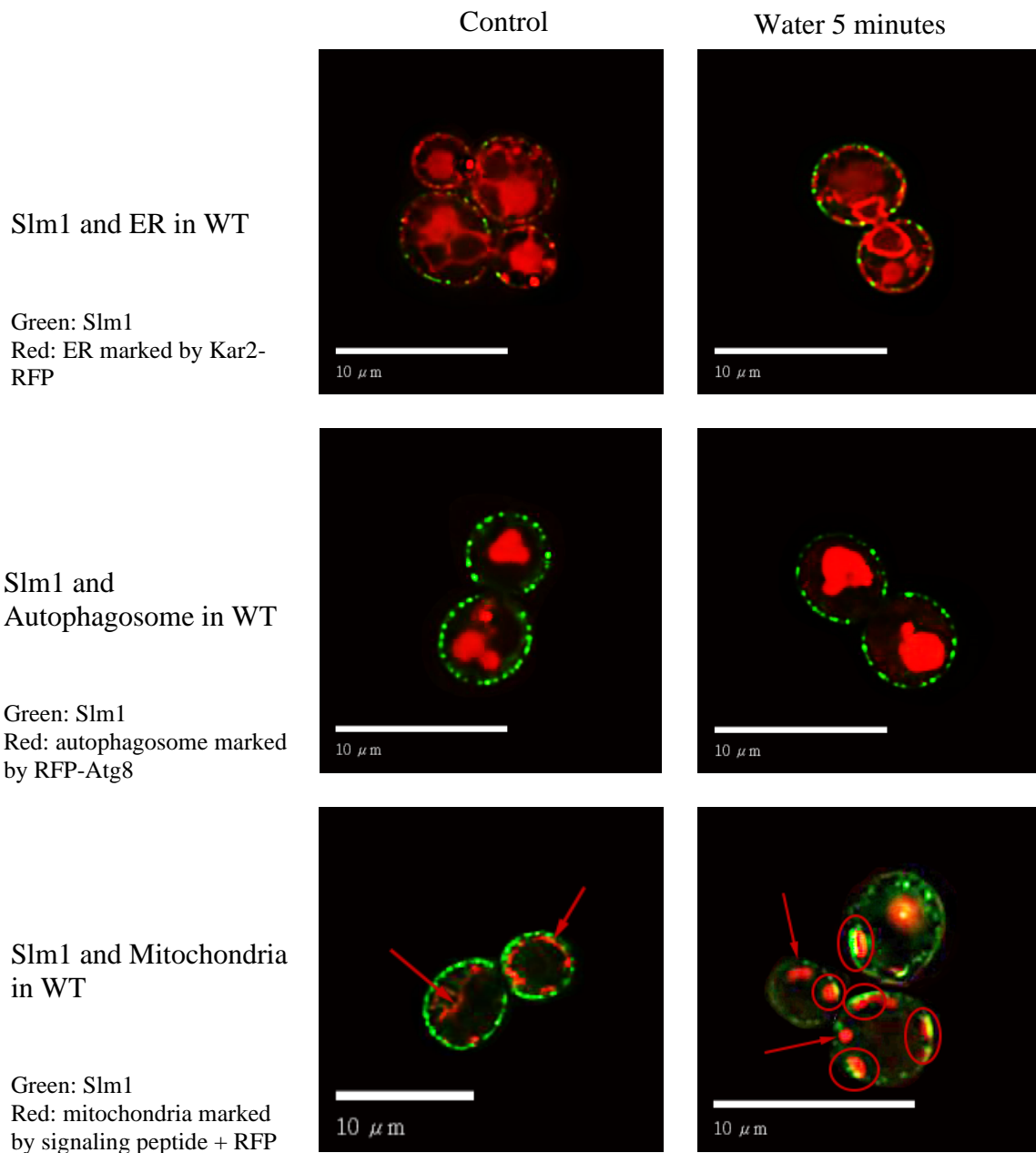
Each strain was grown in synthetic complete medium with corresponding nutrient selection to maintain the plasmids (YNB -URA -LEU, refer to Methods section.). The cells were grown to exponential phase with an OD of ~0.6 before the experiment, followed by a complete media change into pure water to stress the cells. After applying the water treatment for 5 minutes at 30˚C, cells were observed using fluorescent microscopy.

Figure 5 exhibits the resulting pictures for Slm1-GFP and RFP-tagged eisosomes. Slm1 seemed to localize to small puncta on the plasma membrane both before and after the water treatment. However, by carefully comparing the localization of Slm1 with the location of eisosomes, it was obvious that Slm1 moved out of eisosomes into a novel location on the plasma membrane. The signal of Slm1 overlapped with that of the eisosomal marker before the treatment, as indicated by the overlapping yellow signal. And yet, Slm1's signal and the eisosome marker no longer overlapped after water treatment, as indicated by the distinct red and green puncta. This experiment informed us that water treatment causes Slm1 to leave eisosomes and relocate to other structures.

Figure 5. Slm1 Moves Out of Eisosomes Under Water Stress. Slm1 is genomically tagged with GFP. Pil1, an eisosomal protein important for the integrity of eisosomes, is tagged with RFP genomically. The resulting starin (LGY31) is treated with water stress for 5 minutes, or untreated for control. After water stress, the green signal of Slm1 become distinct from the red signal of Pil1, indicating that Slm1 is no longer in the eisosomes.

Green: Slm1
Red: eisosomes marked by Pil1-RFP

	Control	Water 5 minutes

Slm1 and ER in WT

Green: Slm1
Red: ER marked by Kar2-RFP

Slm1 and Autophagosome in WT

Green: Slm1
Red: autophagosome marked by RFP-Atg8

Slm1 and Mitochondria in WT

Green: Slm1
Red: mitochondria marked by signaling peptide + RFP

Figure 6. Slm1 Re-localize to Mitochondria Under Water Stress and Causes Mitochondrial Fragmentation. Slm1 is tagged by GFP in a plasmid. Organelles of interest are marked by RFP-tagged proteins or signaling peptide that localize to the organelle, also constructed in plasmid. The resulting strain (LGY31) is treated with water stress for 5 minutes, or untreated for control. No co-localization of Slm1 is observed before the treatment, as indicated by the separate green and red signals. After water stress, Slm1 and mitochondria show co-localization, as indicated by the yellow signal resulting from the blend of red and green (circled in red). Mitochondria also fragment after water stress. As pointed out by the arrows, the mitochondria before water treatment are longer and fewer in pieces, whereas mitochondria after treatment are shorter and more in pieces.

Figure 6 shows the location of Slm1 before and after the 5-minute water stress, in cells expressing markers for ER, autophagosome, and mitochondria. Other than eisosomes, Slm1 was found to co-localize with no other organelle of interest before the treatment. But after water treatment, Slm1's signal partially overlapped with mitochondria. This indicates the re-localization of Slm1 from eisosomes to mitochondria during the treatment.

In addition to Slm1 co-localization, mitochondria were found fragmented when experiencing water stress, which could suggest that Slm1 might affect mitochondrial morphology.

Mitochondrial Fragmentation is Concentration-dependent to N-terminus of Slm1

To study the role of Slm1 in mitochondrial function, we first aimed to identify the domain that is responsible for the mitochondrial fragmentation. To do so, we divided the sequence of Slm1 into two segments, the 173 amino acid N-terminus that contains the polyglutamine repeats, and the C-terminal region that contains the two well-studied BAR domain and PH domain. The N-terminus contains sites of regulation, as described in the introduction, but have not been carefully studied, whereas the C-terminus is relatively well-studied, but have never been reported to relate to the mitochondria.
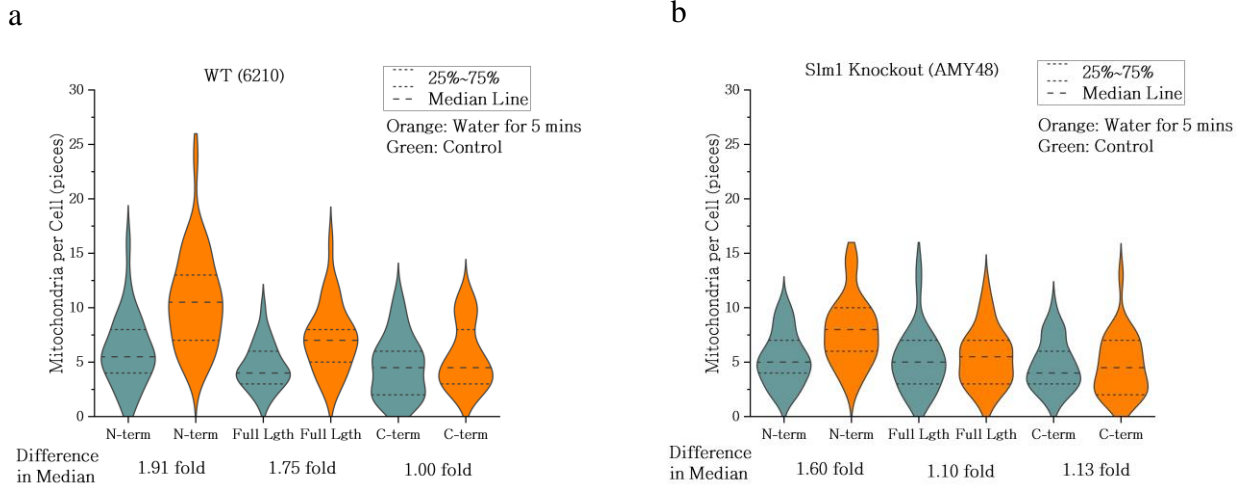
The N-terminus, the C-terminus, and the full length Slm1 were genetically tagged with GFP on their C-terminus in a plasmid respectively (pYL1, pYL3, and pYL4). Each plasmid was then co-transformed into wild type strain (SEY6210) with the mitochondrial marker plasmid (pLG13) used in this study previously. To account for the effect of genomic Slm1, the same combination of plasmids was transformed into a Slm1-knockout strain (AMY48) as a control.

The strains were treated with water stress for 5 minutes, as described previously. Then microscopy pictures were taken. The number of mitochondria in each of the random 30 cells was manually counted before and after water stress is applied, and the median number of mitochondria per cell is calculated accordingly, as presented in Figure 7a, 7b. The median number of mitochondria per cell after the water stress is divided by that before stress to obtain a ratio by which mitochondrial fragmentation has been increased, as presented in Figure 7c (For detail, please refer to the Methods section.).

The resulting ratios of mitochondrial fragmentation formed two distinct groups, one group with ratios within ±0.3 of 1, the other with ratios much greater, between 1.5 and 2. We assigned ratios that are within 0.3 range from 1 to be insignificant, and ratios that deviate from 1 by more than 0.5 to be significant. Rationale to this assignment can be found in the Methods section.

In the wild type strain (SEY6210), a 1.91-fold increase in mitochondrial fragmentation was observed in presence of the N-terminus if Slm1 (pYL1). In the case of full length Slm1 (pYL3), a 1.75-fold increase was observed. In contrast to the significant increase in the previous two cases, the C-terminus of Slm1 (pYL4) caused no increase (1.00-fold) mitochondrial fragmentation. This result indicates that mitochondrial fragmentation under water stress in only observed in presence of additional copies of the Slm1 N-terminus, either just the N-terminus or in context of the full-length protein (Figure 7a and 7c).

In Slm1 knockout strain (AMY48), the N-terminus induced a 1.60-fold increase in mitochondrial fragmentation, whereas full length and the C-terminal region of Slm1 did not induce a significant increase (1.10- and 1.13-fold increase, respectively; Figures 7b and 7c).

a

WT (6210)

········ 25%~75%
– – Median Line

Orange: Water for 5 mins
Green: Control

Mitochondria per Cell (pieces)

| N-term | N-term | Full Lgth | Full Lgth | C-term | C-term |

Difference in Median   1.91 fold   1.75 fold   1.00 fold

b

Slm1 Knockout (AMY48)

········ 25%~75%
– – Median Line

Orange: Water for 5 mins
Green: Control

Mitochondria per Cell (pieces)

| N-term | N-term | Full Lgth | Full Lgth | C-term | C-term |

Difference in Median   1.60 fold   1.10 fold   1.13 fold

Figure 7. Mitochondrial Fragmentation is Concentration-dependent to N-terminus of Slm1. Water treatment for 5 minutes is applied to wild type (SEY6210) or Slm1 knockout strain (AMY48) with N-terminus, full length, or C-terminus of GFP-tagged Slm1 transformed in a plasmid; mitochondrial marker plasmid (pLG13) is co-transformed for imaging mitochondria. (a) (b) present the raw data of mitochondria per cell counted from the original 30 cells in microscopic pictures. (c) present the increase in median of mitochondrial fragmentation in each group. An increase >1.5 fold is considered significant.
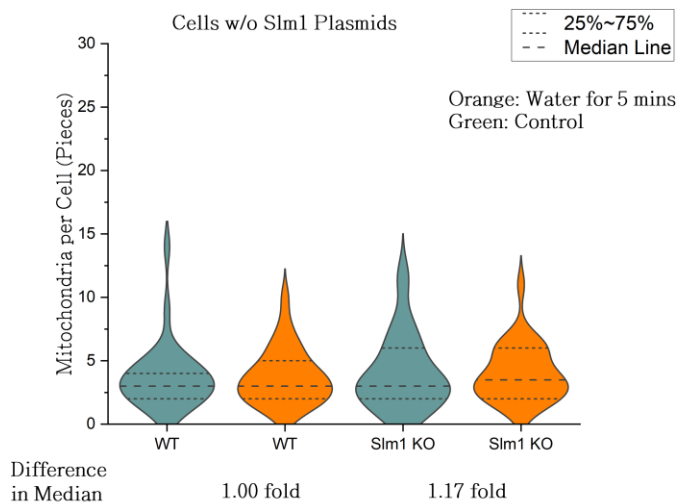
c

Slm1 KO (AMY48)
WT (6210)
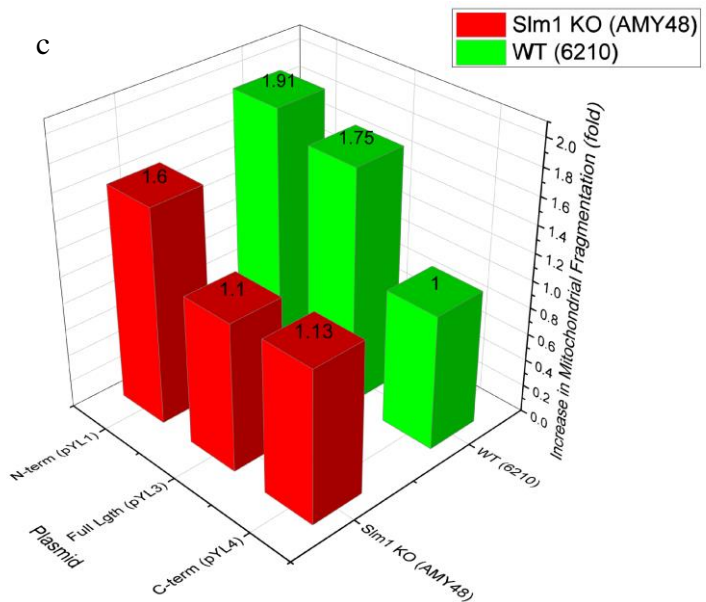
In the wild type strain, which contained the genomic copy of Slm1, mitochondrial fragmentation occurred under water shock as long as an additional copy of Slm1 N-terminus was present, with or without the its C-terminus. In the Slm1 knockout strain (AMY48), however, the genomic copy of Slm1 was not present. The full length Slm1 plasmid (pYL3) complemented the loss of genomic SLM1 and but did not yield significant mitochondrial fragmentation. The only difference between the wild type strain (SEY6210) and the Slm1 knockout strain (AMY48) was the presence or absence of genomic Slm1, thus it could be deduced that the concentration of either Slm1 as a whole or the N-terminus of Slm1 is necessary to drive mitochondrial fragmentation.

If the concentration of full length Slm1 was responsible for mitochondrial fragmentation, the N-terminus of Slm1 plasmid (pYL1) alone in the Slm1 knockout strain (Amy48) would not be sufficient to increase mitochondrial fragmentation, for that the genomic full-length version of Slm1 was lacking in the cell. Our data in Slm1 knockout strain (AMY48) clearly did not agree with such assumption.

On the other hand, if the concentration of the N-terminus of Slm1 alone was sufficient to cause mitochondrial fragmentation, the N-terminus Slm1 plasmid (pYL3) transformed into Slm1 knockout strain (AMY48) might be enough to hit the critical concentration that triggers mitochondrial fragmentation. And that was what we observe in the experiment. As a result, we hypothesize that the N-terminus of Slm1 is the key factor for occurrence of mitochondrial fragmentation.

As for why the full length Slm1 plasmid (pYL3) in the Slm1 knockout strain (AMY48) was not enough to increase mitochondrial fragmentation, we reason that the plasmid copy of full length Slm1 was not enough to achieve critical concentration locally. But with both the genomic copy and the plasmid copy of full length Slm1 present, Slm1 seemed to be sufficiently overexpressed to cause the mitochondrial phenotype. This aligns with our observation that full length Slm1 plasmid cannot cause mitochondrial fragmentation in the Slm1 knockout strain (AMY48) but can cause mitochondrial fragmentation in the wild type strain (SEY6210).

To further confirm our hypothesis, we perform 5-minute water stress on wild type (SEY6210), Slm1 knockout (AMY48), and Slm2 knockout (AMY71) strains, with only the mitochondrial marker plasmid but not any Slm1 plasmid. If the concentration of the N-terminus of Slm1 is really the key to mitochondrial fragmentation, none of the strains should be able to cause mitochondrial fragmentation, for that the innate Slm1 copy is not enough to reach the critical concentration to cause mitochondrial fragmentation. The data, as shown in Figure 8, aligns completely with our prediction by

Figure 8. Mitochondrial Fragmentation in Cells without Slm1 Plasmids. Water stress for 5 minutes is applied to cells with only the mitochondrial marker plasmid (pLG13). There is no significant increase in mitochondrial fragmentation. This result aligns with our hypothesis that mitochondrial fragmentation results from high local concentration of the N-terminus of Slm1.

showing a median of 3 to 3.5 pieces of mitochondria per cell that is typical of normal cells, with no significant increase in mitochondrial fragmentation across any of the groups.
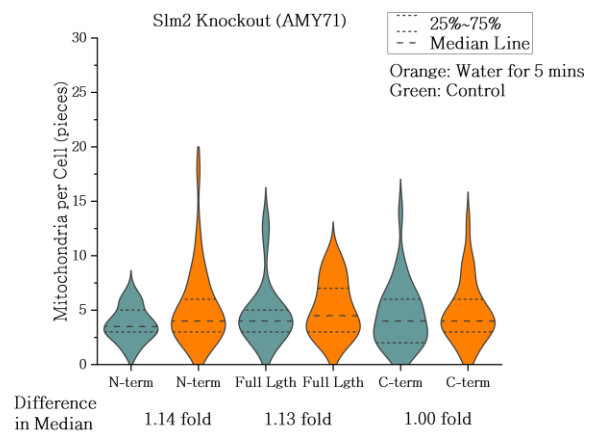
Slm2 is a Co-requisite for Mitochondrial Fragmentation

As mentioned in the introduction, Slm1 has a paralog in yeast, Slm2, that is thought to function together with Slm1. To test whether Slm2 also plays a role in mitochondrial fragmentation, the same experiment was performed in a Slm2 knockout strain (AMY71). We transformed the Slm2 knockout strain (AMY71) with the same three GFP-tagged Slm1 segments. The transformed cells were treated with water for 5 minutes and viewed under the microscope. The increase in mitochondrial fragmentation was then quantified as previously described.

As shown in Figure 9, no significant increase in mitochondrial fragmentation is observed in any of the Slm2 knockout strain. This result indicates that Slm2 is a co-requisite for the increase of mitochondrial fragmentation to occur under water stress.

Modeling of the Stress Response Pathways

The results so far had led us to a model, in which different domains of Slm1 promotes different localization, competing and reaching different equilibria under different environmental stresses (Figure 10). This model agrees with most previous research. Under normal condition, the BAR and PH domains of Slm1 recognizes eisosomes (Olivera-Couto et al., 2011). Eisosomes sense plasma membrane stresses and flatten in response (Appadurai, et al., 2020; Berchtold et al., 2012; Kabeche et al., 2015; Moharir et al., 2018), resulting in releasing Slm1 from the MCC. With mechanical stress or tension on the plasma membrane, Slm1 re-distributes to the MCT which requires the PH domain but not the BAR domain. This re-distribution triggers sphingolipid synthesis, relieving the tension on the plasma membrane in response to the stress (Berchtold et al., 2012). When water brings a combination of stresses altogether to yeast, the effect of the N-terminus of Slm1 becomes dominant and re-localize Slm1 to mitochondria. At mitochondria, Slm1 could potentially tune the metabolism of the cells, or perform some other unknown function. When artificially exacerbated (additional copies of Slm1 or its N-terminus), this effect on mitochondria leads to mitochondrial fragmentation. Fragmentation is known to occur when mitochondrial fusion is impaired or by loss of the mitochondrial membrane potential (references). However, this effect of Slm1 requires the presence of Slm2, suggesting that Slm2 cooperates with Slm1 for its role in mitochondrial regulation.

Figure 9. Slm2 is a Co-requisite for Mitochondrial Fragmentation. 5-minute water stress is applied to Slm2 knockout cells (AMY71) transformed with GFP-tagged N-terminus, full length, or C-terminus Slm1; RFP mitochondrial marker is co-transformed. There is no significant increase in mitochondrial fragmentation, indicating that Slm2 is required for mitochondrial fragmentation.
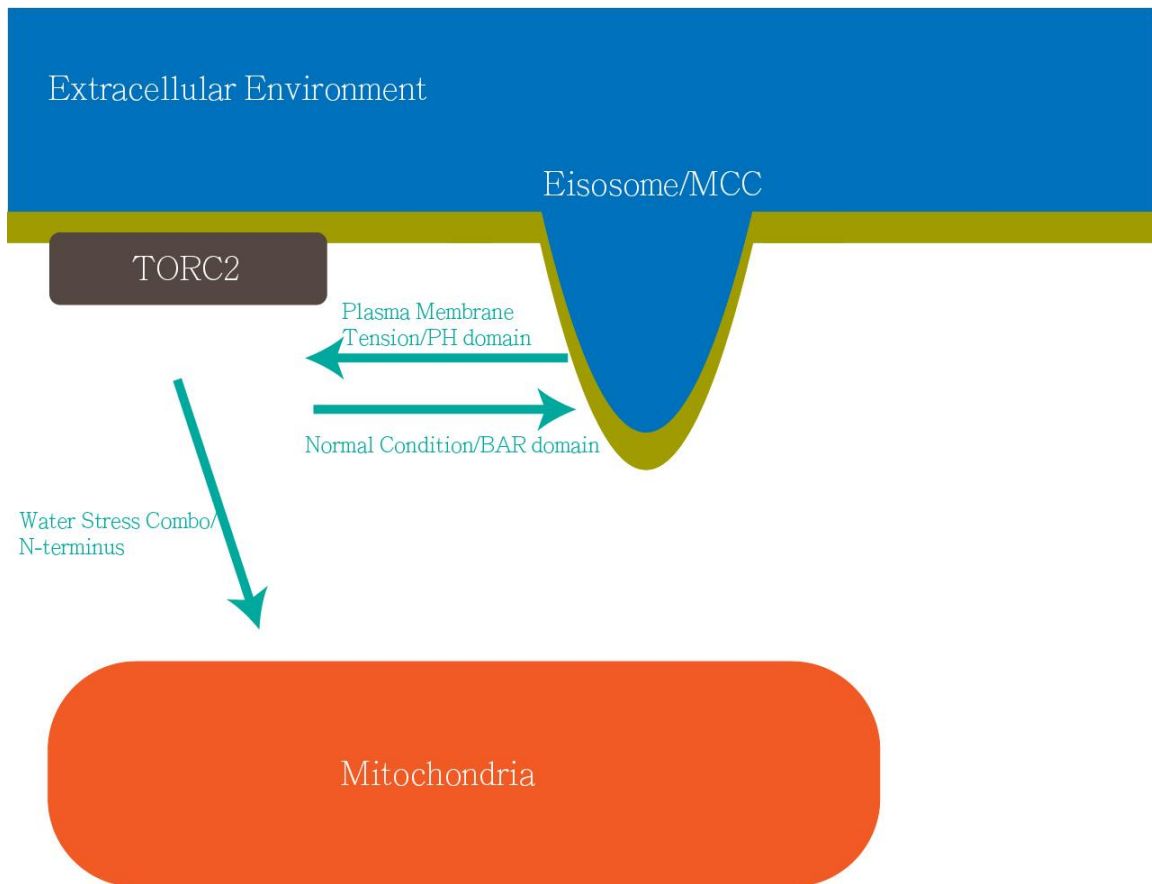
Figure 10. Equilibrium Model of Slm1 Localization. The movement of Slm1 is labelled with green arrows. The text next to the arrow explains the stress that causes the localization, and the domain of Slm1 responsible for the localization. Under normal growth condition, Slm1 localizes to the eisosome, with BAR domain having the dominant effect. When plasma membrane tension is applied, eisosomes flatten, making the PH domain dominant in the equilibrium. This allows Slm1 to go to TORC2, triggering sphingolipid synthesis in response. The combination of stresses in water shifts the equilibrium to favor mitochondria, where Slm1 performs an unknown function.

Hypo-osmolarity and Glucose Starvation Together Trigger the Response that Peaks at 5 Minutes after the Stresses

As we hypothesized in the model, Slm1 could function in the regulation of mitochondrial metabolism. For the model to hold true, the environmental stresses brought by water is unlikely to be as simple as membrane tension, which could be caused by hypo-osmolarity alone. The involvement of change energy source abundance will probably play a role is the combination of stresses.

To understand the exact environmental stress that led to mitochondrial fragmentation by the Slm1 N-terminus, we repeated the microscopy analyses with the same strains as in the previous section, but under different stress conditions.

As an initial control, yeast was stressed with water for 2, 5, and 10 minutes to determine mitochondrial fragmentation over time. In this profile, we observed the highest mitochondrial fragmentation at 5 minutes, peaking at 1.75-fold higher than its control without stress (Figure

11). This profile was then compared with the fragmentation over time profile of various candidate stresses.

Hypo-osmotic stress was the first candidate to be tested, for that hypo-osmolarity can lead to an increase in plasma membrane tension, and that membrane tension has been reported previously to cause the re-localization of Slm1 to TORC2/MCT (Berchtold et al., 2012). For these experiments, we grew the strains in presence of 1M sorbitol, an osmolyte that cannot be metabolized by yeast cells (Refer to "hyper osmotic SD" and "treatments" in the Methods section for details.), and then hypo-osmotically stressed the cells by switching them into growth medium without sorbitol. (For details, please refer to Methods.) This treatment ensured no extra stresses were applied along with hypo-osmotic stress. As expected, hypo-osmolarity alone did not cause mitochondrial fragmentation in the presence of Slm1 at any time point (Figure 11).

Glucose starvation was the next stress to be tested because a change in glucose level is an obvious reason for the cell to tune mitochondrial activity. We applied the stress by replacing the glucose in the medium with the same concentration of sorbitol during the treatment (starvation SD; refer to the Methods section for details.). This treatment maintains the osmolarity in the medium while depleting glucose. The result indicated no increase in mitochondrial fragmentation in 2 and 5 minutes, and even a slight decrease in 10 minutes (Figure 11). Since the ratio was within 0.3 from 1, we reason that the decrease is not significant, and most likely due to experimental errors.

We then tested the combination of hypo-osmotic stress and glucose starvation. The stress was performed by growing the cells in medium containing 1M sorbitol and glucose, then replacing the medium with the medium used in the starvation stress (see Methods). The resulting mitochondrial fragmentation over time profile aligned with that of water stress (Figure 11), indicating that within the arsenal of stresses induced by water, the exact combination of stresses responsible for Slm1's function at mitochondria is hypo-osmolarity combined with glucose starvation.

This result aligned with our model, in which the stress proposed for causing Slm1 to move to mitochondria (and leading to mitochondrial fragmentation) is somewhat related to metabolism, and more complicated than simple plasma membrane tension.
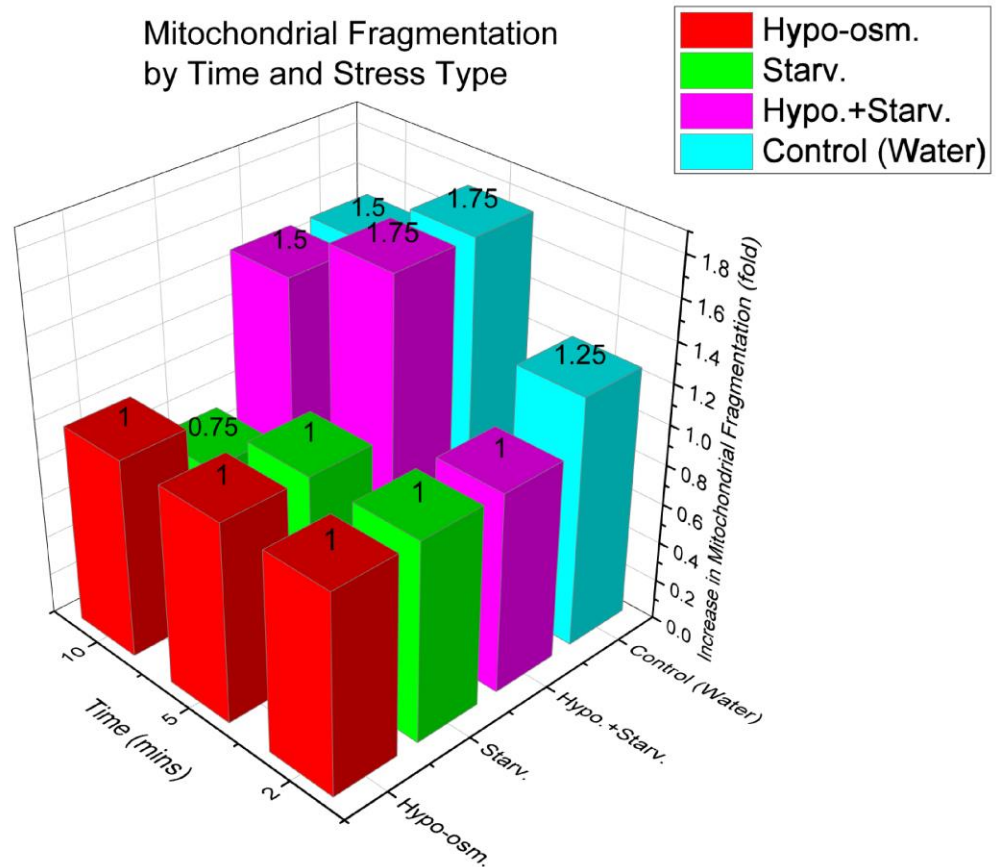
Figure 11. Mitochondrial Fragmentation Profile of Water, Hypo-osmolarity, Glucose Starvation, and Their Combination. Various stresses of interest are applied on wild type cell (SEY6210) with full length Slm1 marker (pYL3) and mitochondrial marker (pLG13). The stresses are applied for 2, 5, and 10 minutes to obtain a fragmentation profile over time. Hypo-osmolarity and glucose starvation alone are unable to cause significant mitochondrial fragmentation, while their combination yields a fragmentation profile almost identical to that of water. This result indicates that hypo-osmolarity combined with glucose starvation is responsible for mitochondrial fragmentation.

DISCUSSION

Our findings on Slm1's N-terminus and the requirement of Slm2 led us to the equilibrium model presented in the result section (Figure 10) that can explain the different Slm1 localizations observed. And yet, physiological relevance of the model still needs to be examined.

Under normal condition, Slm1 localize to eisosomes (Olivera-Couto et al., 2011) as a stress sensor (Figure 12a). Eisosomes flatten under a variety of stresses including proton gradient loss, mechanical tension, etc. The flattening of eisosomes forces proteins containing BAR domain to leave the membrane furrow, including Slm1 (Appadurai, et al., 2020; Berchtold et al., 2012; Kabeche et al., 2015; Moharir et al., 2018). According to our data, we hypothesize that eisosome flattening acts as a stress signal, a signal that Slm1 relays to stress response pathways. However, the target of Slm1 regulation is not determined solely by the loss of BAR domain interaction. The exact stress condition at the time might create a physiological environment that favor one target over the others (Figure 12b). In the case of membrane tension, Slm1 re-localize to the MCT, as reported (Berchtold et al., 2012) (Figure 12c). In the case of hypo-osmolarity and glucose starvation at the same time, Slm1 localizes to mitochondria.

Analogous to the localization to TORC2, it is likely that the localization of Slm1 to mitochondria affects the activity of this target (Figure 12d). However, our study so far has not given insight into the regulation of mitochondria during water stress or the reason why yeast requires to regulate their mitochondria during this stress condition. Furthermore, we cannot rule out the possibility that TORC2 activation might also plays a role in the water stress induced mitochondrial regulation. (Figure 12e).

Finally, even though mitochondrial fragmentation is dependent on artificial overexpression of the Slm1 N-terminus, this phenotype might inform us about the potential function of Slm1 in regulating mitochondria. Over-expression might simply exaggerate the protein's function that resemble its true function in a more perceivable manner. Our data in this study exhibits two distinct groups of mitochondrial fragmentation ratio across all parameters with no obvious outliers, which suggest a specific function rather than a simple artifact. Furthermore, glucose starvation being one of the required stresses has provided logical support to the necessity of recruiting mitochondria. As a result, it is likely that Slm1 has an unrevealed function with mitochondria.

How Slm1 localizes to mitochondria is another topic to be studied. So far, we have shown that the N-terminus of Slm1 is essential to the localization, so is the presence of Slm2. However, similar N-terminus domain study has not been performed on Slm2. Therefore, whether or not Slm2 only requires its N-terminus to cause mitochondrial localization and fragmentation remains unknown. The exact site/sites in the N-terminus that is responsible for Slm1's localization to mitochondria has also not yet been studied. Performing a mutation screening on Slm1's N-terminus may inform us about the exact sequence responsible for the interaction with mitochondria. These studies might also inform us about the mechanism by which Slm1 recruitment to mitochondria is regulated, be it phosphorylation, ubiquitination, or protein phase separation.

Most importantly, the purpose of Slm1's localization to mitochondria is yet to be studied so far. While directly looking for the consequence of Slm1 knockout cells under water stress might inform us about Slm1's purpose at mitochondria, some of the unanswered question mentioned earlier would certainly be the steppingstones to answering the role of Slm1 in mitochondrial function.
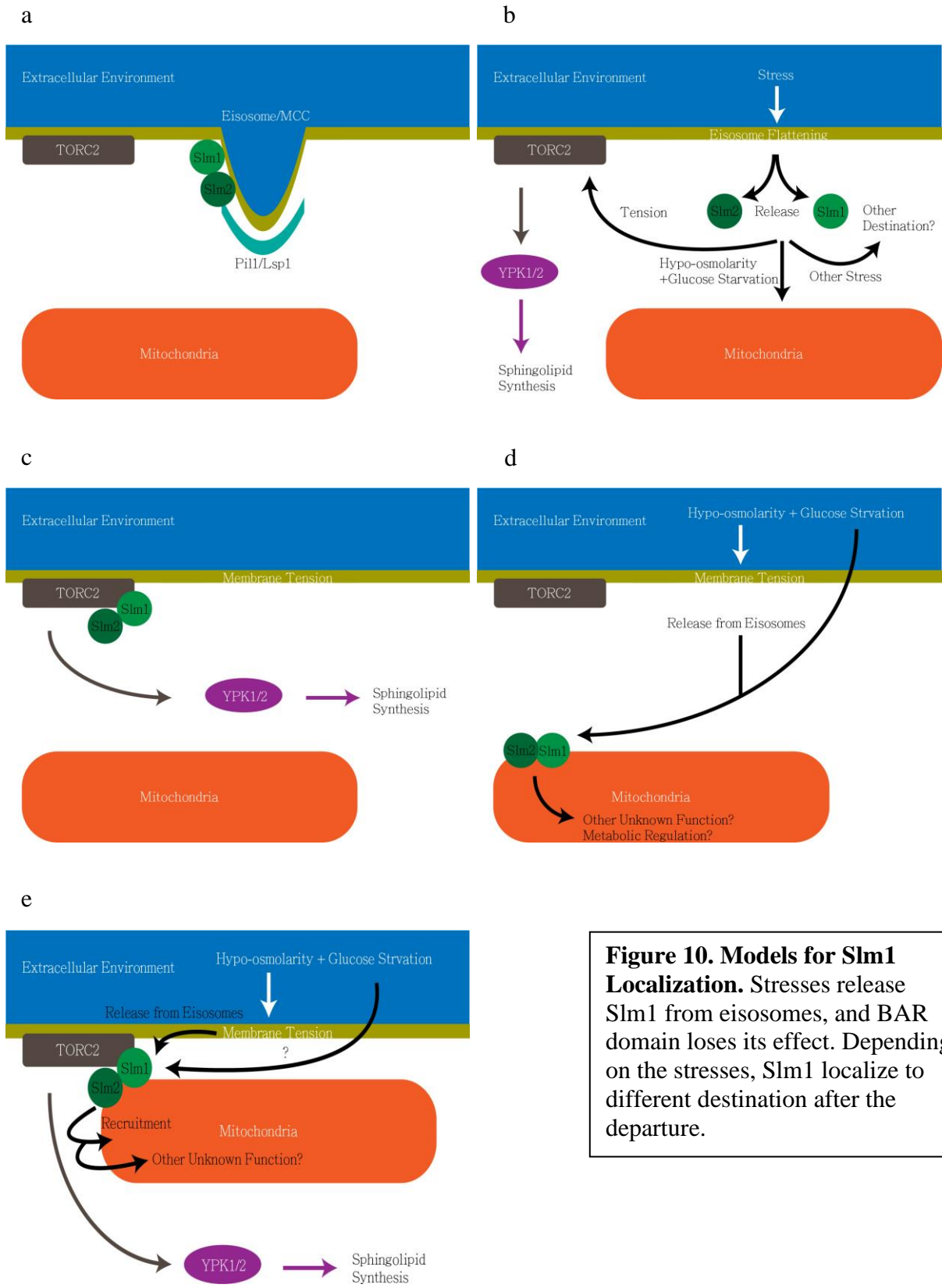
Figure 10. Models for Slm1 Localization. Stresses release Slm1 from eisosomes, and BAR domain loses its effect. Depending on the stresses, Slm1 localize to different destination after the departure.

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