UNIVERSITY OF CALIFORNIA, SAN DIEGO

Activation of the MAP kinase, Slt2, by perturbations in the state of the endoplasmic reticulum

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in

Biology

by

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Committee in charge:

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Professor Colin Jamora
Professor James Wilhelm

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The Thesis of Joel Tourtellotte is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

Arrest of secretion response (ASR)

Caspofungin (Cp)

Cell Wall Integrity (CWI)

Cortical ER (cER)

Dithiothreitol (DTT)

Endoplasmic reticulum (ER)

ER associated protein Degradation (ERAD)

ER surveillance (ERSU)

Eukaryotic initiation factor 2α (eIF2α)

Fluorescence activated cell sorting (FACS)

Guanine nucleotide Exchange Factor (GEF)

Temperature sensitive (ts)

Tunicamycin (Tm)

Unfolded Protein Response (UPR)

UPR Responsive Element (UPRE)

Wild type (WT)

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We thank David Levin for *mkk1/2ΔΔ, bck1Δ,* and *pkc1Δ* strains. As well, we thank Peter Novick for *sec1-1* strain.

Chapter 2 is modified from material that has been submitted for publication. The authors are Anna Babour, Alicia Bicknell, Joel Tourtellotte, and Maho Niwa. In this chapter, I conducted the experiments in Figures 2.2B, 2.3, 2.4 B, 2.5 A, B, 3.1, 3.2, 3.3, and 3.4. Anna Babour conducted experiments in Figures 1.1 A, B, C, and 2.4 C. Alicia Bicknell conducted experiments in Figures 2.2 A, 2.4 A, and 2.5 C. Maho Niwa supervised the work.

ABSTRACT OF THE THESIS

Activation of the MAP kinase, Slt2, by perturbations
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Master of Science in Biology

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Professor Maho Niwa, Chair

The endoplasmic reticulum (ER) is the site of lipid biosynthesis and secretory protein folding, which must be adjusted to accommodate the physiological needs and stresses of the cell. Since ER cannot be made *de novo*, it must be inherited faithfully. During decreased ER function cortical ER and cell cycle progression are delayed by the ER surveillance (ERSU) pathway, which is mediated by the MAP kinase Slt2. We have identified the mechanism through which the accumulation of unfolded

proteins, ER stress, activates Slt2. This response is separate from the canonical ER stress response, the unfolded protein response (UPR). Furthermore, this mechanism is distinct from the cell wall integrity (CWI) pathway, the canonical Slt2 activation pathway. Activated Slt2 has many cytoplasmic and nuclear targets, including the transcription factor Rlm1. We find that Rlm1 aids in cell survival during conditions of decreased ER function, but does not play a role in the ERSU pathway.

INTRODUCTION

The ER and its function

The endoplasmic reticulum (ER) is a membrane-bound, cellular organelle that is continuous with the outer nuclear membrane. The ER has many important cellular functions. First, the ER stores calcium, which important for several signaling cascades (Bonilla 2003). Second, the ER houses lipid biosynthesis enzymes, which can affect the overall abundance of membrane throughout the cell (Daum 1998). Third, the ER associated protein degradation (ERAD) affects the cellular proteosome by removing misfolded proteins from the ER and marking them for destruction (Hampton 2002). Lastly, the ER is the site for protein folding of secretory and plasma membrane proteins. These proteins are translocated into the ER during translation and must be folded properly in order to function. The ER's resident chaperones and reducing environment allows for proper folding to occur. Secretory and plasma membrane proteins are important for physiological functions like neurotransmission, hormone release, and secretions (i.e. antibodies for B cells and digestive enzymes). The cellular demand of the ER function changes depending upon environmental and developmental cues. Thus eukaryotic cells have developed strategies to recognize and respond to changes in the functional demands of the ER in order to maintain ER functionality.

The UPR

The ER has a basal level of ER functionality, which can be overwhelmed when there is an increase in ER functional demand (ER stress). One example of ER

stress, which overwhelms the ER's ability to fold proteins, is an accumulation of unfolded or misfolded proteins. Eukaryotic cells have developed a response that efficiently alleviates this stress. The response to misfolded proteins is termed the unfolded protein response (UPR). In *Saccharomyces cerevisiae* (budding yeast) an ER resident, transmembrane protein, Ire1p, monitors for unfolded proteins. The ER lumenal domain of Ire1p senses an accumulation of unfolded proteins and dimerizes with other activated Ire1p. The cytosolic domain contains a kinase domain that autophosphorylates, which in turn activates an endoribonuclease domain (Cox et al 1993). This endoribonuclease splices a 252-nucleotide intron from HAC1, a cytosolic mRNA. This splicing event allows for efficient translation of the HAC1 into Hac1p. The amount of HAC1 splicing is commensurate with the degree is ER stress. Hac1p is transported into the nucleus where it upregulates transcription of genes (Cox and Walter 1996) with a UPR response element (UPRE) promoter sequence. The gene products alleviate the ER stress. The Ire1/Hac1 system regulates a multifaceted stress response. Folding capacity increases (Travers et al 2000) due to an increase of chaperones and co-chaperones, increase in ER volume by lipid biogenesis, and an increase in ERAD components. The mammalian UPR is more complex. As well as Ire1, which is conserved in all eukaryotes, PERK and ATF6 are proteins that sit in the ER and are activated upon ER stress. PERK inhibits translation by phosphorylating eukaryotic initiation factor 2α (eIF2α), an important translation initiation regulator, while ATF6 is another integral transcription factor.

There are several pharmacological drugs that induce ER stress. In yeast and mammalian cells tunicamycin (Tm) inhibits N-linked glycosylation and dithiothreitol (DTT) disrupts dilsulfide bonds, resulting in accumulation of folding intermediates lacking either N-glycosylation or disulfide bonds, which leads to induction of the UPR. In addition thapsigargin in mammalian cells blocks calcium transport into the ER, and calcium is an important co-factor for chaperone function. In yeast a mutation in the *ERO1* (ER oxidoreductin I), *ero1-1*, gene causes an accumulation of unfolded proteins due to inefficient disulfide bond formation. In this study Tm is the most used ER stress inducer, although all experiments were reproduced in either DTT-treated or *ero1-1* cells.

ER in budding yeast

The peripheral ER of budding yeast forms a cortical network similar to animal cells (Voeltz *et al.* 2002). However, yeast ER consists of two domains, a cortical ER (cER), which is located along the plasma membrane, and a perinuclear ER, which is continuous with the outer nuclear membrane (Rose *et al.* 1989, Preuss *et al.* 1991). Some ER resident proteins are localized throughout both these two domains (Preuss *et al.* 1991, Rossanese *et al.* 1999). It is not known whether these two domains are functionally different.

Many studies have characterized the process by which ER is inherited in budding yeast. In late G1, cER is transported along actin cables by the type V myosin motor, Myo4 (Estrada *et al.* 2003), and attached to the exocyst complex in the daughter bud (Wiederkehr *et al*. 2003). Many of the proteins that are required for

this process have been found, although how each participates is not yet clear. After attachment to the exocyst complex, cER is propagated along the daughter cortex (Fehrenbacher *et al.* 2002). During mitosis, the perinuclear ER is transported into the daughter cell in a microtubule dependent manner (Fehrenbacher *et al.* 2002). The mechanisms that regulate the movement and growth of both cortical and perinuclear ER during the cell cycle is not well known. The importance of regulating ER and its inheritance is exemplified by the fact that it cannot be made *de novo*.

ER function during cell cycle

Traditionally, the UPR has been characterized during conditions of large amounts of unfolded proteins and was thought to be a stress response. However, previously Alicia Bicknell, from our lab has shown that UPR is required for efficient cytokinesis during vegetative growth. It is not known whether there are other cell cycle events that require ER functionality. Bicknell used fluorescence activated cell sorting (FACS) to measure the amount of DNA content within a cell. The fluorescence associated with the yeast haploid cells with one copy of the genome is labeled 1C, while the amount of fluorescence, which corresponds to two copies of genomic DNA is labeled 2C. A 2C amount of DNA indicates that the cell has progressed though S phase. Ultimately, cells undergo cytokinesis and cell separation, which generates cells with 1C of DNA. Wild type (WT) cells treated with an ER stress-inducing drug replicated DNA normally but eventually accumulated DNA content of 3C and 4C. The cells with either 3C or 4C DNA had gone through nuclear division, but not cytoplasmic separation, and each nuclei initiated the next

round of DNA replication even though no cytokinesis took place. Therefore, throughout this study we used the appearance of 3C or 4C DNA peaks as a sign of cytokinesis defect. Based on these studies, we hypothesized that ER stress or elevated demands of the ER functions cause cells to be blocked in cytokinesis.

There are many nuclear checkpoints that ensure that genetic material is transmitted faithfully and error free. Little is known about checkpoints that monitor cytoplasmic elements. ER stress is toxic to the cell, and cannot be made *de novo*. Thus it seemed possible for an ER checkpoint to exist.

Molecular basis for cell cycle arrest

A researcher in our lab, Anna Babour, searched for a molecular basis for this cell cycle arrest by ER stress and found that septins are mislocalized during ER stress (Figure 0.1 WT).

Septins are GTPase proteins that are conserved in fungi and metazoa, and a septin-like protein is found in bacteria (Leipe *et al.* 2002). In yeast, septins form a ring at the mother-daughter budneck (Longtine, Bi. 2003). There are five mitotic subunits in budding yeast: Cdc3, Cdc10, Cdc11, Cdc12, and Shs1 (Versele, Thorner. 2005). How these subunits interact to make polymers is still unclear, although x-ray crystallography has been performed on certain polymers (Bertin *et al.* 2008). In yeast septin forms a dynamic ring at the bud site late in G1. When the bud emerges, septin forms stable collar. Upon cytokinesis, the ring splits and becomes dynamic and can fully disperse (Caviston *et al.* 2003, Dobbelaere *et al.* 2003). The septin complex in yeast acts as a diffusion barrier by restricting movement of ER membrane proteins

through the bud neck (Luedeke *et al.* 2005). As well septins have been shown to function as a scaffold for recruitment of other proteins (Kinoshita 2006). In addition, its best-known function is in cytokinesis (Longtine *et al.* 2003). The function of septin varies tremendously from organism to organism.

As mentioned above, Dr. Babour found that in addition to a delay in cytokinesis, ER stress caused an alteration of septin. Under normal growth conditions, septin is localized to the bud neck, but under ER stress conditions septins were observed distal from the bud neck.

cER inheritance

What was the function of delaying cytokinesis and altering septin morphology during ER stress? In order to further investigate this question, Dr. Babour tagged ER resident proteins, and observed that cER inheritance is delayed under ER stress conditions. Perinuclear ER inheritance was unaffected by the addition of ER stress. Thus it appeared as though, the delay in cell cycle was in place in order to prevent the stressed cER from entering the daughter bud. cER eventually enters the daughter cell, presumably after the ER is returned to homeostasis.

The UPR is not responsible for the cytokinesis defect, alterations in septin, and delay in cER inheritance

Cytokinesis, septin morphology, and cER delay were all being observed under ER stress conditions. Surprisingly, the canonical UPR consisting of Ire1 does not mediate the defect in cytokinesis, the septin alteration, or the cER inheritance delay that is observed during ER stress conditions. FACS analysis of *ire1Δ* cells

illustrates that there is no appearance of the 3C and 4C peaks, indicating that these cells are undergoing efficient cytokinesis. In addition, septin GFP tagged *ire1Δ* cells displayed aberrant septin morphology (Figure 0.1 *ire1Δ*). Lastly, cER inheritance was not transmitted immediately to the daughter cell in *ire1Δ* cells. They therefore conclude that Ire1 is not mediating these cellular changes. Ire1 is the only known sensor that responds to the state of the ER, what, then, could be mediating the above phenotypes?

Slt2 regulates the cytokinesis defect, alterations in septin, and delay in cER inheritance

Taken together, Drs. Babour and Bicknell found that during ER stress, WT cells display three phenotypes: a delay of both cytokinesis and cER inheritance, as well as alteration of septin structures. They theorized that these phenotypes were not separate phenomena, but a mechanism that monitors the state of the ER and delays cER inheritance during periods of decreased ER function. Since Ire1 does not mediate these three phenotypes we searched for candidates that might. Our selection criteria for these candidates consisted of components that were connected to the ER or UPR and involved in any of the ER stress associated phenotypes: cER inheritance, cytokinesis, and septin structure regulation. One protein that fit our criteria was a MAP kinase, Slt2. Slt2 provides resistance to the ER stress inducing drug Tm (Chen *et al.* 2005), plays a role in cER inheritance (Du *et al.* 2006), and when knocked out is synthetically lethal with a septin mutation (Longtine *et al.* 1998). While these previous studies point to a genetic interaction of Slt2, little has been shown the

molecular mechanisms of Slt2 in each case. We predicted that a candidate would have 1C and 2C peaks by FACS indicating a normal cytokinesis progression, as well as septin localized to the budneck as seen in untreated WT cells, and lastly normal cER inheritance kinetics. When *slt2Δ* cells were treated with an ER stressor, this is what was seen: cytokinesis occurs normally, septin is localized to the budneck (Figure 0.1 *slt2Δ*), and cER is transmitted to the daughter cell. They hypothesized that these alterations during ER stress were initiated in order to ensure that a functional ER was inherited in the newly formed cell. This inheritance is required for cell survival, which is evident in a growth assay that measures the ability of a strain to grow. In this case, cells are plated in a series of fold dilutions on a YPD plate containing 0.4 μg/ml of Tm. This was verified by staining *slt2Δ* cells with a vital dye that measures the metabolic activity of a cell. *slt2Δ* populations were undergoing high amounts of cell death during ER stress compared to WT. It appears that this mechanism monitors the condition of the ER and prevents its transmission into the newly formed daughter cell. Only once the ER stress has been alleviated can the ER be transmitted into the daughter cell. We term this pathway ER sureveillance (ERSU).

Slt2p is a MAP kinase and its ability to be phosphorylated and phosphorylate other proteins is required for its function. This protein is known to be activated during disruptions in the cell wall (cell wall stress) by the cell wall integrity (CWI) pathway. Ultimately Slt2 affects the genetic expression of the cell in order to cope with the cell wall stress. The CWI pathway is initiated by plasma membrane

receptors that sense the cell wall disruptions through Wsc1 and Mid2 mainly, but also through Wsc2, Wsc3, and Mtl1 (Rajavel *et al.* 1999, Verna *et al.* 1997, Ketela *et al.* 1999). The receptors bind with a Rho1 GEF, Rom2 and Rom1, which allows the GEF to switch Rho1 into its active form Rho1-GTP (Ozaki *et al.* 1996). Rho1 in turn activates the kinase cascade by activating Protein kinase c (Pkc1) (Nonaka *et al.* 1995). Pkc1 phosphorylates Bck1, which phosphorylates Mkk1 and Mkk2. Mkk1 and Mkk2 directly activate Slt2. One of the known targets of Slt2 is Rlm1, a transcription factor that regulates cell wall constituents and repair enzymes. One of Rlm1's target genes is *SLT2*, representing a positive feedback loop (Jung, Levin 1999). Whether ER stress affects the cell wall and the CWI signaling has not been well characterized.

In addition the CWI components Wsc1, Wsc2, and Pkc1 participate in response to an arrest of secretion (arrest of secretion response: ASR). Upon secretion arrest, Wsc1 and Wsc2 activate Pkc1, which mediates the translocation of nuclear proteins and reversibly perturbs nuclear import. It is theorized that this biosynthetic economy helps the cell cope during inhibited secretion (Nanduri, Tartakoff 2001). More importantly to this study, the current model of Slt2 activation by CWI is due to plasma membrane stretch. The drug chlorpromazine causes membrane stretch and is a potent activator of Slt2 (Kamada *et al.* 1995). In addition, increased extracellular osmolarity, which decreases outward membrane stretch by neutralizing turgor pressure, diminishes Slt2 activation by various inducers (de Nobel *et al.* 2000). Presumably a block of secretion, which prevents the plasma

membrane from replenishing lipids, would cause plasma membrane stretch. The relationship between an arrest of secretion and CWI has not been well characterized. Furthermore, a connection between ER stress and arrest of secretion has never been shown.

Summary

This study examines the mechanism of Slt2p activation during ER stress. Since UPR has been ruled out because ER stress induction in *ire1Δ* cells caused alterations in cytokinesis, septin morphology, and cER inheritance at the extent similar to WT cells. We investigate whether ERSU is distinct from other signaling pathways that may be activated during ER stress. Furthermore, we begin to characterize a transcription factor, Rlm1, which is activated directly by Slt2p.

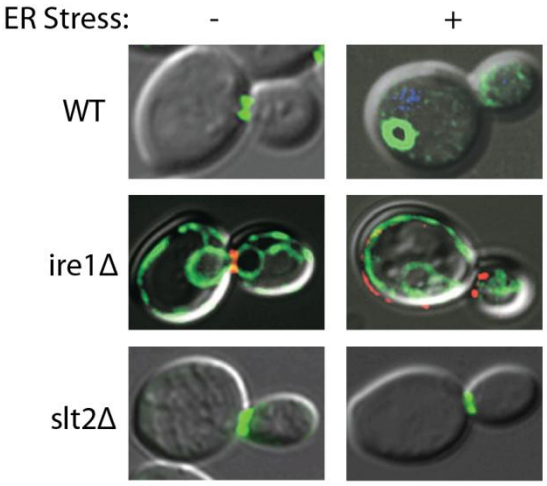


Figure 0.1. Septin alteration during ER stress is Slt2 dependent.
Aberrant septin morphology was seen in cells treated with a variety of ER stress inducers. Genomic loci encoding septin subunits, either SHS1 or CDC11, were C-terminally tagged with GFP for wild type and *slt2Δ* cells and with mCherry for *ire1Δ* cells. *ireΔ* cells also have the ER protein, Hmg1, tagged with GFP. ER stress was induced in asynchronous populations of cells with 1 μg/m1 Tm or 2 mM DTT, or in *ero1-1* cells by shifting to the non-permissive temperature 37 degrees Celsius. GFP merged with a DIC image is shown.

CHAPTER 1

ERSU is distinct from the CWI and ASR signaling pathways and require Wsc1, Pkc1, Bck1, Mkk1 and Mkk2 for activation

Introduction

MAP kinases affect many activities of a cell including responding to stress. A MAP kinase is activated by an extracellular signal, which transduces the signal to a MAP kinase via a kinase cascade. In yeast, the MAP kinase Slt2 is activated by a disruption in the cell wall, which is detected by plasma membrane sensors Wsc1, Wsc2, Wsc3, Mid2, and Mtl1 (Gray 1997, Verna 1997, and Ketela 1999, respectively). Wsc1 and Mid2 appear to be the most important (Rajavel 1999). These sensors activate Rom2 and Rom1, guanine nucleotide exchange factors (GEF) for Rho1, which loads Rho1-GDP with GTP. When Rho1 is loaded with GTP it is in its active form, and this active Rho1 thereby activates Protein kinase C (Pkc1) (Kamada *et al*. 1996). Pkc1 begins the kinase-activating cascade by phosphorylating Bck1, a MAP/ERK kinase kinase (MEKK). This activating phosphorylation allows Bck1 to activate Mkk1 and Mkk2, which are two redundant MAP/ERK Kinases (MEK). Mkk1 and Mkk2 activate slt2 by phosphorylation. This activation pathway is illustrated in Figure 1.1. Activated slt2 activates transcription factors like Rlm1 by phosphorylation, which upregulate target genes. The expressed target genes carry out different cellular processes, in the case of Slt2, cell wall maintenance and repair. Because of its function in repairing and maintaining the cell wall, this response has been termed the cell wall integrity pathway (CWI). A recent report suggests that

CWI activation leads to UPR induction (Scrimale 2009). Slt2 is activated during ER stress (Chen 2005), but little is known about how it is activated and what it does upon activation. The latter is beginning to be elucidated. Our lab has shown that Slt2 mediates ER surveillance (ERSU) by blocking cytokinesis and cER inheritance, as well as, altering septin (from Introduction). These processes are required for cell survival under ER stress causing conditions. What factors mediate each phenotype and how each is connected to another are still unknown.

In addition to the CWI pathway described above, the arrest of secretion response (ASR) utilizes similar components for signaling. When secretion is blocked, the ASR reversibly inhibits nuclear export, and downregulates transcription of components of the ribosome and tRNA (Nanduri 2001). In the same study, ASR was mediated by known CWI components (discussed above) Wsc1 to a lesser extent, Wsc2 and Pkc1. Whether arrest of secretion occurs during ER stress has not been investigated.

We first investigate whether the well-known CWI is the pathway through which Slt2 is activated during ER stress. Since Slt2 is involved in multiple pathways, we examined if ER stress causes activation of Slt2 via any of the known signaling cascades

Results

Slt2 activation is known to occur through the CWI, which consists of the upstream receptors Wsc1, Wsc2, Wsc3, Mid2, and Mtl1. Upon activation these receptors bind Rom proteins, which convert Rho1 into its active GTP-bound form. Rho1 initiates a kinase cascade by activating Pkc1. Pkc1 directly activates Bck1, and Bck1 activates Mkk1 and Mkk2, two redundant MEKs (Figure 1.1). These MEKs activate Slt2, which in turn activates transcription via Rlm1. In order to test whether these upstream components are also involved in Slt2 activation during ER stress, we analyze by western whether null mutant strains are able to phosphorylate Slt2.

Slt2 is a MAP kinase, and it requires the ability to be phosphorylated and kinase activity for its function. Dr. Bicknell tested growth during Tm treatment with strains carrying either Slt2 that is incapable of being phosphorylated or kinase dead. She found that these cells were very sensitive to Tm and concluded that phosphorylation and kinase activity were required for ERSU signaling. Using this same growth assay, we see that *mkk1/2ΔΔ, bck1Δ,* and *pkc1Δ* cells are very sensitive to ER stress due to their inhibited growth on Tm plates (Figure 1.2 A). Sorbitol is an osmotic stabilizer that assists in growth in CWI deficient cells. Although WT's colonies also decrease in robustness slightly, each null mutation shows little to no growth. In addition we tested whether these null mutations had any affect on phosphorylation of Slt2. We find that WT cells had a robust induction of Slt2 phosphorylation, while the upstream kinase mutants showed no increase in the amounts of phosphorylated Slt2 (Figure 1.2 B). Since *mkk1/2ΔΔ, bck1Δ,* and *pkc1Δ*

cells display sensitivity to Tm and are not able to phosphorylate Slt2 during ER stress, we conclude that Mkk1, Mkk2, Bck1, and Pkc1 are required for slt2 activation during ER stress.

Recall that in the CWI pathway, plasma membrane receptors bind Rom2 and Rom1, which allow GTP exchange in Rho1. Rho1-GTP, its active form, activates Pkc1 (Figure 1.1). In order to test whether Rho1 is involved in Slt2 activation during ER stress, we are currently running a Tm sensitivity assay as well as measuring Slt2 phosphorylation in *rho1-2* and *rho1-5* temperature sensitive (ts) mutants. We further ask whether Rom1 and Rom2 are involved in Slt2 activation during ER stress. We tested *Δrom1* and *Δrom2* on plates with Tm to assess if these strains display defective growth during ER stress. We see that they grow well under unstressed and ER stressed conditions (Figure 1.3 A). We also checked the amount of phosphorylated slt2 in these strains and see that Slt2 is highly phosphorylated (Figure 1.3 B). Thus we conclude that neither Rom2 nor Rom1 are playing a role in Slt2 activation during ER stress.

Rom2 has been shown to interact with the CWI membrane receptors. When this interaction was disrupted, the amount of activated Rho1-GTP was significantly decreased (Philip 2001), which activates Pkc1 (Figure 1.1). Even though Rom1 and Rom2 were shown to have no effect on Slt2 phosphorylation, we asked whether the known upstream receptors of the CWI had any effect on Slt2 activation during ER stress. The known receptors for the slt2 pathway are Wsc1, Wsc2, Wsc3, Wsc4, Mid2, and Mlt1 (Figure 1.1). In order to assess each receptors contribution to slt2

activation we obtained knockouts for each component and analyzed Tm sensitivity and phosphorylated Slt2 as above. Surprisingly, only *Δwsc1* showed a notable growth defect when grown on Tm plates (Figure 1.4 A). Furthermore, *Δwsc1* displayed diminished Slt2 phosphorylation during ER stress, while the absence of the other known receptors had no effect on Slt2 phosphorylation (Figure 1.4 B). In summary, Wsc1, but not Wsc2, Wsc3, Wsc4, Mid2, or Mtl1, is involved in Slt2 activation during ER stress. In addition the upstream components that are required for Slt2 activation during ER stress are Pkc1, Bck1, Mkk1, Mkk2, while Rom1 and Rom2 are not.

The above MAP kinase cascade was initially characterized as a response to disruption in the cell wall. Not only do drugs induce cell wall stress, but also cellular functions like vegetative growth or pheromone induced morphogenesis (Errede 1995). It has been hypothesized that ER stress causes activation of CWI (Scrimale 2009). We asked whether ERSU was activated by ER stress-induced cell wall stress. First we compared the components that activate Slt2 during ER stress (from above) to the activators during cell wall stress. Recall that cell wall stress activates Slt2 via the pathway in Figure 1.1. Notably, cell wall stress is transduced into the cell via Wsc1 and Mid2. As illustrated in 1.3, activation of slt2 during ER stress is dependent on Wsc1 only; *Δmid2* cells induced with ER stress do not show any effect on Slt2 activation. This implies that ERSU is triggered by a distinct set of sensors. Furthermore, the signals from the plasma membrane receptors activate Rom1 or Rom2, and therefore converge unto Rho1. Surprisingly, Figure 1.4 shows that the

activation of Slt2 is not dependent on Rom1 or Rom2. Therefore the mechanism of signaling is different for ERSU and CWI. Lastly, we use sorbitol in conjunction with Tm for growth assays for the upstream kinases. Sorbitol stabilizes the cell wall, and thereby inhibits CWI signaling. It has been shown that these mutant strains regain growth when treated with a cell wall stress inducing drug and Sorbitol (Chen 2009). From Figure 1.2 A, we see that Sorbitol does not rescue the growth defect during Tm treatment. We conclude this growth defect is not caused by CWI, but ERSU. In addition, there are differences in the components that ERSU and CWI utilize in order to activate Slt2, which leads us to conclude that ERSU and CWI are distinct signaling pathways.

Another signaling pathway that utilizes components of this cascade is the arrest of secretion reponse (ASR). When secretion is blocked or arrested, ASR is activated, which translocates many nuclear proteins into the cytoplasm and inhibits ribosomal RNA synthesis until secretion is restored. Activation of the ASR requires Wsc2 and Pkc1 (Nanduri 2001). Also it is not known whether any of the ER stress inducers cause an arrest of secretion.

In order to determine whether ERSU is distinct from the ASR, we obtained the ts mutant of *SEC1*, *sec1-1*, which blocks secretion from the golgi to the plasma membrane. At the permissive temperature (23 degrees), secretion occurs normally, but at the restrictive temperature (37 degrees) secretion is blocked. As mentioned earlier, an arrest of secretion was shown to downregulate rRNA, thus in order to assess whether secretion was indeed blocked, we measured the amount of mRNA for

a protein component of the large ribosomal subunit Rpl32 in the WT and *sec1-1* strains. We see that the amount of *RPL32* RNA decreases in *sec1-1* cells but not in WT (Figure 1.5 A), which indicates that secretion is arrested in *sec1-1* only. Since we are testing to see whether ASR and ERSU, which is caused by ER stress, are distinct, it is important that these cells are not undergoing ER stress. UPR has been shown to be active during heat shock (Kamada 1995), and in order to affect the *sec1-1* phenotype we shift the temperature to 37 degrees. We see that HAC1 is not being spliced in these cells, and thus we conclude that these cells do not have stressed ERs (Figure 1.5 B). In addition, we test to see if septin is altered, which is a downstream effect of Slt2 activation during ERSU. Under normal conditions, septin is localized to the bud site or bud collar during the cell cycle, except just before cytokinesis (Figure 1.5 C). On the other hand, ER stress causes an aberrant septin morphology where septins can be found distal from the bud collar. From this we conclude that ASR and ERSU are distinct signaling pathways.

In summation, ERSU not only requires Slt2 but also Wsc1, Pkc1, Bck1, MKK1 and MKK2, and it is a signaling pathway that is distinct from CWI.

Discussion and Future Directions

In this study we show that Slt2 activation during ER stress requires Mkk1, Mkk2, Bck1, Pkc1, and Wsc1, but not Rom1 and Rom2. Slt2 is activated during cell wall stress, and we describe a novel, distinct pathway that also activates Slt2 but by ER stress (ERSU). Currently there are no studies that describe cell wall stress-induced activation of Slt2 that causes a cytokinesis defect, an alteration in septin structure, and cER inheritance delay. Thus Slt2 is able to affect many different activities depending on the activating stimulus. How can different stimuli cause different activities in the same protein? Some proteins function different depending on which of its phosphorylation sites get modified. Perhaps the same sort of differential activation is occurring with Slt2. Another explanation is that other components are being activated or upregulated depending on the stress, which work in concert with Slt2 and can affect very different downstream effects. In addition, we see that phosphorylated Slt2 is not abolished in *wsc1Δ*, but increases slightly during ER stress. This indicates that there may be other components that are not sufficient to phosphorylate Slt2 alone but in concert with Wsc1 allows for a high efficiency of Slt2 phosphorylation.

Another quite puzzling feature is the involvement of Wsc1, but not Rom1 and Rom2. The CWI signaling model has Wsc1 and all the cell wall sensors binding Rom, which activates Rho1. It will be interesting to find out whether Rho1 is involved in Slt2 activation. A third Rho1 GEF has been described, but has not been shown to be playing a major role in Rho1 activation during cell wall stress (Shmelzle

et al. 2002). Wsc1 not only signals from the plasma membrane (during CWI), but also along the secretory pathway (during ASR). Wsc1 is a plasma membrane protein that is able to interact with the ER lumen, perhaps it is then that Wsc1 is able to detect fluctuations in the ER functionality.

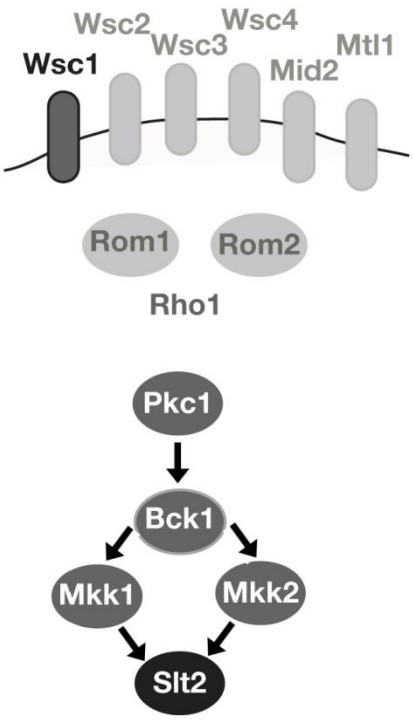
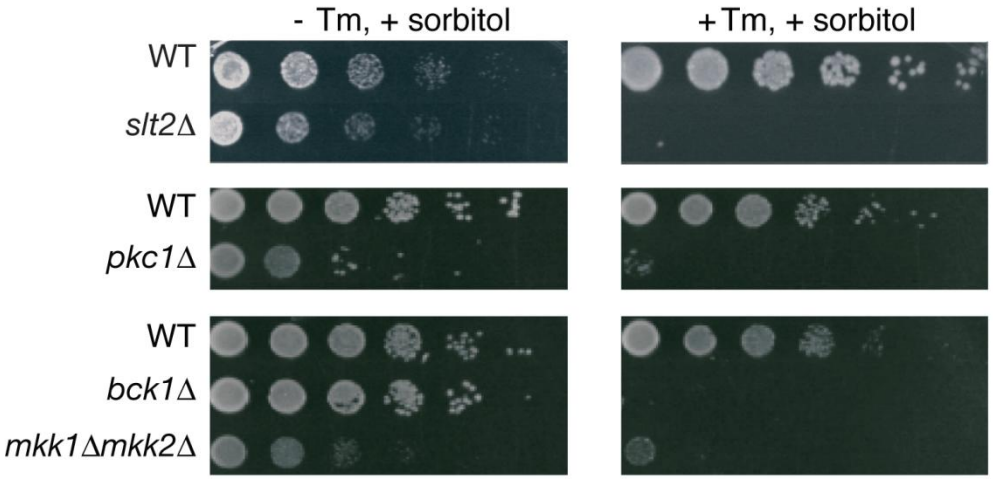


Figure 1.1. Components of the canonical Slt2 activation pathway.

A.



B.

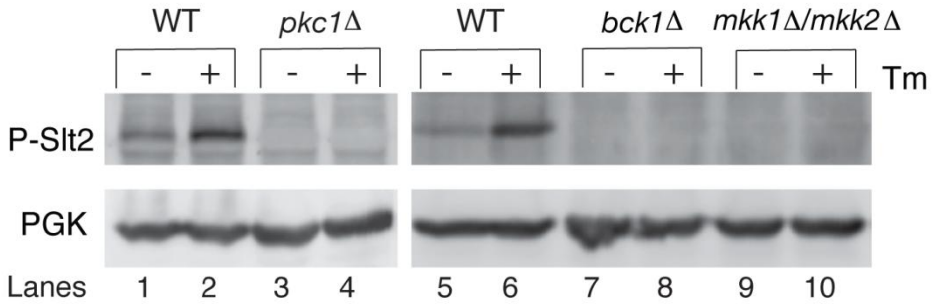
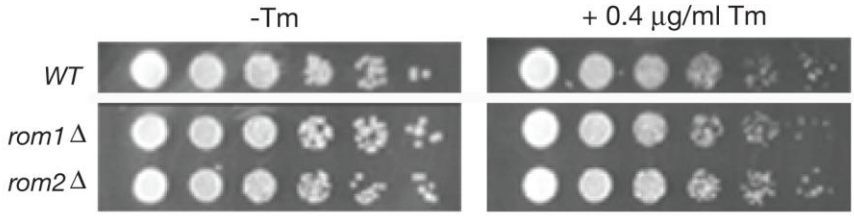


Figure 1.2. The kinases Pkc1, Bck1, Mkk1 and Mkk2 are required for Slt2 phosphorylation during ER stress.
(A) Cells of the indicated genotype were grown to log phase in YPD + 1M sorbitol, diluted serially 5 fold, and spotted onto plates containing 1M sorbitol or 1M sorbitol + 0.2 μg/m1 Tm.
(B) *pkc1Δ* and corresponding wild type cells were grown in the presence of 1M sorbitol, while *bck2Δ, mkk1Δ, /mkk2Δ* and corresponding wild type cells were grown in the absence of sorbitol. Cells were treated with 1 μg/m1 Tm for 2 h, and total cell extracts underwent immunoblot analysis to detect levels of phospho Slt2 and PGK (loading control).

A.



B.

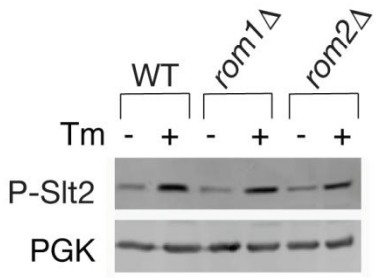
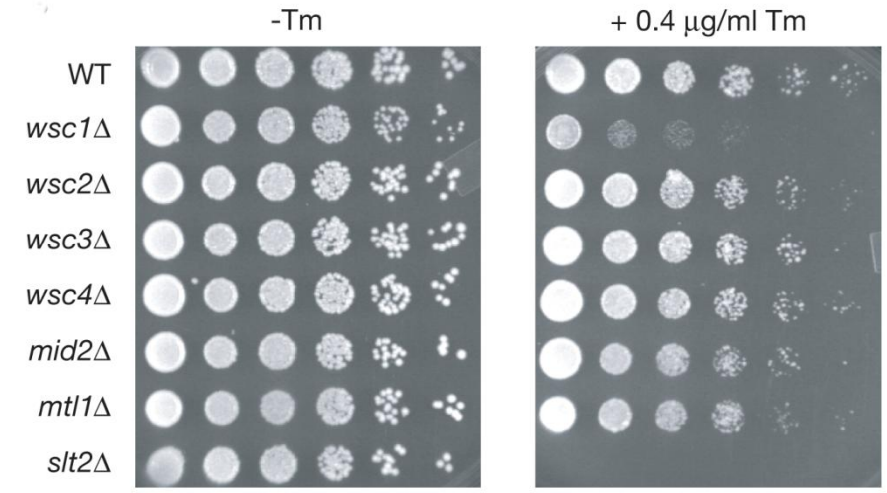


Figure 1.3. ROM1 or ROM2 is not involved in the ERSU pathway.
(A) Either *rom1Δ* or *rom2Δ* cells can support growth on a YPD plate containing 0.4 µg/m1 Tm. Five-fold serial dilutions of the indicated mutant cells were grown on medium with and without Tm.
(B) Either *rom1Δ* or *rom2Δ* cells can phosphorylate S1t2 upon ER stress at the level similar to ER stressed WT cells. Samples were collected and analyzed by western blot for S1t2 phosphorylation (top), and PGK (loading control, bottom).

A.



B.

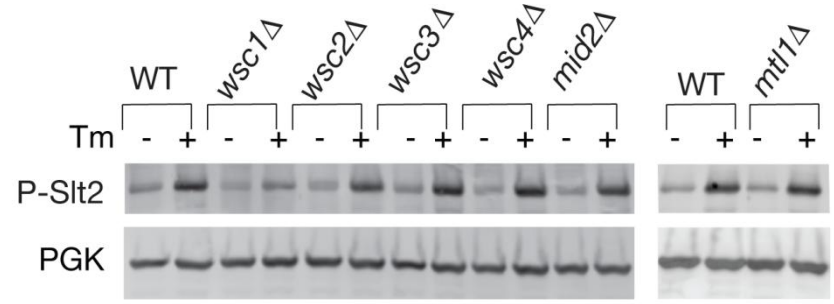


Figure 1.4. Wsc1 is required for ERSU.
(A) The absence of *WSC1* did not support growth on a YPD plate containing 0.4 µg.m1 Tm. Five-fold serial dilutions of the indicated mutant cells were grown on medium with and without Tm.
(B) *WSC1* is required for phosphorylation of Slt2 upon ER stress. The indicated mutants in the BY4741 backgrounds were grown in YPD and treated with 1 µg.ml Tm for 2 hrs. Samples were collected and analyzed by western blot for Slt2 phosphorylation (top), and PGK (loading control, bottom).

C.

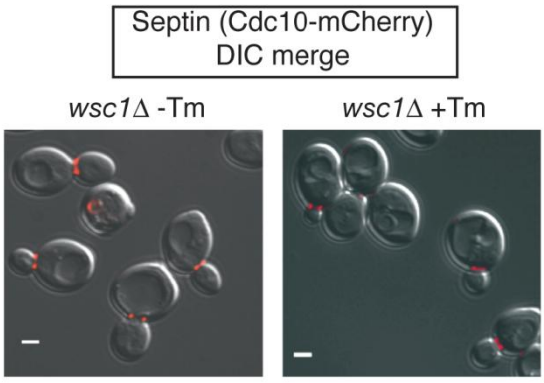


Figure 1.4 Continued. Wsc1 is required for ERSU.
(C) *WSC1* is involved in ERSU pathway. In the absence of WSC1, septin rings did not show altered morphology in response to ER stress induced by tunicamycin (Tm), and were observed only at the bud neck (a similar septin phenotype as what was seen in *slt2Δ* cells). The septin ring morphologies were monitored in wsc1Δ cels expressing a Cdc10-mCherry fusion protein upon growth in YPD (left) or YPD + 1 μg/m1 Tm.

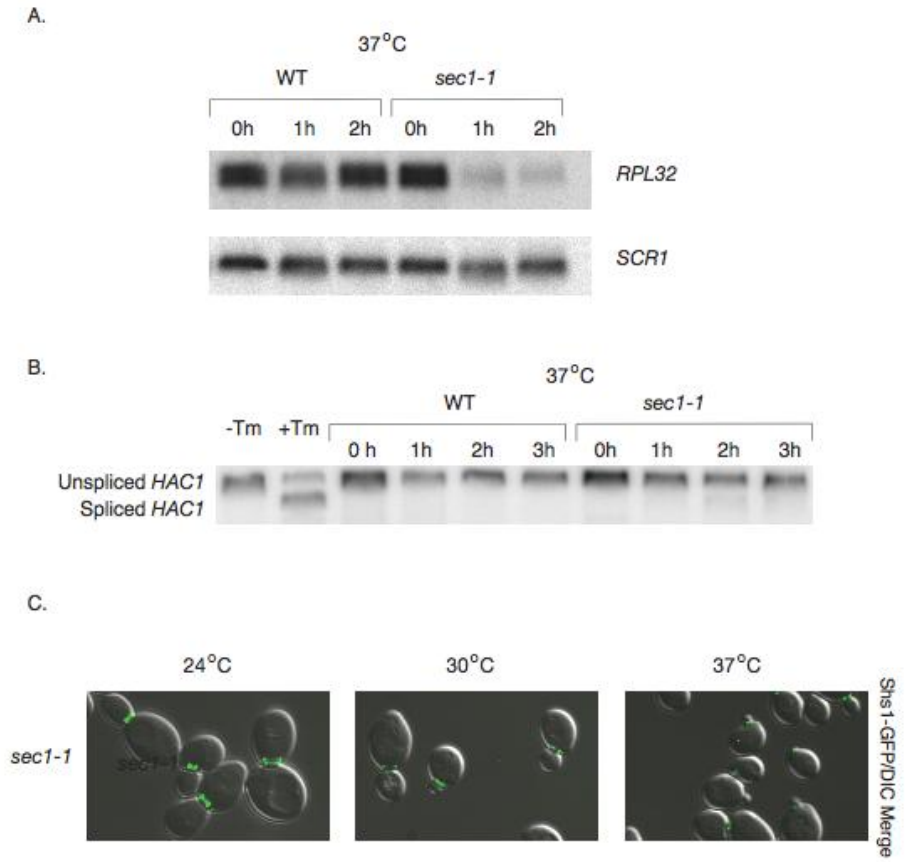


Figure 1.5. ERSU is not caused by a block of secretion.
(A) Imposition of a secretory block in S. cerevisiae is known to repress transcription of the mRNA transcript coding for the ribosomal protein *RPL32* through the arrest of secretion response. After shifting both WT and *sec1-1* cells to the non-permissive temperature 37 degrees Celsius for the indicated amount of time, total RNA samples from each time point were analyzed by northern blots for the level of *RPL32* mRNA and as a loading control, *SCR1*. A dramatic loss of *RPL32* RNA confirmed that a secretory block was induced upon shifting *sec1-1* cells to 37 degree Celsius.
(B) The UPR pathway was not activated during a secretory block induced in sec1-1 cells. UPR activation was monitored by splicing of *HAC1* mRNA. Upon tunicamycin (+Tm) treatment, northern analysis showed appearance of spliced form of *HAC1* mRNA. In contrast, the lack of *HAC1* splicing during the secretory block induced in *sec1-1* cells grown at 37 degrees Celsius indicates that ER stress was not induced.
(C) Septin ring was monitored in *sec1-1* cells genomically expressing the septin reporter Cdc10-GFP at permissive (24 degree Celsius), semi-permissive (30 degree Celsius), and non-permissive temperature (37 degree Celsius). A *sec1-1*-induced secretory block affected the size of the daughter cell, but the septin ring target to its normal position at the bud neck.

CHAPTER 2

Rlm1 affects cell survival to tunicamycin

and regulates transcription of Slt2 during ER stress

Introduction

An extracellular signal or stress is transduced to a MAP kinase (or to multiple MAP kinases) via a kinase cascade, and the MAP kinase activates nuclear and cytoplasmic targets by phosphorylation. The activation of these targets elicits a change in the activity of the cell, for example, when cell wall stress is transduced into yeast cells the MAP kinase, Slt2, causes the cell to manufacture constituents and enzymes that will repair the cell wall by (Jung, Levin 1999).

As far as Slt2 targets are concerned, Slt2 activates the Cch1/Mid1 calcium channel, which regulates intracellular calcium. An activation in Cch1/Mid1 leads to an accumulation of intracellular calcium (Fischer 1997). Slt2 also affects two of its four regulatory phosphatases in a feedback loop. The first phosphatase, Msg5, is phosphorylated by Slt2, which disrupts its binding with Slt2. This disruption decreases the rate with which Msg5 can de-activate Slt2 (Flandez 2004). The second affected phosphatase is Ptp2. The affect is an indirect one; the activation of Slt2 causes an induction of *PTP2* mRNA partially under the control of Rlm1 (Hahn 2002).

In addition to affecting its regulatory phosphatases either by direct phosphorylation or by indirect induction, genetic evidence suggests that Slt2 downregulates Mih1 (Harrison *et al.* 2001). Mih1 is a phosphatase that regulates the

activity of Cdc28, a cell cycle dependent kinase (CDK) (Lew 2003). Cdc28 is phosphorylated by perturbations in the actin cytoskeleton by the kinase Swe1, which causes the cell cycle to arrest in G2 and is known as the morphogenesis checkpoint (McMillan 1999). Thus Slt2 promotes the morphogenesis checkpoint because it downregulates Mih1.

Slt2 also regulates nuclear targets. Slt2 activates the transcription factor Rlm1, which induces *SLT2* transcription and transcription of proteins and enzymes that repair the cell wall (Jung, Levin 1999). It is not known whether Rlm1 is activated during ER stress. In addition to Rlm1, Slt2 activates two other transcription regulators, Swi4 and Swi6. Swi4 and Swi6 form a complex that regulates late G1 specific genes (Breeden 2003). Since Rlm1 regulates *SLT2* mRNA induction in cell wall stress, we focus our investigation on this particular Slt2 target in order to see if this is also true during ER stress.

Previously our lab has discovered that Slt2 regulates cytokinesis, septin structures, and cER inheritance during ER stress. However, the mechanisms through which these events occur remain unknown.

There are many nuclear and cytoplasmic targets of Slt2 as mentioned above, but this study specifically investigates if the Slt2 target, Rlm1, is playing a role during ERSU. As well, this study investigates whether Rlm1 regulates the induction of both Slt2 protein and mRNA.

Results

From a previous study in our lab, we found that Slt2 regulates cytokinesis, septin structures, and cER inheritance. As well, in Chapter 1 we characterized the mechanism through which Slt2 is activated. But what are the downstream effectors that mediate each of these phenotypes? One target of Slt2, which is responsible for a majority of the transcriptional changes during cell wall stress, is Rlm1 (Jung 1999). We wondered whether Rlm1 was playing a role in ERSU. We tested this possibility by series diluting *rlm1Δ* cells on Tm plates (Figure 2.1). We see that *rlm1Δ* cells are more sensitive to Tm than WT. This requirement for Rlm1 in cell survival when treated with Tm implied that Rlm1 might be playing a role in ERSU function. Since Rlm1 has been shown to regulate Slt2 mRNA induction (Jung, Levin 1999), we looked at *SLT2* mRNA production as a control to ensure that Rlm1 was not present in *rlm1Δ* cells. We see that *SLT2* mRNA is induced in WT cells, whereas there is no induction in *rlm1Δ* cells (Figure 2.2). In addition, Hac1 does not play a role in regulating SLT2 mRNA during ER stress (Figure 2.2). We conclude that SLT2 mRNA regulation during ER stress is dependent on Rlm1 and not the canonical UPR. Although *SLT2* mRNA production is abolished in *rlm1Δ* cells, we decided to test whether this had any affect on the protein levels of Slt2 by running a Western and probing with Slt2 specific antibodies. In WT we observe that Slt2p levels increase after Tm treatment, while *rlm1Δ* cells do not increase their Slt2p levels (Figure 2.3 Slt2 lanes). This indicates that Rlm1 is required for Slt2p induction. We

wondered whether an increase in Slt2p was required for activation of Slt2. In order to test this we measured phosphorylated Slt2 levels in WT and *rlm1Δ* cells treated with Tm by Western with a phospho-Slt2 specific antibody (same as in Chapter 1). We see an increase of phosphorylated Slt2 in WT (as seen in Chapter 1), as well as in *rlm1Δ* cells (Figure 2.3 p-Slt2 lanes). We conclude that although Rlm1 regulates an increase in Slt2p, there is enough Slt2p present to become activated to a similar extent as WT. Is this activation of Slt2 in *rlm1Δ* cells sufficient to activate ERSU? In order to test this we measured ERSU activation by monitoring septin morphology in *rlm1Δ* cells in a similar manner as in Chapter 1. We fused a septin subunit, Shs1, with GFP in *rlm1Δ* cells and monitored septin morphology in an asynchronous population. We observe that untreated WT cells have septin localized to the budneck (Figure 2.4 WT untreated panel), whereas the treated WT have aberrantly localized septin (Figure 2.4 WT ER stress panel). Likewise, untreated *rlm1Δ* display septin collars located at the mother-daughter budneck (Figure 2.4 *rlm1Δ* untreated panel), while treated *rlm1Δ* have aberrantly localized septin (Figure 2.4 *rlm1Δ* ER stress panel). In addition to septin localization, we analyzed cER inheritance and found that *rlm1Δ* cells treated with Tm have delayed cER inheritance (data not shown). Since septins continued to be mislocalized distal from the budneck and cER inheritance was delayed in *rlm1Δ* cells, we conclude that ERSU is activated in *rlm1Δ* cells.

In this study we find that Rlm1 regulates Slt2 mRNA and Slt2p levels, but does not have an effect on the induction of phosphorylated Slt2 after Tm treatment. Furthermore, this amount of phosphorylated Slt2 is sufficient to activate ERSU.

Discussion and Future Directions

We report here that Rlm1 is required for cell survival when treated with Tm. As well, Rlm1 regulates Slt2 mRNA and Slt2p induction during ER stress (Figure 2.3 and Figure 2.4, respectively). However, this does not significantly affect the activation of Slt2 as septins are mislocalized and cER inheritance is delayed (Figure 2.2 and data not shown, respectively).

How is that Slt2p levels are higher in *rlm1Δ* cells when they have decreased levels of SLT2 mRNA? One explanation is that Rlm1 serves two functions: (i) upregulating *SLT2* transcription (as seen in Figure 2.2) and (ii) inhibiting basal Slt2 translation (as seen in Figure 2.3).

Another interesting feature of this study is that *rlm1Δ* cells have defective growth on Tm plates. Why is Rlm1 required for cell survival during ER stress treatment? We see that ERSU is still active in disrupting septin structures in *rlm1Δ* cells, might Rlm1 be important for other aspects of ERSU like the delay of cytokinesis or cER inheritance? Another possibility for the defective growth of *rlm1Δ* cells on Tm is Rlm1 regulates genes that necessary to alleviate ER stress, which is toxic for the cell.

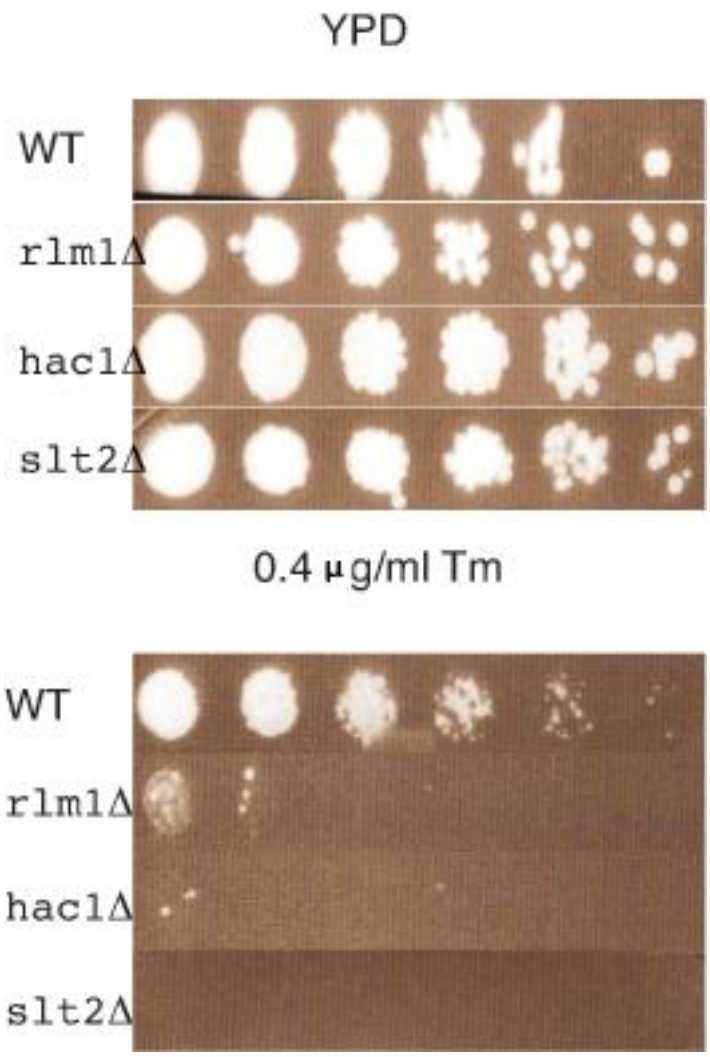


Figure 2.1. The absence of Rlm1 inhibits growth during ER stress.
rlm1Δ cells were not able to sustain growth on a plate containing 0.4 µg/ml Tm. Five fold serial dilutions of wild type and *rlm1Δ* cells were spotted on plates containing no drug (YPD) or +Tm. *slt2Δ* and *hac1Δ* cells are included as negative controls.

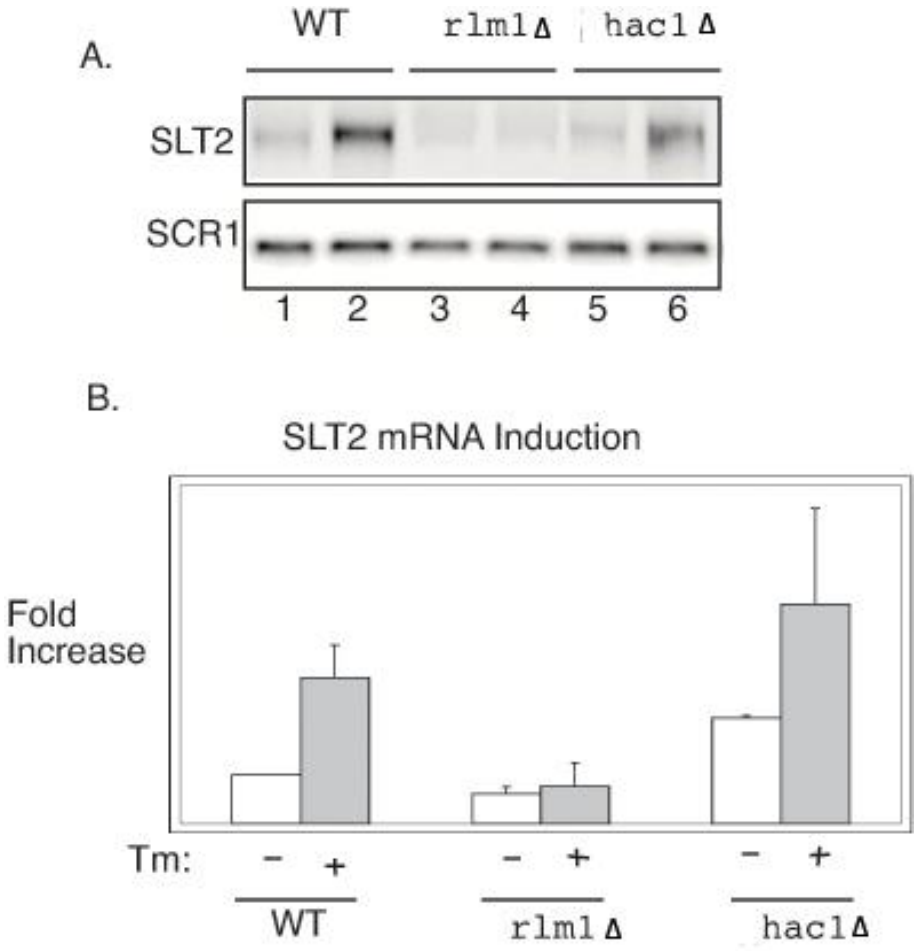


Figure 2.2. Rlm1 regulates *SLT2* transcription.
(A) *SLT2* induction was monitored in *rlm1Δ* and *hac1Δ* cells by *SLT2* mRNA. Upon tunicamycin treatment, *SLT2* mRNA levels increased in wild type and *hac1Δ* cells. In contrast, *SLT2* mRNA levels remained the same in *rlm1Δ* cells.
(B) *SLT2* mRNA Northerns from (A) were quanitated by phosphorimager. Error bars were generated from SD.

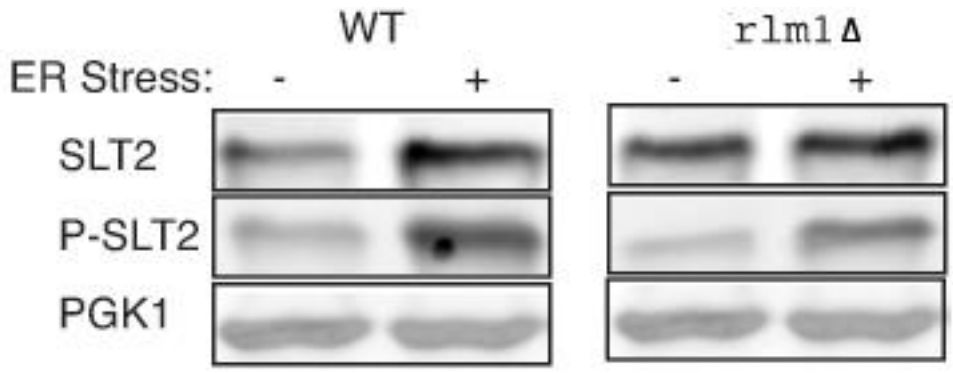


Figure 2.3. Slt2p induction is Rlm1 dependent while phosphorylation of Slt2p is not.
The levels of Phosphorylated Slt2p levels increase in WT as well as *rlm1Δ*. Samples were collected and analyzed by western blot for Slt2 (top), phosphorylated Slt2 (middle), and PGK (loading control, bottom).

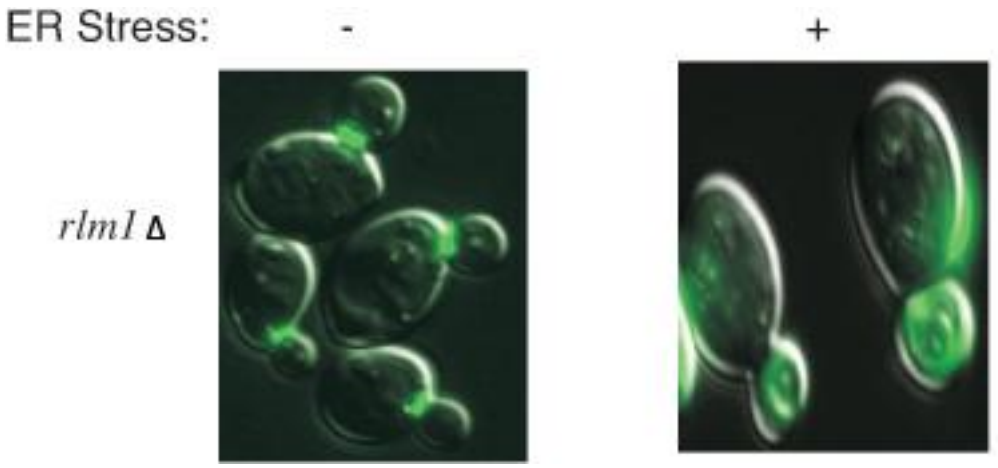


Figure 2.4. ERSU is Rlm1-independent.
Similar to ER-stressed wild type cells, the septin ring was not located at the budneck in ER-stressed *rlm1Δ* cells. Visualization of septin by Shs1-GFP reporter was performed in *rlm1Δ* that had been grown with or without 1 μg/mL Tm.

MATERIALS AND METHODS

Strains, media, and growth conditions

All yeast strains were generated using standard genetic methods and are listed in Table 1.1. Deletion and epitope tagged strains were constructed using a one-step recombination-mediated technique (Longtine 1998).

Cells were grown in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) at 30°C unless otherwise noted. During DTT treatments, YPD was pH 5.4. For Slt2 immunoblot experiments, cells were grown to an OD of 1, then diluted to an OD of 0.25 before treatment. To induce ER stress, Tm (Calbiochem) was added at a final concentration of 1 mg/ml, or DTT (Fisher) was added at a final concentration of 2 mM, 3 mM, or 4 mM as indicated. Tm was stored as a 10 mg/ml stock in DMSO, and DTT was stored as 1 M stock in H_2O. For ER stress growth assay, cells were grown to approximately 3 x 10^7 cells before being 5-fold series diluted and spotted unto YPD plates (1% yeast extract, 2% bactopeptone, 2% glucose, 2% bactoagar) containing 0.4 μg/ml Tm.

Immunoblotting

For immunoblot analysis, approximately 3 x 10^7 cells were harvested by centrifugation at 4°C, washed with 1 ml H_2O, frozen with liquid N_2 and stored at –80°C. Pellets were resuspended in 100 μl lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM sodium pyrophosphate, 1 mM PMSF, 1 mM sodium orthovanadate, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 20 mM NaF, 5

μg/ml aprotinin, 1.75 mM ß glycerophosphate). 100 μl of acid washed glass beads were added and cells were vortexed at 4°C for 5 min. Lysates were centrifuged at 13K for 8 min at 4°C and the supernatant was collected. Protein concentration was determined using BCA protein assay kit (ThermoScientific). 20 μg of protein (phospho Slt2) or 40 μg of protein (total Slt2) were denatured at 95°C in 2X loading buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 50% glycerol, 12% BME, .02% bromophenol blue) and then separated on an 8% (Slt2) SDS-polyacrylamide gel, and transferred to nitrocellulose. Primary antibodies and antisera were phospho p44/p42 MAP Kinase (NEB) at a 1:1000 dilution overnight, total Slt2 (Santa Cruz Biotechnology, Inc) at a 1:100 dilution overnight, PGK (Molecular Probes) at a 1:10,000 dilution for one hour, or phospho-p38 (Cell Signaling) at a 1:1000 dilution overnight. Secondary antibodies were HRP-conjugated donkey anti-rabbit (GE Healthcare) at a 1:10000 dilution for phospho p44/p42 and phospho p38, rabbit anti-goat (Zymax) at a 1:5000 dilution for total Slt2, and goat anti-mouse (Biorad) at a 1:10000 dilution for PGK. Membranes were developed with ECL Plus Western blotting detection reagent (GE Healthcare), imaged using a typhoon phosphorimager (GE Healthcare), and analyzed using ImageQuant software (GE Healthcare).

Northern blotting

RNA isolation and northern blotting were carried out as previously described (5). Briefly, RNA was isolated using a modified hot phenol method, and 5 mg (*RPL32* northerns), 10 mg (*HAC1* and *SLT2* northerns) or 25 mg (*HSP12* northerns) of RNA were loaded on a 1.5% agarose gel with 6.7% formaldehyde, and transferred

to zeta probe membrane (BioRad) in 10x SSC by capillary action overnight. Following UV-crosslinking, membranes were probed overnight with a DNA probe generated by random primed DNA labeling. Blots were scanned on a typhoon phosphorimager (GE Healthcare) and analyzed using ImageQuant software (GE Healthcare).

Microscopy

Cells were imaged live and sonicated briefly before analysis. For Septin visualization genomic Shs1 was fused with a C-terminal GFP tag. Cells were visualized using a Zeiss Axiovert 200M microscope 100X 1.3 NA objective. Images were captured using an Axiocam monochrome digital camera (Carl Zeiss) and analyzed using Axiovision software (Carl Zeiss).

Table 1. Yeast strains used in this study.

Strain	Relevant Genotype	Source
MNY1050	*MATa, his3D1, leu2D0, met15D0, ura3D0, rlm1D::KanMX*	Yeast KO collection
	MATa, his3D1, leu2D0, met15D0, ura3D0:: rlm1D:URA3, SHS1-GFP::KanMX	This study
MNY1051	*MATa, his3D1, leu2D0, met15D0, ura3D0, ire1D::KanMX*	Yeast KO collection
MNY1052	*MATa, his3D1, leu2D0, met15D0, ura3D0, slt2D::KanMX*	Yeast KO collection
MNY1053	*MATa, his3D1, leu2D0, met15D0, ura3D0, wsc1D::KanMX*	Yeast KO collection
MNY1054	*MATa, his3D1, leu2D0, met15D0, ura3D0, wsc2D::KanMX*	Yeast KO collection
MNY1055	*MATa, his3D1, leu2D0, met15D0, ura3D0, wsc3D::KanMX*	Yeast KO collection
MNY1056	*MATa, his3D1, leu2D0, met15D0, ura3D0, wsc4D::KanMX*	Yeast KO collection
MNY1057	*MATa, his3D1, leu2D0, met15D0, ura3D0, mid2D::KanMX*	Yeast KO collection
MNY1058	*MATa, his3D1, leu2D0, met15D0, ura3D0, mtl1D::KanMX*	Yeast KO collection
MNY1060	*MATa /MAT*a, *trp1-1/trp1-1, leu2-3,112/leu2-3,112, ura3-52/ura3-52, his4/his4, bck1D::G418/bck1D::G418*	Kim 2008
MNY1061	*MATa /MAT*a, *his3D1/his2D1, leu2D0/leu2D0,LYS2/lys2D0, met15D0/MET15,ura3D0/ura3D, mkk1D::G418/mkk1D::G418 mkk2D::G418/mkk2D::G418*	Kim 2008
MNY1063	*MATa, leu2-3,112, ura3-52, trp1-1, his4, can1r, pkclD::LEU2*	Watanabe 1994

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