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A Surveillance Pathway Monitors the Fitness of the Endoplasmic Reticulum to Control Its Inheritance

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SUMMARY

The endoplasmic reticulum (ER) plays an essential role in the production of lipids and secretory proteins. Because the ER cannot be generated de novo, it must be faithfully transmitted or divided at each cell division. Little is known of how cells monitor the functionality of the ER during the cell cycle or how this regulates inheritance. We report here that ER stress in *S. cerevisiae* activates the MAP kinase Slt2 in a new ER stress surveillance (ERSU) pathway, independent of the unfolded protein response. Upon ER stress, ERSU alters the septin complex to delay ER inheritance and cytokinesis. In the absence of Slt2 kinase, the stressed ER is transmitted to the daughter cell, causing the death of both mother and daughter cells. Furthermore, Slt2 is activated via the cell surface receptor Wsc1 by a previously undescribed mechanism. We conclude that the ERSU pathway ensures inheritance of a functional ER.

INTRODUCTION

The endoplasmic reticulum (ER) functions as a gateway for newly synthesized secretory or membrane proteins. Upon synthesis, polypeptides coding for such proteins are targeted and translocated into the lumen of the ER as linear unmodified polypeptides. In the ER, the polypeptides undergo folding and modification processes to become their native functional structures. Only properly folded proteins can exit from the ER to their target sites (Bicknell and Niwa, 2009; Mori, 2000; Ron and Walter, 2007; Rutkowski and Kaufman, 2004). Misfolded proteins are toxic to the cell and become marked within the ER and targeted for degradation by a process termed ER-associated protein degradation (ERAD) (Hampton, 2002; Bukau et al., 2006; Vembar and Brodsky, 2008). Additionally, the ER is the primary site of lipid biosynthesis and thus influences the relative composition and overall abundance of lipids throughout the cell (McMaster, 2001). Essentially, the ER serves as a master regulator for the complex and error-prone process of protein maturation, quality control, and trafficking.

Morphologically, the ER is a continuous tubular-reticular network that is contiguous with the outer membrane of the nuclear envelope (Koning et al., 2002; Preuss et al., 1991). In yeast, the ER is comprised of two subdomains: the perinuclear ER/nuclear envelope and the reticulum of cortical ER (cER) tubules, which is found near the plasma membrane or the cortex of the cell. ER tubules, approximately 50–100 nm in diameter, connect the cER to the perinuclear ER (Voeltz et al., 2002). Most ER proteins analyzed to date can migrate freely between perinuclear ER and cER. The functional implication for the distinction between these ER domains is unclear.

Paradoxically, the mechanism of inheritance of perinuclear ER and cER appears to be different. The perinuclear ER remains closely associated with the nucleus and becomes segregated and partitioned between the two cells in a microtuble-dependent manner. In contrast, the inheritance of cER is actin-based and powered by a myosin motor (Du et al., 2001; Estrada et al., 2003; Prinz et al., 2000). Recent genetic studies have begun to elucidate the molecular events of cER inheritance. Very early in the cell cycle, ER cytoplasmic tubules align along the mother-bud axis and extend to the newly developed bud. These tubules become anchored at the bud tip (Du et al., 2001; Huffaker et al., 1988; Jacobs et al., 1988), followed by the tubules spreading along the periphery of the bud to form the cER of the daughter cell (Estrada et al., 2003, Estrada de Martin et al., 2005; Prinz et al., 2000, Wiederkehr et al., 2003). While interesting molecular components and mechanisms involved in ER transmission have been identified, the extent of regulation imposed on this process remains largely unexplored.

Proper segregation of cellular components is the essence of cell division and is critical to sustain life. In addition to genomic materials in the nucleus, cytoplasmic components must also be separated properly so that the newly generated daughter cells can autonomously carry out cellular events immediately after cell division (Peng and Weisman, 2008). Given the critical nature of ER function in the cell and as ER is not synthesized de novo but arises only from existing ER, we reasoned that a surveillance mechanism may exist to ensure that a minimal threshold of ER functional capacity is inherited by each daughter cell during the cell cycle.

In *S. cerevisiae*, we have previously shown that ER stress causes cell-cycle delay with high DNA content, large buds, and divided nuclei. Further analyses have revealed that ER stress

does not alter mitotic events, including the major cell-cycle regulator Clb2 production/degradation, mitotic phosphatase Cdc14 release into the cytoplasm, and mitotic spindle formation/depolymerization. ER stressed cells are unable to divide even after lyticase treatment, revealing that ER stress causes a cytokinesis defect rather than a problem in cell separation (Bicknell et al., 2007).

Here, we set out to investigate molecular events leading to the ER stress-induced cytokinesis delay. We find that the cytokinesis delay is part of a multifaceted cell-cycle response to ER stress, including septin alteration and ER inheritance delay. Furthermore, this ER surveillance response ensuring cER inheritance is independent of the canonical unfolded protein response (UPR) pathway, but rather coordinated by MAP kinase Slt2.

RESULTS

ER Stress Alters the Localization and Morphology of the Septin Ring

Previously, we showed that cytokinesis block in cells experiencing ER stress is not due to delayed or altered actin patch distribution. To probe the molecular basis of blocked cytokinesis in ER-stressed cells, we examined the localization dynamics of the septin complex during the cell cycle after treating cells with tunicamycin (Tm). Tm is a well-characterized inducer of ER stress that inhibits N-linked glycosylation, leading to an accumulation of unfolded protein in the ER. As currently understood, assembly of the five septin complex subunits, Cdc3, Cdc10, Cdc11, Cdc12, and Shs1, at the bud neck establishes a septation site for cytokinesis and is thought to act at one of the most upstream levels of the yeast cytokinesis pathway (Bertin et al., 2008; Gasper et al., 2009; Gladfelter et al., 2001; Iwase et al., 2006; Kim et al., 1991; McMurray and Thorner, 2009). We monitored the localization of the septin subunit Cdc10-GFP (Cid et al., 1998) in synchronized cells after treatment with Tm. In unstressed cells, we observed septin ring formation at the bud neck of each mother cell during bud formation with conversion to hourglass structures over time. (Caviston et al., 2003; Dobbelaere et al., 2003; McMurray and Thorner, 2009) (Figure 1A). ER stress did not affect septin subunit targeting or ring formation, which were similar in normal and stressed cells. In unstressed cells, the septin ring went on to disperse toward the end of the cell cycle as cytokinesis progressed (Figure 1A; 60 min after release from mating pheromone). In contrast, in stressed cells, the septin ring did not disperse, and cytokinesis was not observed even after 90 min (Figure 1A; compare −Tm and +Tm at 60 and 90 min). Ultimately, the septin fluorescence was observed distal from the bud neck in stressed cells, a localization that was never seen in unstressed cells. The aberrant behavior of the septin complex in Tm-treated cells was a general consequence of ER stress. This behavior was also observed when ER stress was induced by two other well-characterized means: DTT treatment, which disrupts disulfide bonds, or inactivation of the Ero1 protein (endoplasmic reticulum oxidoreductin I) through expression of the *ero1-1* temperature sensitive allele by shifting from permissive temperature (at 24°C) to nonpermissive temperature (at 37°C) (Frand and Kaiser, 1998; Pollard et al., 1998) (Figure 1B). This effect of ER stress that we observed on septin was not specific to the Cdc10-GFP reporter, as we observed similar changes in strains expressing Cdc11-GFP and Shs1-GFP fusion proteins.

The morphology of and choreographed changes in the septin ring that are observed in normal cells as the cell cycle progresses are known to be regulated by posttranslational modifications that affect the stability of interactions between septin subunits (Dobbelaere et al., 2003). To test the possibility that ER stress stabilizes the septin complex, giving rise to the persistent septin ring appearance observed (Figure 1A), we examined cells bearing the *cdc12-6* mutation. This temperature-sensitive mutation of the septin subunit *CDC12* is known to cause septin ring disassembly at the restrictive temperature (at 30°C), presumably by destabilizing interactions between septin subunits (Dobbelaere et al., 2003). Thus, we reasoned that ER stress might stabilize the septin ring sufficiently to rescue *cdc12-6* cell growth at the restrictive temperature. Growth of the *cdc12-6* mutant at the restrictive temperature is known to result in the formation of elongated cells that fail to undergo cytokinesis (Figure 1C, 30°C −Tm) (Dobbelaere et al., 2003; Kim et al., 1991). Remarkably, addition of the ER stress inducer Tm to *cdc12-6* cells at 30°C resulted in cells with a normal septin ring morphology and, ultimately, normal cell shape and cytokinesis (Figure 1C, 30°C +Tm), resulting in the rescue of overall cell growth (Figure 1D, 30°C +Tm).

Thus, ER stress suppressed the cytokinesis defect due to the *cdc12-6* mutation. Similarly, we found that Tm treatment also rescued aberrant septin localization and morphology, and elongated shape and growth of cells deleted for *SHS1* (Figures S1A and S1B available online), which encodes a subunit of the septin ring. These observations suggest that ER stress stabilizes the abnormal septin rings of *cdc12-6* and *shs1Δ* cells sufficiently to allow normal septin behavior and cytokinesis to occur. Furthermore, this observation suggests that in WT cells ER stress delays cytokinesis by stabilizing the septin ring.

ER Stress Induces an Inhibition of Cortical ER Inheritance

Since ER stress delays cell-cycle progression, we asked whether ER stress also affects ER inheritance. Using the ER marker GFP-HMG CoA reductase (Hmg1-GFP) (Du et al., 2001; Hampton et al., 1996), we examined the distribution of both cortical and perinuclear ER in mother and daughter cells in the presence and absence of ER stress. In the absence of stress, cortical ER (cER) was delivered to the daughter cell very early in the cell cycle, consistent with previous reports (Estrada de Martin et al., 2005). As soon as a bud was visible, 96% of buds contained some cER (Figure 2A; yellow arrows, no stress or *ero1-1* at 24°C, class I). As the bud grew, the cER began to spread along the cortex of the bud (Figure 2A; no stress or *ero1-1* at 24°C, class II and III). Perinuclear ER was inherited later in the cell cycle, during mitosis, along with the nucleus (Figure 2A; red arrows, no stress or *ero1-1* at 24°C, class III).

When ER stress was induced, whether with Tm, DTT, or the *ero1-1* allele grown at 37°C, cER entry into the daughter cell was significantly inhibited (Figure 2A; quantified for DTT- and Tm-induced ER stress in Figure 2B and Figure S2A, respectively). Early in the cell cycle, prior to nuclear division, only

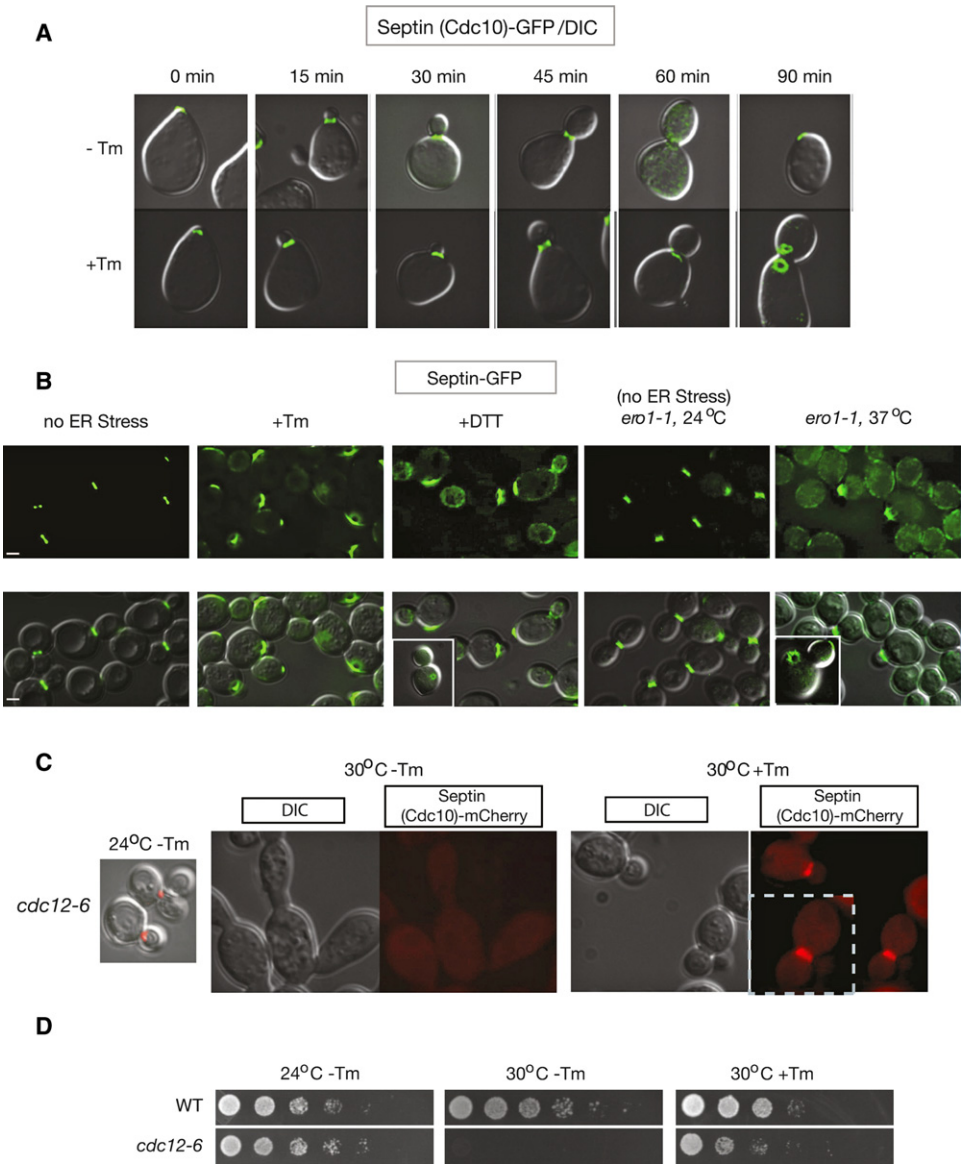

Figure 1. ER Stress Alters the Localization and Morphology of the Septin Ring

(A) Septin ring morphology during normal growth (−Tm) and under ER stress (+Tm). Upon release, α factor-synchronized cells expressing genomically GFP-tagged *CDC10* (Jiménez et al., 1998) were treated with or without tunicamycin (Tm) for the indicated amount of time (Bicknell et al., 2007).

(B) Genomic loci encoding septin subunits, either *SHS1* or *CDC11*, were C-terminally tagged with GFP. ER stress was induced in asynchronous populations of WT cells with 1 μg/ml Tm (3 hr) or 2 mM DTT (3 hr), or in *ero1-1* cells by shifting from the permissive temperature (24°C) to the nonpermissive temperature (37°C) for 3 hr. Note that similar conditions were used for ER stress induction throughout this study unless otherwise stated. GFP alone (upper panels) and GFP merged with a differential interference contrast (DIC) image (lower panel) are shown. Scale bars represent 2 μm.

(C and D) Septin morphology in *cdc12-6* cells carrying *CDC10*-mCherry in YPD or YPD+Tm at the indicated temperature. Upon shift to the nonpermissive temperature without addition of Tm in the growth media (30°C, 3 hr, −Tm), cells showed loss of normal septin structure (dispersed) and abnormal elongated morphology. However, addition of Tm (1 μg/ml, 3 hr at 30°C) caused recovery of the septin ring and normal cell morphology to majorities of *cdc12-6* cells, consistent with the partial recovery of cell growth with Tm at 30°C as shown in (D). *cdc12-6* cells were not able to sustain their growth at 30°C without Tm. Cells were spotted on YPD or YPD+Tm (0.2 μg/ml) plates after 5-fold serial dilutions and incubated at the indicated temperature for 3 days.

See also Figure S1.

13% of ER-stressed cells with small buds (Figure 2A left panels, +DTT, +Tm, *ero1-1* at 37°C, defined as class I) and 30% of cells with medium buds (Figure 2A middle panels, +DTT, +Tm, *ero1-1* at 37°C, class II) contained any cER. Even after mitosis (i.e., completed nuclear division; class III), 27% of ER-stressed cells still contained no visible cER in the bud (Figure 2A, right panels). In contrast, the perinuclear ER was inherited normally during ER stress. The inhibition of cER inheritance was also seen after Tm

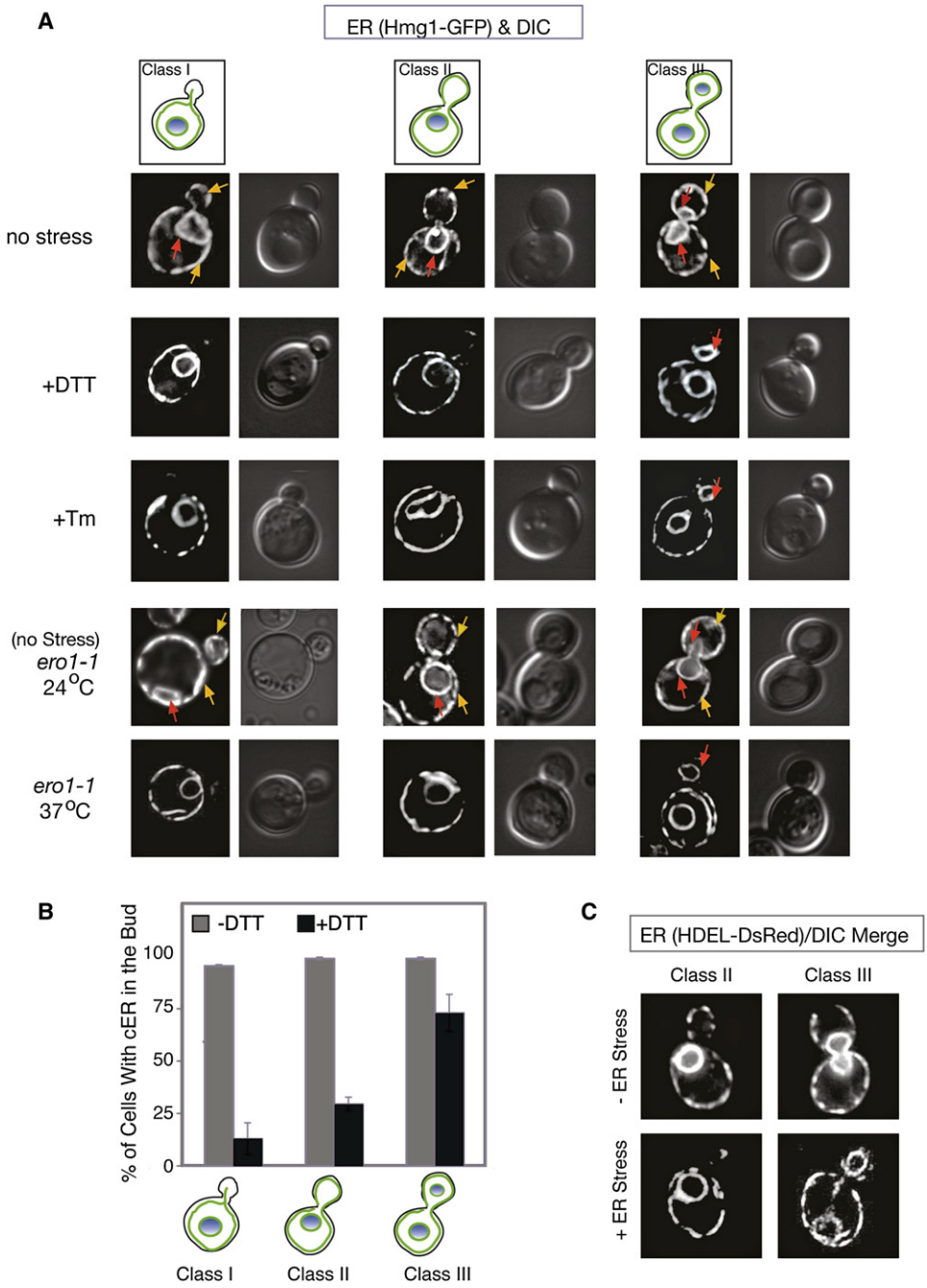

Figure 2. ER Stress Induces a Delay in Cortical ER Inheritance

(A) ER stress was induced (by Tm, DTT, or *ero1-1*) in cells expressing the ER membrane marker, Hmg1-GFP, for examination by fluorescence microscopy. Three categories of cells are depicted for each condition and as described in Results section. Yellow arrow shows cortical ER (cER), while red arrow represents perinuclear ER.

(B) For each of the three classes of cells, the number of cells containing elements of cER in the daughter cell was counted (n = 300) during growth under normal conditions (gray bars) or in the presence of 2 mM DTT (3 hr; black bars). The average of three independent experiments is depicted; error bars represent the standard deviation (SD).

(C) ER visualized by a HDEL-DsRed ER reporter confirmed that cER inheritance was delayed when ER stress was induced by Tm. Images shown are HDEL-DsRed alone.

See also Figure S2.

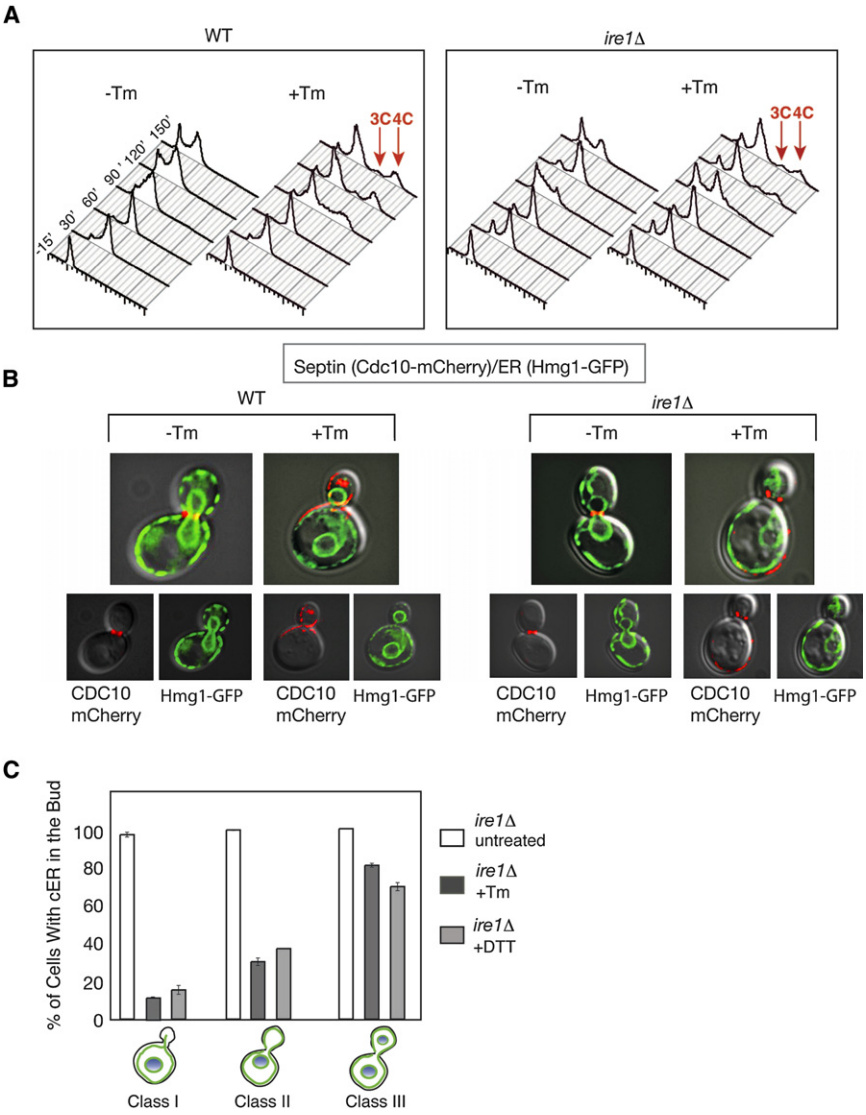

Figure 3. The UPR Does Not Signal Septin Stabilization, Cytokinesis Block, or ER Inheritance Delay during ER Stress

(A) DNA content of α factor-synchronized wild-type and *ire1Δ* cells were monitored by FACS upon Tm treatment as described in experimental procedures. After 90 min, both cell types showed populations of cells with 3C or 4C DNA content (red arrows), indicating a cytokinesis delay (Bicknell et al., 2007).

(B) Cortical cER inheritance (visualized by Hmg1-GFP) and septin morphology (by Cdc10-mCherry) were perturbed in both WT and *ire1Δ* cells when grown in Tm. DIC pictures merged with either Cdc10-mCherry or Hmg1-GFP are shown for both WT and *ire1Δ* cells.

(C) Quantitation of *ire1Δ* cells with cER in the bud under ER stress inducing conditions (n = 300 for each class, under normal conditions (white bars) or in the presence of 1 μg/ml Tm (dark gray bars) and 2 mM DTT (light gray bars). Error bars represent the SD of three independent experiments.

treatment of cells bearing another ER reporter, HDEL-DsRed, which marks the lumen of the ER (Figure 2C and Figure S2B), demonstrating that the effect is independent of the ER reporter used. We conclude that ER stress inhibits the inheritance of cER. Finally, we found that ER stress did not affect the inheritance of the vacuole (Figure S2C) and the mitochondria (Figure S2D), although their morphologies differed between ER-stressed and unstressed cells.

The UPR Is Not Involved in the Septin Stabilization, Cytokinesis Block, or ER Inheritance Delay Observed during ER Stress

ER stress impacts the cell cycle in three ways: it alters septin structures, inhibits cER inheritance, and delays cytokinesis. We reasoned that these events might be a part of a surveillance mechanism to monitor the ER's functional capacity and to prevent propagation of a compromised ER. Because of the physical location and functional roles of the septin complex, we reasoned that it might provide a pivotal point for integrating the functional state of the ER, its inheritance, and cell-cycle progression. To date, the only signaling pathway known to be initiated by ER stress is the UPR pathway. In *S. cerevisiae*, the UPR is set in motion by Ire1p, an ER transmembrane receptor kinase/riboendonuclease (RNase) that senses ER stress and signals to downstream components in order to help cells cope with the stress (Cox et al., 1993; Mori et al., 1993). Surprisingly, we found that cells lacking *IRE1* (*ire1Δ*) continued to exhibit a delay in cytokinesis, observed as an increase in the 3C/4C DNA content (Bicknell et al., 2007) (Figure 3A) and aberrant septin morphology (Figure 3B, Cdc10-mCherry). Furthermore, a delay in cER inheritance still occurred in the bud of ER stressed *ire1Δ* cells (Figure 3B, quantified in Figure 3C) and did so at levels similar to those of ER stressed WT cells. Thus, the ER surveillance mechanism responsible for these events must be signaled via a mechanism independent of the *IRE1*-mediated UPR pathway.

Slt2 MAP Kinase Is Required for ER Stress Surveillance

In search of a regulatory molecule controlling the observed cell-cycle defects above, we tested a number of candidate mutants. These included mutations in genes encoding proteins residing on the ER membrane, proteins involved in cER movement, and canonical signaling molecules such as kinases and phosphatases (Table S1). We screened for mutants that had lost the characteristic responses to ER stress described above. Our search revealed a linkage to the Slt2 MAP kinase. Slt2 was originally included in our screen as it was known to be phosphorylated during ER stress (Bonilla and Cunningham, 2003;

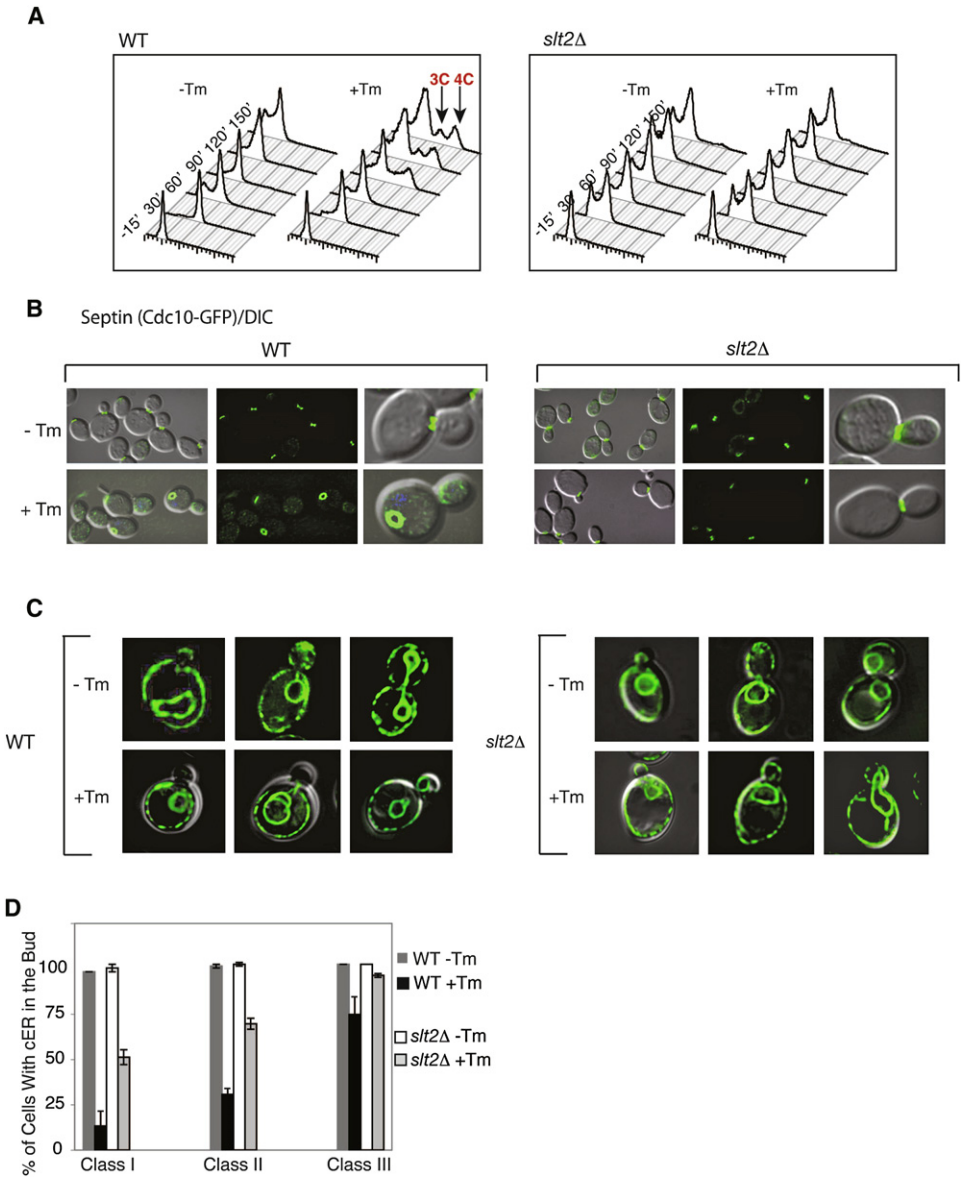

Figure 4. Slt2 MAP Kinase Mediates Septin Alterations, Cytokinesis Delay, and ER Inheritance Delay during ER Stress

(A) ER stress did not induce a cytokinesis delay in *slt2*Δ cells, shown by no *slt2*Δ cells with 3C or 4C DNA content FACS peaks, in contrast to ER stressed wild-type cells (red arrows). Experimental conditions were as described in Figure 3A.

(B) Unlike in ER-stressed WT cells, the septin ring remained at a normal position in ER-stressed *slt2*Δ cells. Septin was visualized by Cdc10-GFP in WT (left) and *slt2*Δ cells (right) grown with or without 1 μg/ml Tm, as indicated. Additional genes tested are shown in Table S1.

(C) cER entered the bud of *slt2*Δ cells treated with Tm (1 μg/ml) as visualized by Hmg1-GFP.

(D) Quantitation of wild-type and *slt2*Δ cells with elements of cER in the daughter cell under normal (−Tm) or ER stress (+Tm; 1 μg/ml) conditions in each class (n = 300). The average of three independent experiments is depicted; error bars represent the SD.

See also Figure S3.

Chen et al., 2005) and had been genetically, albeit separately, linked to septins (Longtine et al., 1998a) and to ER inheritance (Du et al., 2006), although the functional significance was unknown.

If Slt2 were mediating the ER surveillance mechanism, we expected that *slt2Δ* cells exposed to ER stress would not induce a cytokinesis defect, septin alterations, or delay the inheritance of cER. Indeed, synchronized *slt2Δ* cells did not exhibit 3C/4C DNA content after Tm treatment (Figure 4A; *slt2Δ* +Tm). The septin ring of *slt2Δ* cells also appeared to be normal after Tm treatment (Figure 4B). Moreover, cER inheritance was delayed significantly less in *slt2Δ* cells than in WT cells exposed to ER stress (Figure 4C, quantified in Figure 4D). The failure to induce these events was not caused by an inability of Tm to induce

ER stress in *slt2Δ* cells because *slt2Δ* cells efficiently activated the separate UPR pathway under these conditions (Figure S3A, lanes 3 and 4). Taken together, the data indicate that *SLT2* mediates the previously undefined *ER surveillance* (ERSU) pathway that links ER stress with the cell cycle and ER inheritance.

ER Surveillance Signaling Is Necessary for Survival of ER Stress

Induction of ER stress arrests the cell cycle. However, this arrest is not permanent; over a longer period of time, wild-type cells can recover and are observed to grow on Tm or DTT plates (Figure 5A). We hypothesized that the ERSU response, like the UPR response, allows cells to first adapt to ER stress, allowing them to grow under constant stress. Consistent with this hypothesis, *slt2Δ* cells failed to grow on plates containing Tm or DTT (Figure 5A, +Tm or +DTT). This suggests that the output of the ERSU pathway allows cells to cope with long-term stress.

Slt2 Phosphorylation and Kinase Activity Are Essential for ER Stress Surveillance

Slt2 was phosphorylated upon ER stress (Figure 5B, lanes 1and 2). Phosphorylation of Slt2 still occurred in *ire1Δ* cells (Figure 5B, lanes 3 and 4), revealing that Ire1 is not required for Slt2 phosphorylation and providing further support that ERSU is independent of UPR. It should be noted that in addition to Slt2 phosphorylation, the total Slt2 protein level also increased upon ER stress. This was the consequence of a transcriptional increase of *SLT2* during ER stress that was, as previously reported (Chen et al., 2005) independent of Hac1, a UPR transcription factor (Figure S3B, lanes 1 and 2 versus, lanes 3 and 4). Instead, Rlm1, one of the transcription factors reported to be downstream of Slt2 (Levin, 2005) was found to be responsible for the transcription increase in Slt2 (Figure S3B). Furthermore, in *rlm1Δ* cells, while ER stress did not induce *SLT2* mRNA transcript levels, the increase in Slt2 phosphorylation still took place, revealing that Slt2 phosphorylation is independent of *SLT2* mRNA or protein increase (see Figure S3C, lanes 3 and 4). We therefore tested whether Slt2 phosphorylation and/or Slt2 kinase activity are important for surviving ER stress. *slt2Δ* cells transformed with wild-type *SLT2* regained their ability to grow on Tm plates. However, *slt2Δ* cells transformed with either the kinase-dead *slt2*-K54R mutant or the phosphorylation site *slt2-T190A/Y192F* mutant (Kim et al., 2008; Watanabe et al., 1994) failed to grow on Tm plates (Figure 5C). Therefore, we conclude that both Slt2 kinase activity and Slt2 phosphorylation are required to survive ER stress.

We next investigated the mechanism of Slt2 phosphorylation. Slt2 is a MAP kinase, and multiple upstream activators of Slt2 have been identified (Levin, 2005). We found that both ER stress-induced phosphorylation of Slt2 (Figure 5D) and growth in the presence of Tm (Figure 5E) require Pkc1 (MEKK activator), Bck1 (MEKK), and either Mkk1 or Mkk2 (redundant MEKs). Furthermore, septin ring and daughter cell cER inheritance of *pkc1Δ* and *bck1Δ* cells were not disturbed, even upon ER stress induction, similar to what we observed in *slt2Δ* cells (Figure S4). This suggests that the ERSU signaling pathway is activated upstream of Pkc1.

The ERSU Pathway Is Activated by Wsc1 and Is Distinct from the Cell Wall Integrity and Arrest of Secretion Response Pathways

The Pkc1-Slt2 pathway can be activated by any one of six upstream components, Wsc1, Wsc2, Wsc3, Wsc4, Mid2, and Mtl1, which reside in the plasma membrane (de Nobel et al., 2000; Gray et al., 1997; Ketela et al., 1999; Philip and Levin, 2001; Verna et al., 1997; Zu et al., 2001). We found that, of these six sensors, only *wsc1Δ* cells displayed reduced Slt2 phosphorylation during Tm treatment (Figure 6A) and Tm sensitivity on plates (Figure 6B). Furthermore, *wsc1Δ* cells did not display any aberrant septin morphology (Figure 6C) and no delay in cER inheritance (Figure S5A) during Tm treatment, indicating that ERSU relies on Wsc1 activation.

Wsc1 is known to mediate the cell wall integrity (CWI) pathway that allows a cell to respond to excess turgor pressure against the cell wall. Its activation during cell wall stress results in phosphorylation of Slt2 via Pkc1 (Levin, 2005). We therefore asked whether the ERSU signal originates as a defect in CWI. We found that stimulation of cell wall stress induced by the chitin antagonist calcoflour white (CFW) did not affect septin dynamics, even though it induced Slt2 phosphorylation as previously reported (Figures 6D and 6E). Thus, Slt2 phosphorylation is not sufficient to activate the ERSU response, and the ERSU pathway is not induced by cell wall stress. Moreover, a recent report has found that CFW-induced cell wall stress activates the UPR response (Scrimale et al., 2009). In this case, however, UPR was mediated by Mid2, and not by Wsc1, differentiating the ERSU pathway from cell wall stress.

Additional support for the distinction between the ERSU pathway and the CWI pathway came from our observation that sorbitol, an osmotic stabilizer known to suppress signaling through the CWI pathway (Verna et al., 1997), did not alter Tm sensitivity of *slt2Δ* cells (compare Figure 5A, no sorbitol, and Figures 5E and 6B, with sorbitol). Furthermore, it has been observed that in the CWI pathway, the GDP exchange factors Rom1 and Rom2 mediate signaling from the cell surface components (Philip and Levin, 2001). We found, however, that they were not involved in the ERSU pathway, as neither *rom1Δ* nor *rom2Δ* cells were sensitive to Tm, and Slt2 underwent phosphorylation at similar efficiency in these mutant cells as in WT cells (Figures S5B and S5C). Lack of Rom1 and Rom2 involvement also provides further support for difference in the CWI pathway and the ERSU signaling.

A previous report has shown that the arrest of secretion response (ASR) is caused by secretory block and is mediated by Pkc1 and Wsc proteins trapped along the secretory pathway (Nanduri and Tartakoff, 2001). Because the ER plays roles in maturation of secretory proteins, ER stress may indirectly cause secretory block and, therefore, ER surveillance may be induced by activation of the ASR pathway. To distinguish between the ERSU and ASR pathways, we induced a secretory block that was independent of ER stress, using the *sec1-1* temperature sensitive allele (Figure S5D) and asked whether this caused visible defects in the septin ring. *sec1-1* is one of the best-characterized secretory block mutants (Novick and Schekman, 1983). Shifting of *sec1-1* cells to the restrictive temperature (37°C) has been shown to result in a reduction of mRNA transcripts coding

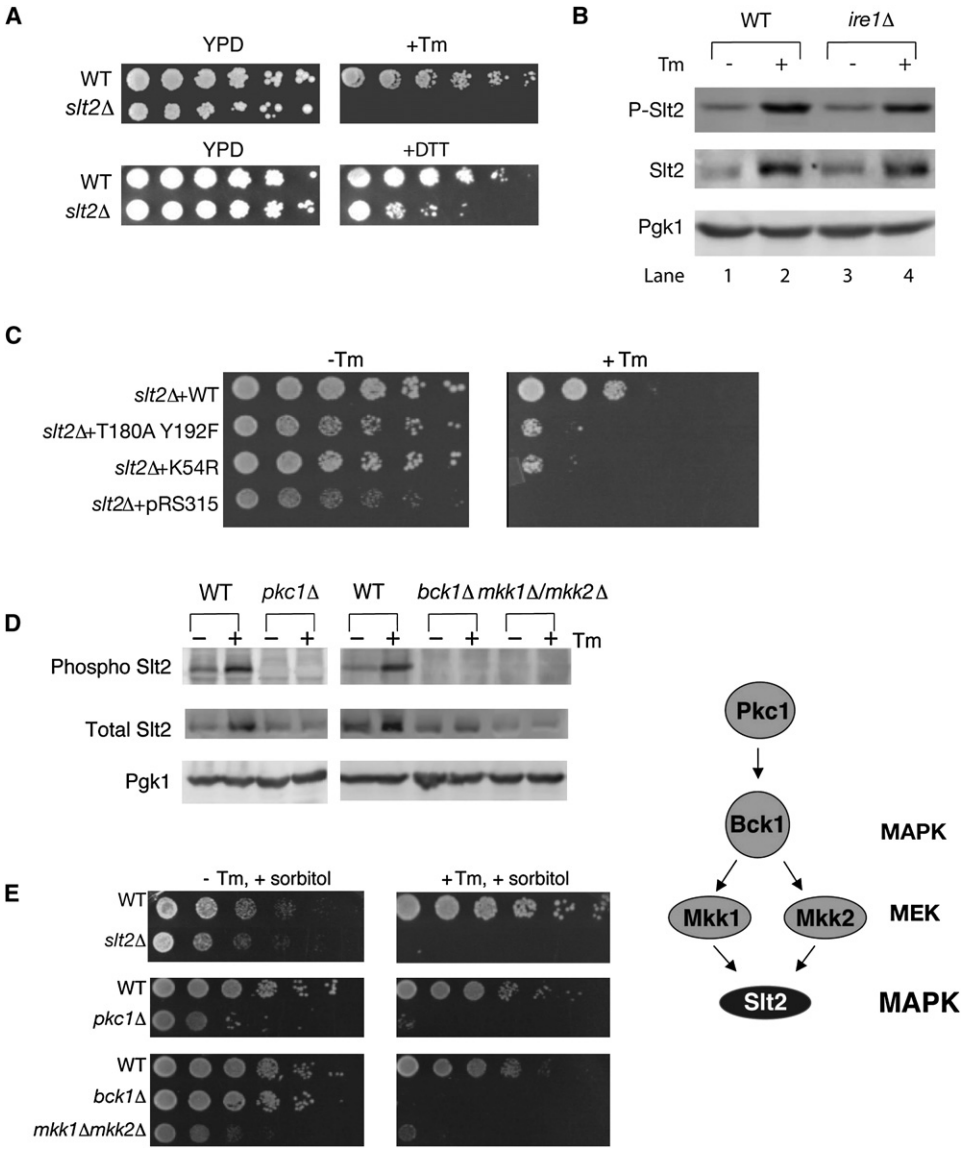

Figure 5. Slt2p Phosphorylation and Kinase Activity Are Essential for ER Stress Surveillance

(A) *slt2*Δ cells were not able to sustain growth on a plate containing Tm or DTT. 5-fold serial dilutions of WT and *slt2Δ* cells were spotted on plates containing no drug (YPD) or +Tm (0.1 μg/ml), or +DTT (4 mM).

(B) Slt2 MAP kinase was phosphorylated upon ER stress in WT (lanes 1 and 2) and *ire1Δ* cells (lanes 3 and 4). Total cell lysate of either WT or *ire1Δ* cells with (+) or without (–) Tm treatment were blotted with anti-phospho-specific Slt2, total Slt2, or Pgk1 (loading control) antibodies. In addition to increase in phosphorylation of Slt2, total protein level was also increased, although ER stress-induced phosphorylation does not require increase in Slt2 protein levels (see Figure S3B).

(C) *slt2Δ* cells were transformed with empty plasmid, or plasmid containing WT *SLT2*, T180AY192F *slt2*, or K54R *slt2*. Cells were grown to log phase in synthetic complete (SC)-leu medium, diluted serially 5-fold, and spotted onto plates with or without 0.1 μg/ml Tm.

(D) *pkc1Δ* and corresponding WT cells were grown in the presence of 1 M sorbitol, while *bck1Δ*, *mkk1Δ /mkk2Δ*, and corresponding WT cells were grown in the absence of sorbitol.

(E) Cells of the indicated genotype were grown to log phase in YPD + 1 M sorbitol, diluted serially 5-fold, and spotted onto 0.2 μg/ml Tm or no Tm plates containing 1 M sorbitol. Sorbitol had no effect on sensitivity of *slt2Δ* on Tm plate (compare Figure 5E versus Figure 5A), revealing lack of the CWI pathway involvement. See also Figure S4.

for ribosomal proteins including RPL32 (Nanduri and Tartakoff, 2001). Although we confirmed reduction in *RPL32* mRNA transcript when *sec1-1* cells were grown at 37°C (Figure S5E), septin morphology and localization remained normal (Figure S5F), distinguishing ERSU signaling from ASR pathway. Additionally, ASR signal is mediated via Pkc1 but it does not involve Slt2, while ERSU is mediated by Wsc1, Pkc1, and Slt2 upon ER stress, providing further distinction from the ASR pathway.

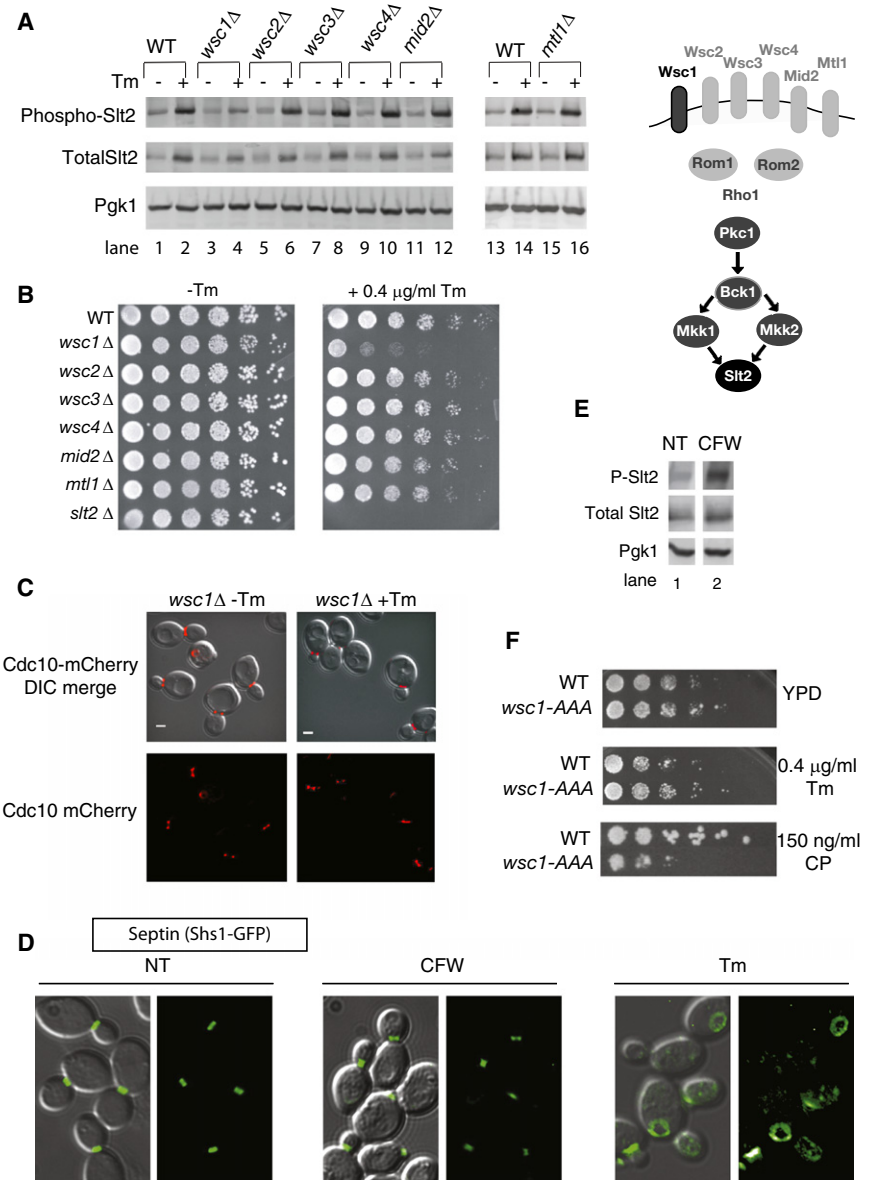

Figure 6. The ERSU Pathway Is Activated by Wsc1 and Is Independent of the Cell Wall Integrity and Arrest of Secretion Response Pathways

(A) *WSC1* is required for phosphorylation of Slt2 upon ER stress. Tm treated (+Tm) or untreated (−Tm) cells of indicated genotypes were collected and analyzed by western blot for Slt2 phosphorylation, total Slt2, and Pgk1.

(B) *wsc1Δ* cells did not support growth on a YPD plate containing 0.4 μg/ml Tm. 5-fold serial dilutions of the indicated mutant cells were grown on medium with and without Tm.

(C) In the absence of WSC1, septin rings (Cdc10-mCherry reporter) did not show altered morphology upon ER stress induced by Tm (1 μg/ml for 3 hr), but were only observed at the bud neck (a similar septin phenotype as what was seen in *slt2Δ* cells).

(D) Not all stresses that induce Slt2 activation affect septin ring dynamics. Wild-type cells expressing Shs1-GFP were visualized after 2 hr of treatment with 10 μg/ml calcofluor white (CFW) or 1 μg/ml Tm for 3 hr. NT, no treatment. GFP alone and GFP merged with a DIC image are shown.

(E) Samples from the experiment described in (D) were collected and analyzed by western blot for Slt2 phosphorylation.

(F) Endocytosis of Wsc1 does not play a role in the Wsc1 function in ERSU signaling, although it is known to be involved in the cell wall integrity (CWI) pathway. 5-fold serial dilutions of WT cells and cells with the genomic copy of *WSC1* replaced by the endocytosis mutant *wsc1-AAA* (Piao et al., 2007) were spotted onto plates with and without Tm (0.4 μg/ml) and the CWI pathway activator, caspofungin (CP; 150 ng/ml). Growth was monitored after 2 days.

See also Figure S5.

Finally, during cell wall stress, Wsc1's function in sensing the stress relies on its localization to sites of polarized growth on the plasma membrane (Piao et al., 2007). This localization requires constitutive endocytosis of Wsc1 from the cell surface. Indeed, *wsc1* mutants defective in endocytosis (*wsc1*^{AAA}) cannot establish a polarized localization and cannot sense cell wall stress (Piao et al., 2007). They are therefore hypersensitive to caspofungin (CP) treatment, which induces cell wall stress (Figure 6F, compare WT and *wsc1-AAA* with CP) (Piao et al., 2007). In contrast, we found that cells expressing an endocytosis-defective mutant form of *WSC1* (*wsc1*^{AAA}) were able to grow in the presence of Tm at the rate similar to that of wild-type cells (Figure 6F). Therefore, taken together, the data indicate that Wsc1 senses ER stress by mechanisms distinct from cell wall stress and that the ERSU represents a previously uncharacterized utilization of this MAP kinase cascade.

ERSU Promotes Mother Cell Viability during ER Stress

We have shown that Slt2's function in linking the cell cycle with ER stress is important for long-term survival during ER stress (Figure 5A). To further determine whether the output of the ERSU pathway that prevents stressed cER from entering into the daughter cell is protective, we asked whether we could mimic the ERSU response in the *slt2Δ* mutant by inhibiting cER entry into the daughter cell. Since both cER movement and septin morphology are actin dependent (Estrada et al., 2003; Kozubowski et al., 2005), we asked whether treatment of *slt2Δ* cells with the actin depolymerizing agent Latrunculin B (LatB) (Spector et al., 1983) would prevent ER inheritance, alter septin structure, and allow growth in Tm of *slt2Δ* cells. Remarkably, LatB treatment suppressed the cER inheritance (Figure 7A; Hmg1-GFP) and altered the septin morphology (Figure 7A; Shs1-GFP). Quantification indicated that the treatment of *slt2Δ* cells with LatB + Tm reduced the number of daughter cells containing cER (Figure S6B, class I and II) to numbers similar to wild-type cells treated only with Tm. Furthermore, LatB also rescued

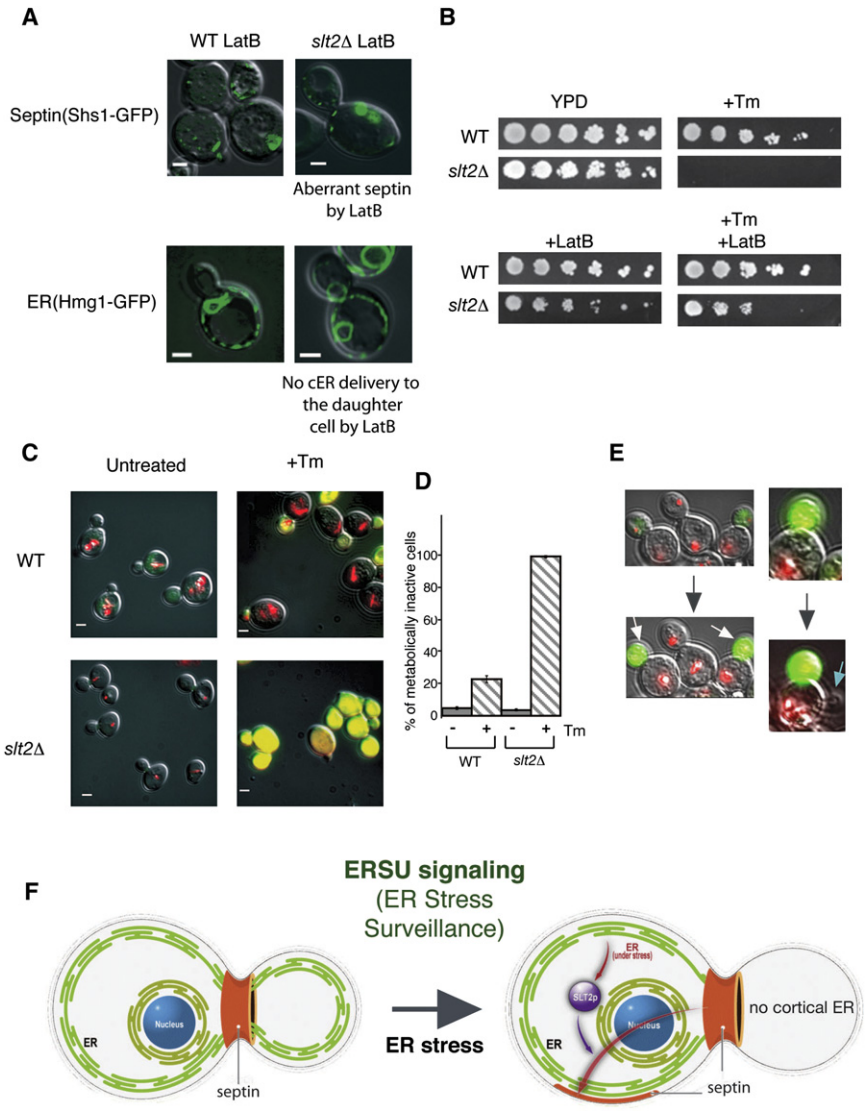

Figure 7. ERSU Signaling Protects Mother Cells during ER Stress

(A) The effect of Latrunculin B (LatB) on ER and septin distribution was visualized with the septin reporter Shs1-GFP and the ER reporter Hmg1-GFP in wild-type (left) or *slt2*Δ cells (right) treated with 1 μg/ml Tm and 400 μM LatB for 2 hr. In both cell types, septin morphology was altered and cER inheritance was inhibited by the presence of LatB. Scale bars represent 2 μm.

(B) Lat B rescues growth of *slt2Δ* cells during ER stress. 5-fold serial dilutions of WT and *slt2*Δ cells were spotted on plates containing no drug (YPD), +Tm (0.1 μg/ml) alone, +LatB alone (6 μM), or +Tm (0.1 μg/ml)/+LatB (6 μM). See also Figure S6.

(C and D) Untreated and Tm treated WT and *slt2Δ* cells were stained with FUN-1. Upon ER stress induction by Tm treatment (+Tm; 1 μg/ml, 3 hr), the daughter cells showed yellow staining, while the larger mother cells showed intravacuolar red rod-like structures (CIVSs), indicating that they were metabolically active. In contrast, *slt2Δ* cells after Tm treatment were stained yellow for both mother and daughter cells. Untreated WT and *slt2Δ* cells showed red CIVSs. Quantitation of yellow metabolically inactive cells is shown in (D). For WT and *slt2Δ* cells, the numbers of cells with yellow staining were counted. Scale bars represent 2 μm.

(E) Continuous observation of mother and daughter cell fate during ER stress. After staining with Fun-1, Tm-treated cells (1 μg/ml for 30 min) were spread onto a gelatine-YPD pad containing 0.1 μg/ml Tm for microscopic observation. Initially, both mother and daughter cells were alive (stained red), although the daughter cell (white arrow) became metabolically inactive. Furthermore, a blue arrow indicates a new daughter cell started to emerge from the original mother cell, showing asymmetric protection of the mother cell. See also Figure S7.

(F) During the cell cycle, the functional capacity of the ER is under surveillance. When ER stress is detected, ERSU activates Slt2, leading to septin reorganization and alteration so that the aberrant septin ring (shown in orange) is found in unusual places and is no longer at the bud neck. Concomitantly, ER inheritance, and cytokinesis are delayed. Thus, ERSU plays a critical role in correct transmission of a functional ER into the progeny cell.

the Tm sensitivity of *slt2Δ* cells (Figure 7B), i.e., *slt2Δ* cells were able to grow on Tm plates when LatB prevented cER entering into the daughter cell. Similarly, prevention of cER inheritance by *MYO4* gene deletion (Estrada et al., 2003) mimicked the ability of LatB to rescue *slt2Δ* cell growth on Tm (Figure S6C). In addition, mild actin defect helps cER inheritance delay upon induction of ER stress, allowing *act1-1* to grow better than WT cells (Figure S6E). Taken together, these results are consistent with the idea that ERSU signaling ultimately protects cells from deleterious effects of inheriting stressed ER.

We next asked whether this survival is achieved through specific preservation of either the mother or daughter cell. To distinguish the viability of mother and daughter cells, we stained both WT and *slt2Δ* cells with the vital dye FUN-1 after Tm treatment (Figures 7C and 7D). This dye generates differential staining patterns in metabolically active and inactive cells (Millard et al., 1997): metabolically active cells exhibit red-fluorescent cylindrical intravacuolar structures (see Figure S7A, part b, upper panel), whereas metabolically inactive cells display diffuse bright green and red cytoplasmic fluorescence, which appears yellow when merged (see Figure S7A, part d, lower panel). Upon induction of ER stress, 22% of cells became metabolically inactive, while less than 5% were metabolically inactive in an asynchronous population of normally grown WT cells (Figure 7C, quantified in Figure 7D [n = 300]). Strikingly, in budded cells, the mother cell remained metabolically active, whereas the daughter cell appeared metabolically inactive. In contrast, in the *slt2Δ* mutant, ER stress caused a much higher fraction of the cells to become metabolically inactive (Figures 7C and 7D), and there was no preference for the daughter over the mother. Similar results

were obtained with WT and *slt2Δ* cells when nonviable cells were enumerated by propidium iodide (PI) dye, a well-established nucleic acid binding agent that is only permeable to dead cells (Figures S7B and S7C). Furthermore, when ER stress was induced by *ero1-1* cells grown at nonpermissive temperature (37°C), we observed a similar increase of PI stained cells (Figure S7D). We also examined the viability of WT and *slt2Δ* cells treated with Tm in the presence of LatB and compared this to treatment with Tm alone (Figures S7B and S7C). Whereas ~95% of *slt2Δ* cells treated with Tm alone were stained by PI (both mother cells and daughter cells), only ~25% of cells were stained (mostly daughter cells with some both mother and daughter cells) in the presence of Tm and LatB. These results are in agreement with the growth rescue of *slt2Δ* cells observed on YPD medium containing Tm and LatB (Figure 7B). Finally, the selective inactivation of the daughter cell was further confirmed by continuous observation of live cells with FUN-1 staining after ER stress induction (Figure 7E). We observed FUN-1-stained daughter cells attached to a viable mother cell from which, ultimately, a new bud started to emerge. This suggests that ERSU signaling specifically promotes mother cell viability, at the expense of the daughter cell.

DISCUSSION

A minimum level of ER functionality is required for cell viability. Thus, the functional capacity and timing of cell division and cER inheritance may be coordinated by a checkpoint that ensures a minimum ER functionality before cell division. We report here the identification of an ERSU pathway that may function as the gatekeeper for this checkpoint and monitors the functional capacity of the ER during cell division. When ER stress is induced, ERSU causes cytokinesis delay and cER to be retained in the mother cell until it replenishes ER function (Figure 7F). The delay in cytokinesis correlates with, and is likely caused by, altered dynamics of the septin complex. ERSU is independent of UPR signaling and instead relies upon the MAP kinase Slt2, ensuring that only functional ER is transmitted to daughter cells. Thus, ER stress activates both the ERSU pathway, which controls cell-cycle progression, and UPR pathways, which re-establish ER functions. Once the ER capacity is re-established, we expect that the ERSU pathway will be turned off. Thus, although the ER may not be delivered to the daughter cell during ER stress, subsequent daughter cells will receive functional ER. In ERSU deficient cells (for example, *slt2Δ* cells), this regulation is lost, and mother cells distribute cER into the daughter cells irrespective to functional state of the ER. As a consequence, the level of ER functionality in the mother cell may drop below the minimum requirement, causing both cells to undergo cell death. Thus, during ER stress, in *slt2Δ* cells both the mother and daughter cell are inviable, whereas in WT cells, when the ER is retained in the mother cell, only the daughter cell is inviable. In support of this model, inhibiting ER inheritance in *slt2Δ* cells through treatment with LatB allowed restoration of mother cell viability (Figures 7A and 7B). While we described in this report conditions in which ER stress is highly induced, we believe that the ERSU pathway may also function during the normal cell cycle, serving as a cell-cycle "checkpoint" that assures generation of progeny cells with functional ER.

One intriguing observation is that the ERSU pathway causes stressed cER to be preferentially retained in the mother cell. This type of mother cell retention has also been seen for factors that contribute to cell aging, such as extrachromosomal ribosomal DNA circles (ERCs), maternal nuclear pores, and carbonylated proteins (Tessarz et al., 2009; Shcheprova et al., 2008). Such toxic factors accumulate with age and ultimately lead to cell death; they are observed to be retained in the mother cell presumably to increase the life span of the newly born daughters (Erjavec et al., 2007; Murray and Szostak, 1983; Sinclair and Guarente, 1997). It has been shown that the retention in the mother cells of nuclear pores and ERCs requires a septin-dependent diffusion barrier within the nuclear envelope (Shcheprova et al., 2008). Taken together, these data point to a general evolutionary rationale that uses multiple critical mechanisms to assure the asymmetric distribution of elements to the mother cell. At first glance, ERSU seems to act differently from these aging pathways, because it allows preferential protection of mother cells rather than daughter cells. However, unlike the accumulation of aging factors, ER stress is reversible. Thus, the ERSU pathway ultimately promotes conservation of offspring by protecting the mother cell and allowing it to generate subsequent generations of daughter cells.

Another intriguing feature of the ERSU pathway is the different behavior of cortical and nuclear ER. We observed that perinuclear ER was inherited normally with the nucleus during ER stress, while cER delivery to the daughter cell was inhibited, suggesting a potential distinction between cortical and perinuclear ER. A recent report using an ER stress reporter indicates that ER stress is not transmitted to daughter cells (Merksamer et al., 2008). As we have found that perinuclear ER along with the nucleus is transmitted into daughter cells even during ER stress, such an observation suggests that perinuclear ER is free of ER stress. It prompts one to ask whether ER stress could somehow be partitioned to the cER, which is retained in the mother cell. Future studies will test such ideas and explore the mechanism and functional implications of this distinction between the two subdomains of the ER.

ERSU controls cell cycle in response to ER stress via Wsc1 and Slt2, distinct from the UPR, CWI, and ASR pathways. Currently, we do not know how ER stress is signaled through Wsc1. The mechanism is unlikely to involve gross changes in Wsc1 localization, as we have found that the steady state localization of Wsc1 during ER stress does not change (data not shown). One possibility is that ER stress activates Wsc1 at the cell surface through an unknown mechanism. Alternatively, as Wsc1 transits the ER during its folding process, it might directly detect ER stress and initiate the ERSU pathway. For example, during ER stress, Wsc1 protein might be modified within the ER lumen before exiting from the ER to initiate ERSU. In addition, we do not know how Slt2 kinase activation leads to the cER inheritance delay and septin alteration. Slt2 kinase may directly phosphorylate cER inheritance components or septin subunits. Future studies will be required to uncover the molecular mechanism of Wsc1 and Slt2 activation during ER stress.

In summary, our study has described the discovery of an ERSU pathway in yeast. We have mapped a number of the components of the ERSU pathway but anticipate that future studies will provide additional components involved in the pathway. For example, a recent report describes a novel molecular mechanism involving the polarisome, a multiprotein complex that regulates actin cytoskeleton restricting apical growth of *S. cerevisiae* (Pruyne et al, 2002; Sagot et al., 2002), that prevents protein aggregates from staying in the daughter cell (Liu et al., 2010). It may also be possible that the polarisome functions to establish the cER inheritance delay in response to ER stress. In addition, Ptc1, a phosphatase that is thought to negatively regulate Pkc1, and thus ultimately Slt2 kinase (Huang and Symington, 1995; Nanduri and Tartakoff, 2001; Martín et al., 2005), may also function in the ERSU pathway. A recent genetic screen identified Ptc1 is a component with an as yet unknown role in ER inheritance during normal cell growth (Du et al., 2006). Ptc1 also regulates inheritance of the mitochondria (Roeder et al., 1998) and the vacuole (Jin et al., 2009). Therefore, Ptc1 may be a part of a master regulator choreographing different mechanisms that modulate the transmission of organelles and the cytoplasmic components to the daughter cell.

A mechanism of ER surveillance similar to ERSU may exist in mammalian cells. Since the fundamental mechanisms of cytokinesis differ between yeast and mammalian cells, the details of ERSU may differ between the two cell types. However, the failure to properly regulate ER functional capacity in vertebrate cells is increasingly recognized as contributing to the pathophysiology of a number of human diseases, including diabetes and certain cancers. Thus, further understanding of the cellular mechanisms of the ERSU response that we have reported here, and investigation of the mammalian counterpart may allow for the development of previously unrecognized strategies for therapeutic intervention.

EXPERIMENTAL PROCEDURES

Cell Manipulations

The *S. cerevisiae* strains used in this study are described in Table S2. All cells were grown in YPD at 30°C and were examined during log phage unless otherwise noted. ER stress was induced upon addition of Tm or DTT, or shifting of the temperature of *ero1-1* cells to 37°C, as described detail in figure legends and in the Extended Experimental Procedures.

Northern and Western blots

Both northern and western blotting were performed as described previously (Bicknell et al., 2007).

Microscopy

All cells were visualized with a microscope (Axiovert 200M; Carl Zeiss MicroImaging) with a 100 × 1.3 NA objective. Images were captured with a monochrome digital camera (Axiocam; Carl Zeiss MicroImaging) and analyzed using Axiovision software (Carl Zeiss MicroImaging).

DNA Staining and Flow Cytometry

One million cells were fixed in 70% ethanol overnight, treated with 1 mg/ml RNase A at 37°C for 2 hr, 5 mg/ml pepsin for 20 min at 37°C, and then stained with 10 μM Sytox Green (Invitrogen), as described previously (Bicknell et al., 2007). Data were collected with a flow cytometer (FACSCalibur; BD Biosciences) and analyzed with FlowJo software (TreeStar).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.cell.2010.06.006.

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EXTENDED EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Conditions

Deletion and epitope tagged strains were constructed using a one-step PCR-mediated technique (Longtine et al., 1998b). Plasmids p2188 (pRS315[*MPK1-3xHA*]), p2190(pRS315[*mpk1*(*T190A Y192F*)*-3xHA*]) and p2193 (pRS315[*mpk1*(*K54R*)*-3xHA]*) were transformed into MNY1045. ER localization was monitored using the previously-characterized ER membrane reporter plasmid, pRH 475, and luminal reporter plasmid, pRH 1827 (Du et al., 2001; Hampton et al., 1996). pRH 475 expressed Hmg1-GFP, the transmembrane NH2-terminal domain (1-702) of the ER resident protein HMG-CoA reductase isozyme I fused to GFP and was expressed from the strong, constitutive TDH3 promoter. pRH 1827 contained an HDEL-DsRed fusion protein bearing the signal sequence and an HDEL ER retrieval signal from *KAR2*.

All cells were grown in rich complete medium (YPD) except strains transformed with p2188, p2190 and p2193, which were grown in synthetic complete –LEU medium. Strains were grown at 30°C and examined during log phase, unless otherwise noted. For synchronized experiments, cells were treated with 50 ng/ml α factor (stored as a 1 mg/ml stock in PBS at –20°C) for 2.5 hr, washed twice with an equal volume of fresh medium, diluted to an OD of 0.25, and then allowed to recover for 15 min before treatment with Tm. For Slt2 immunoblot experiments, cells were grown to an OD_{600} of 1, then diluted to an OD of 0.25 before treatment with Tm or CFW.

Stock and final concentrations of drugs used in this study were as follows: Tunicamycin (Tm) (Calbiochem), final 1 μg/ml or as indicated; DTT (Fisher), final 2 mM or 4 mM, as indicated; Latrunculin B (LatB) (Calbiochem), final 400 μM; calcofluor white (CFW) (Sigma), final 10 μg/ml; and caspofungin (CP) (a generous gift from Merck), final 10 ng/ml. For plates, final concentrations were 0.2 μg/ml or 0.4 μg/ml Tm, as indicated, and 6 μM LatB.

Cell Extracts, Northern Blotting and Immunoblotting

For Northern blotting, ten μg of total 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 with a radio labeled DNA probe.

For Western blot analysis, protein was extracted as previously described (Bicknell et al., 2007), and 20 μg of protein were loaded on an 8% SDS-PAGE gel, and transferred to nitrocellulose. Primary antibodies used were anti-phospho p44/p42 MAP Kinase (Slt2) antibody (Cell Signaling) at a 1:1000 dilution overnight and anti-PGK (phosphoglycerate kinase) antibody (Molecular Probes) at a 1:10,000 dilution for one hour, and anti-Slt2 antibody (Santa Cruz Biotechnology) at a 1:1000 dilution for overnight at 4°C. Anti-Secondary antibodies were HRP-conjugated donkey anti-rabbit antibody at 1:10,000 (GE Healthcare) for phosphor Slt2, and goat anti-mouse 1:10,000 (BioRad) for PGK. Membranes were developed with ECL Plus Western blotting detection reagent (GE Healthcare) and imaged using a Typhoon phosphorimager (GE Healthcare).

Microscopy

Tagged septin subunits and Hmg1-GFP were imaged in live log phase cells. To quantitate ER inheritance, 300 budded cells were counted, divided into 3 classes as described in Results, and scored for the presence or absence of cER in the bud. FUN1 staining was performed using FUN-1 dye (Molecular Probes) at a final concentration of 10 μM for 1 ml of $3x10^6$ cells/ml for 30 min at 30°C in the dark. Propidium iodide (PI) staining was performed using a 1 mg/ml stock solution at a final concentration of 3 μg/ml. Cells were imaged immediately after staining. No washing step was included.

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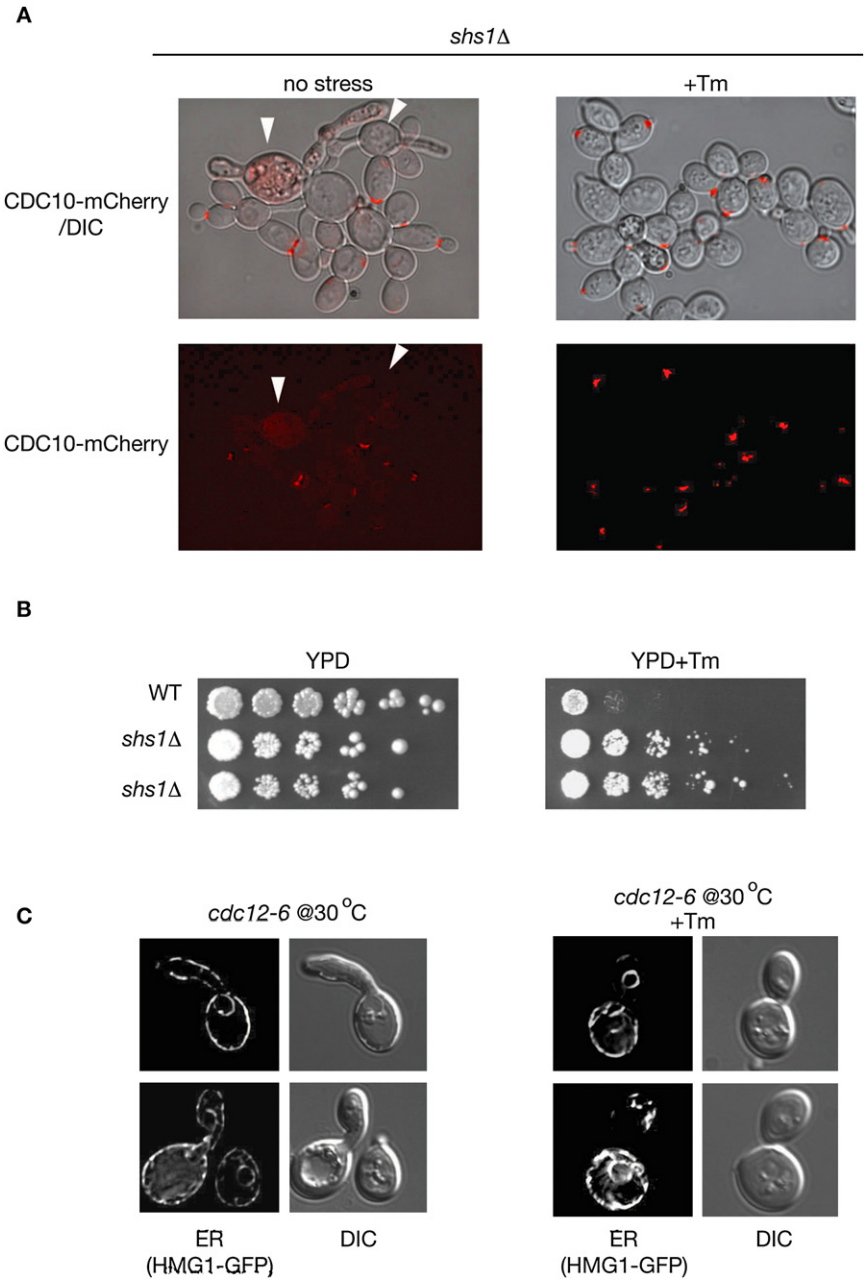

Figure S1. ER Stress Restores Normal Septin Morphology in *shs1Δ* Cells, Related to Figure 1

(A) Septin behavior in *shs1Δ* cells. Deletion of the septin subunit gene *SHS1* causes gross alterations to septin morphology and localization under normal growth conditions. Septin was visualized by integration of CDC10-mCherry septin reporter at the genomic locus. Septin ring of *shs1Δ* cells were dispersed or localized away from the bud neck (white arrowhead). In addition to abnormal septin ring, many *shs1Δ* cells were elongated, similar to *cdc12-6* cells grown at non-permissive temperature (30°C) (Figure 1C). Treatment of *shs1Δ* cells with Tm (1 μg/ml, 3 hr) to induce ER stress restored normal septin morphology and bud neck localization, also similar to the effects of ER stress on *cdc12-6* cells.

(B) Impaired growth of *shs1Δ* cells was rescued by ER stress. The growth of *shs1Δ* cells on YPD and YPD+Tm plates was compared to wild-type (WT) cells. Log phase cells spotted at five fold serial dilutions on YPD or YPD+Tm (0.4 μg/ml) plates were grown for 3 days. *shs1Δ* cell grew better than wild-type cell upon induction of ER stress with Tm, correlating with ER stress induced restoration of normal septin morphology and localization, and of normal cell shape to *shs1Δ* cells (shown in (A)).

(C) Inheritance of cER to *cdc12-6* daughter cells grown at non-permissive temperature (30°C) is significantly reduced by ER stress induction with Tm (1 μg/ml) for 3 hr. These results are in good correlation with the restoration of normal septin morphology and localization, cell morphology, and growth of *cdc12-6* cells under ER stress (growth at 30°C + Tm) as shown in Figures 1C and 1D. The ER in *cdc12-6* cells was visualized using Hmg1-GFP ER reporter.

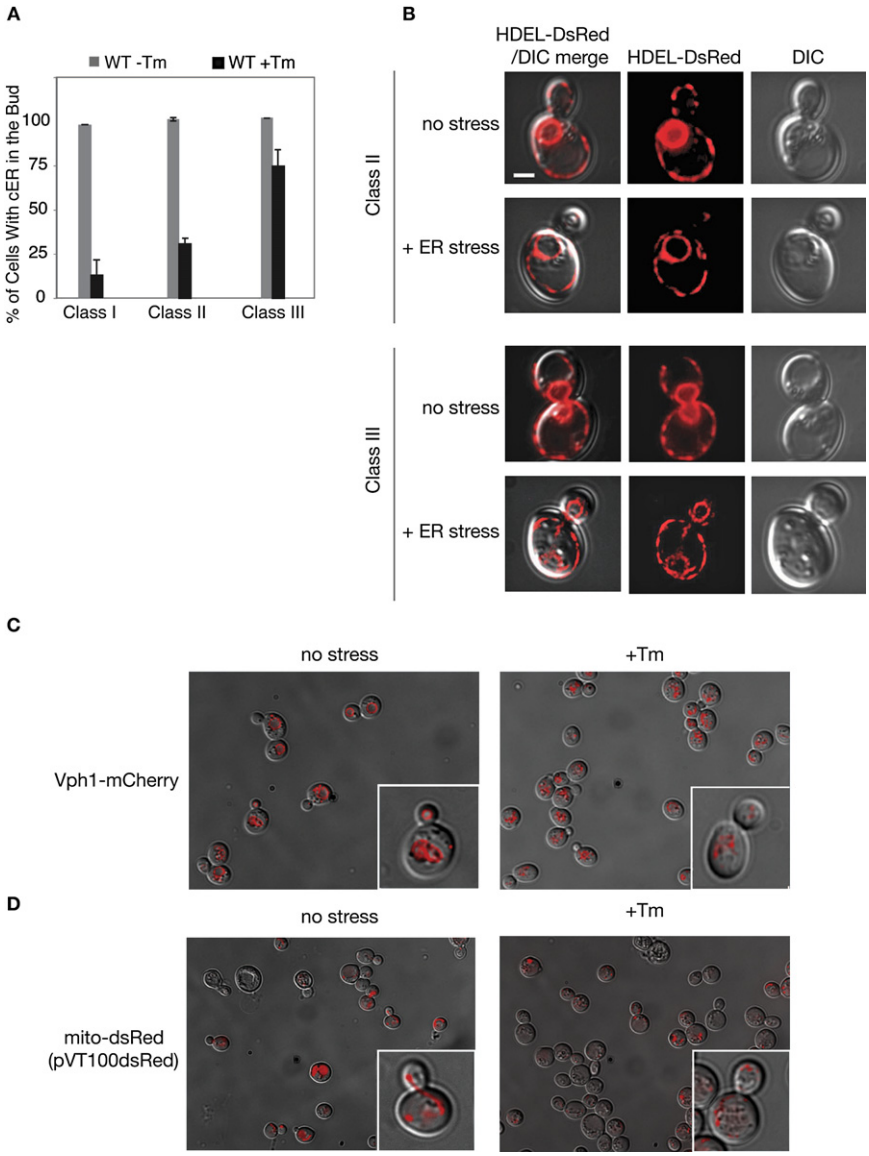

Figure S2. Visualization of ER Using HDEL-DsRed Reporter, of Vacuoles with Vph1-mCherry, and of Mitochondria Using Mito-dsRed, during ER Stress, Related to Figure 2

(A) Quantitation of cells with cER in the daughter cell (bud) in WT and ER-stressed populations. For each class of cells (see Figure 2 for definition), at least 300 cells were counted to score the numbers containing cER in the daughter cell under normal conditions (gray bars) and in the presence of 1 μg/ml Tm for 3 hr (black bars). The standard deviation was calculated from three independent experiments.

(B) Visualization of ER using the HDEL-DsRed ER reporter instead of Hmg1-GFP confirms delayed inheritance of cER in ER-stressed (1 μg/ml Tm for 3 hr) cells. HDEL-DsRed alone, DIC alone, and a merged (HDEL-DsRed/DIC) image are shown.

(C and D) Inheritance of vacuoles and mitochondria are not significantly affected by ER stress induction. Wild-type cells expressing a vacuolar marker Vph1-mCherry integrated at the genomic locus (Singh et al., 2008) (C), and a mitochondrial matrix marker pVT100-dsRedT1 (fusion of a mitochondrial targeting sequence and *dsRed-T1*) under the *ADH1* promoter (Meeusen et al., 2006, Suda et al., 2007) (D), were grown under non-stressed (YPD) conditions or in the presence of Tm (1 μg/ml) for 3 hr, before visualization under the microscope.

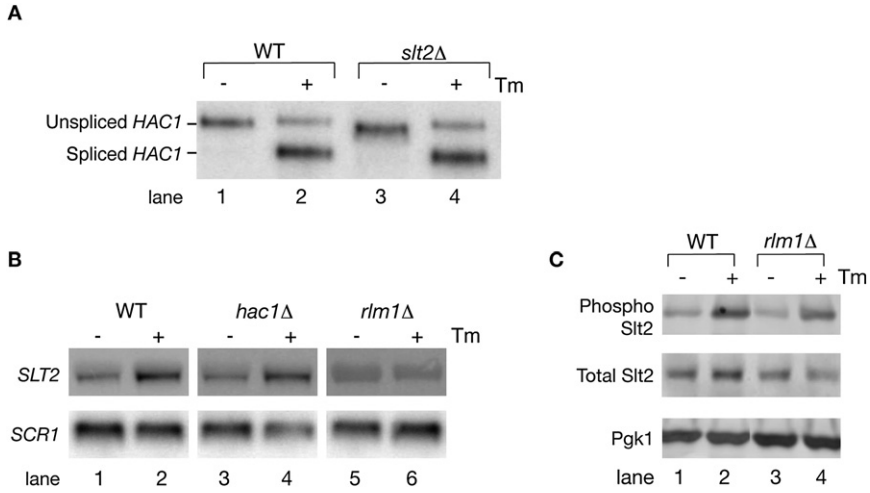

Figure S3. *slt2Δ* Cells Are Capable of Induction of the Unfolded Protein Response (UPR) in Response to ER Stress and ER Stress-Induced Phosphorylation of Slt2 Occurs Independent of the Increase in Total Slt2 Protein Level, Related to Figure 3

(A) Tm treatment induced splicing of *HAC1* mRNA in both WT and *slt2Δ* cells at similar levels. Positions of unspliced and spliced *HAC1* mRNA on Northern blot are indicated.

(B) Northern blot of *SLT2* mRNA. Total RNA of WT, *hac1Δ*, and *rlm1Δ* cells were analyzed by Northern blot, probing with *SLT2* coding sequence. The same blot was re-probed with *SCR1* as a loading control. Increase in Slt2 protein (see (C)) in response to ER stress was caused by increase in mRNA (lanes 1 & 2). *SLT2* mRNA level was also increased in the absence of the UPR transcription factor, Hac1 (lanes 3 & 4), consistent with the previous report (Chen et al., 2005). However, in *rlm1Δ cells*, *SLT2* mRNA level was minimum and did not increase after ER stress induction by Tm (lanes 5 & 6), suggesting that Rlm1, but not Hac1, is the transcription factor responsible for ER stress induced transcription of SLT2.

(C) ER stress induced phosphorylation of Slt2 and increased overall levels of Slt2 protein. WT and *rlm1Δ* cells were treated with Tm (1 μg/ml) for 2 hr before preparation of total cell extract for Western blot analyses. Phosphorylated Slt2 (phospho-Slt2) was visualized by probing with anti-phospho Slt2 specific antibody, while total Slt2 was analyzed with anti-Slt2 antibody. Pgk1 level examined by anti-Pgk1 antibody was used as a loading control. In addition to phosphorylation, total level of Slt2 increased upon ER stress induction. *RLM1* codes for a MADS-box transcription factor similar to serum response factor-like protein and is thought to be a target for Slt2 MAP Kinase (Levin, 2005). The increase in total Slt2 protein level was diminished in *rlm1Δ* cells, suggesting that Rlm1 is responsible for increase in Slt2 protein level (compare WT in lanes 1 & 2 with *rlm1Δ* cells, lanes 3 & 4). Note that phosphorylation of Slt2 increased even in *rlm1Δ* cells upon ER stress (lane 4, phospho-Slt2), revealing that ER stress induces both phosphorylation and increase in overall protein level of Slt2 and that phosphorylation does not require increase in Slt2 protein level.

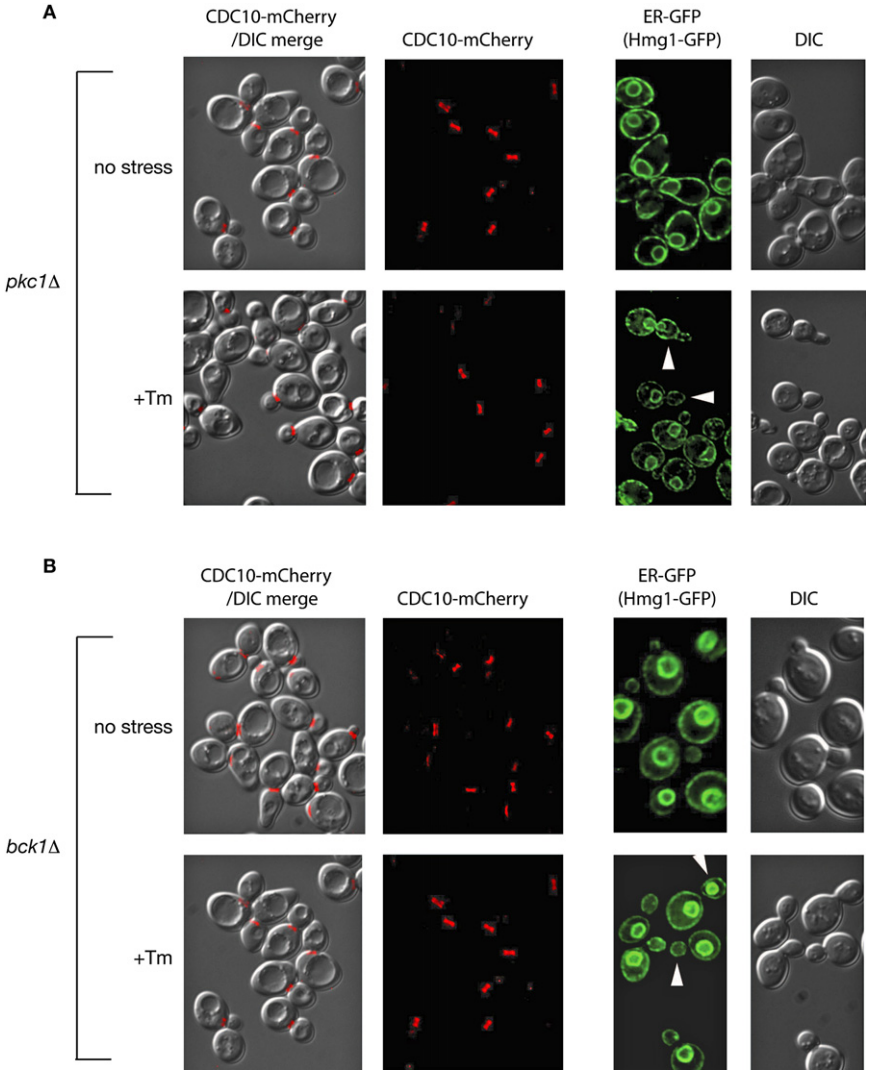

Figure S4. Kinases Upstream of Slt2 Are Essential for ER Stress Surveillance (ERSU), Related to Figure 4

pkc1Δ (A) and *bck1Δ* (B) cells carrying ER reporter (Hmg1-GFP) and septin reporter (Cdc10-mCherry) integrated at the corresponding genomic loci were analyzed for ER behavior and septin ring morphology by fluorescence microscopy. Under non-stressed conditions (YPD), ER distribution and septin ring localization (at the bud neck) are normal. Following ER stress (by Tm 1 μg/ml, 3 hr), normal septin ring morphology and normal ER behavior persisted (Examples of daughter cells with cER are shown with white arrows), indicating that septin and cER in these knockout cells are unaffected by ER stress. Similar results were seen with *slt2Δ* cells in response to ER stress (Figure 4). *pkc1Δ* cells were grown in YPD in the presence of 1M sorbitol. 1M sorbitol does not influence ERSU phenotypes (see Figures 5A versus 5E). *bck1Δ* cells were grown in YPD.

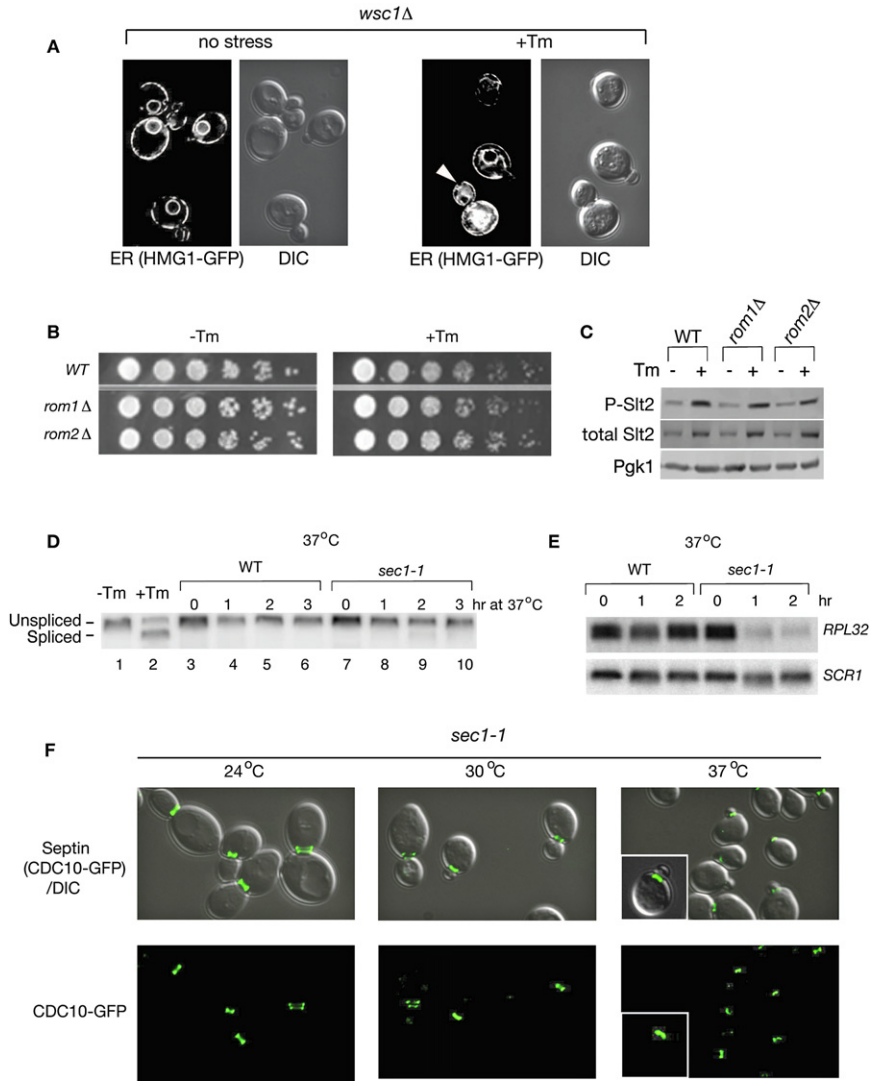

Figure S5. Involvement of Wsc1 in ERSU Is Distinct from Its Functioning in Previously Reported Signaling Pathways, Related to Figure 5

(A) In the absence of *WSC1*, cER enters daughter cell following ER stress induction. *wsc1Δ* cells carrying Hmg1-GFP reporter were grown in YPD or under ER stress inducing condition (YPD + Tm 1 μg/ml for 3 hr). A white arrowhead shows a daughter cell with cER.

(B) Neither *ROM1* nor *ROM2* is not involved in the ERSU pathway. Either *rom1Δ* or *rom2Δ* cells can support growth on a YPD plate with Tm. Five-fold serial dilutions of the indicated mutant cells were grown on medium with and without Tm (0.4 μg/ml).

(C) Both *rom1Δ* and *rom2Δ* cells can phosphorylate Slt2 upon ER stress at a level similar to ER stressed WT cells. Samples were collected and analyzed by Western blot for Slt2 phosphorylation (top), total Slt2 (middle) and Pgk1 (loading control, bottom) as described in Figure S3C.

(D) 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 (lane 2). In contrast, the lack of *HAC1* splicing during the secretory block induced in *sec1-1* cells grown at 37°C indicates that ER stress was not induced (lanes 7-10). Shifting temperature to 37°C in WT cells did not induce *HAC1* mRNA splicing (lanes 3-6).

(E) 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 (Carr et al., 1999). After shifting both WT and *sec1-1* cells to the non-permissive temperature 37°C for the indicated amount of time, total RNA samples from each time point were analyzed by Northern blot for the level of *RPL32* mRNA and *SCR1* RNA as a loading control. A dramatic loss of *RPL32* RNA confirmed that a secretory block was induced upon shifting *sec1-1* cells to 37°C.

(F) The *sec1-1* mutation induces a secretory block, but no ER stress. Septin ring was monitored in *sec1-1* cells genomically expressing Cdc10-GFP at permissive (24°C), semi-permissive (30°C), and non-permissive temperature (37°C). A *sec1-1*-induced secretory block affected the size of the daughter cell, but the septin ring targeted to its normal position at the bud neck.

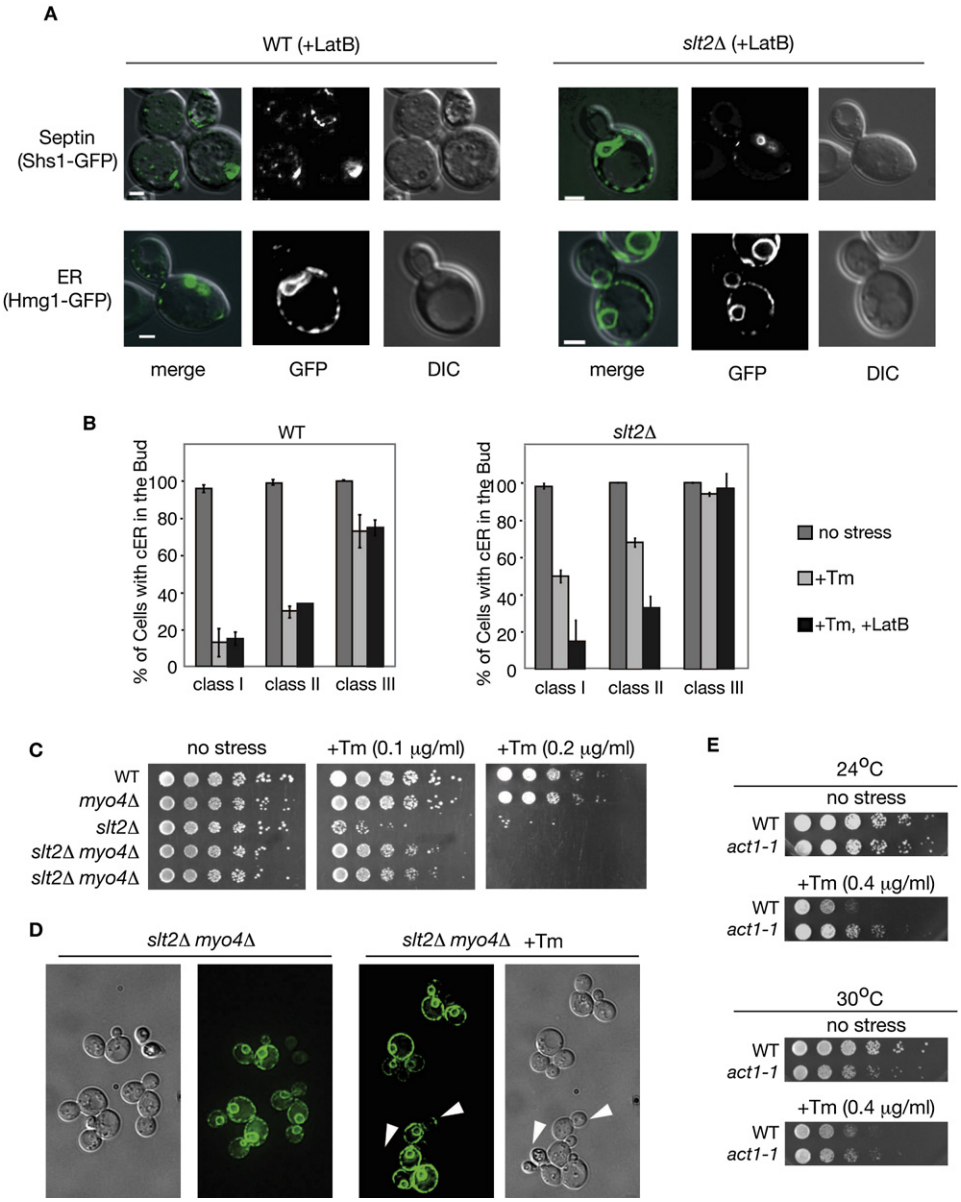

Figure S6. Inheritance of the Stressed cER into the Daughter Has Deleterious Consequences to the Cell, Related to Figures 6 and 7

(A) The effect of Latrunculin B (LatB) on cER and septin distribution was visualized with the ER Hmg1-GFP reporter and the septin reporter Shs1-GFP, respectively. GFP/DIC merged images shown Figure 7A were separated to GFP or DIC alone. Both wild-type (left) or *slt2*Δ cells (right) were treated with 1 μg/ml Tm & 400 μM LatB for 2 hr.

(B) Quantitation of Lat B effect on cER in the daughter cell. For each of the three classes of cells, the number of wild-type and *slt2*Δ cells containing cER in the daughter cell was counted (n = 300) during growth under normal conditions (dark gray bars) or in the presence of 1 μg/ml Tm (2 hr) (light gray bars) or 1 μg/ml Tm & 400 μM LatB (2 hr) (black bars). The average of three independent experiments is depicted; error bars represent standard deviation (SD). cER inheritance in Tm-treated WT cells was not significantly affected by inclusion of LatB (WT, compare Tm to LatB+Tm).

(C) Prevention of cER inheritance by *MYO4* gene deletion (Estrada et al., 2003), instead of LatB, rescues *slt2*Δ cell growth on Tm plates (0.1 μg/ml Tm + 1M sorbitol). *myo4*Δ *slt2*Δ cells (two different isolates) were able to grow better than *slt2*Δ cells or *myo4*Δ cells on Tm plates at the specific concentration range of Tm (0.1 μg/ml). Deletion of *MYO4* in *slt2*Δ cells mimicked the rescue effect of LatB seen for *slt2*Δ cells grown with Tm (Figure 7B). Curiously, however, we found that neither *myo4*Δ*slt2*Δ nor *slt2*Δ cells were able to grow at higher concentration of Tm (0.2 μg/ml + 1M sorbitol).

(D) cER in *myo4*Δ*slt2*Δ cells carrying Hmg1-GFP under no stress and ER stressed conditions (+Tm, 1 μg/ml for 3 hr). Examples of daughter cells with little cER are indicated by white arrowheads. Under normal growth conditions, most *myo4*Δ*slt2*Δ cells did not exhibit cER inheritance defects. This was surprising since *MYO4* was reported to impair cER inheritance under normal growth conditions (Estrada et al., JCB *163*: 1255-1266 2003). Further, since *slt2*Δ cells are not defective for cER inheritance under normal growth (Figures 4C and 4D), we anticipated cER inheritance defect in *myo4*Δ*slt2*Δ under the normal growth. Nevertheless, we did observe cER inheritance delay to many *myo4*Δ*slt2*Δ daughter cells upon ER stress induction. Taken together, our result showing the rescued growth of *myo4*Δ*slt2*Δ cells under low concentration of Tm is consistent with the minor delay of cER inheritance seen in *myo4*Δ*slt2*Δ cells.

(E) *act1-1* temperature sensitive mutant grew better than wild-type cells in the presence of Tm (+Tm 0. 4 μg/ml) at permissive (24°C). At semi-permissive temperature (30°C), *act1-1* grew significantly slower than wild-type cell under no ER stress. In the presence of Tm, however, *act1-1* cells grew slightly better than wild-type cells. Similarly, at permissive temperature (24°C), while *act1-1* and WT cells grew similarly under no ER stress, ER stress caused *act1-1* cell to grow better than WT. (*act1-1* is known to display mild actin defect even at permissive temperature). Together, these results are consistent with the idea that mild actin defect helps cER inheritance delay upon induction of ER stress, allowing *act1-1* to grow better than WT cells.

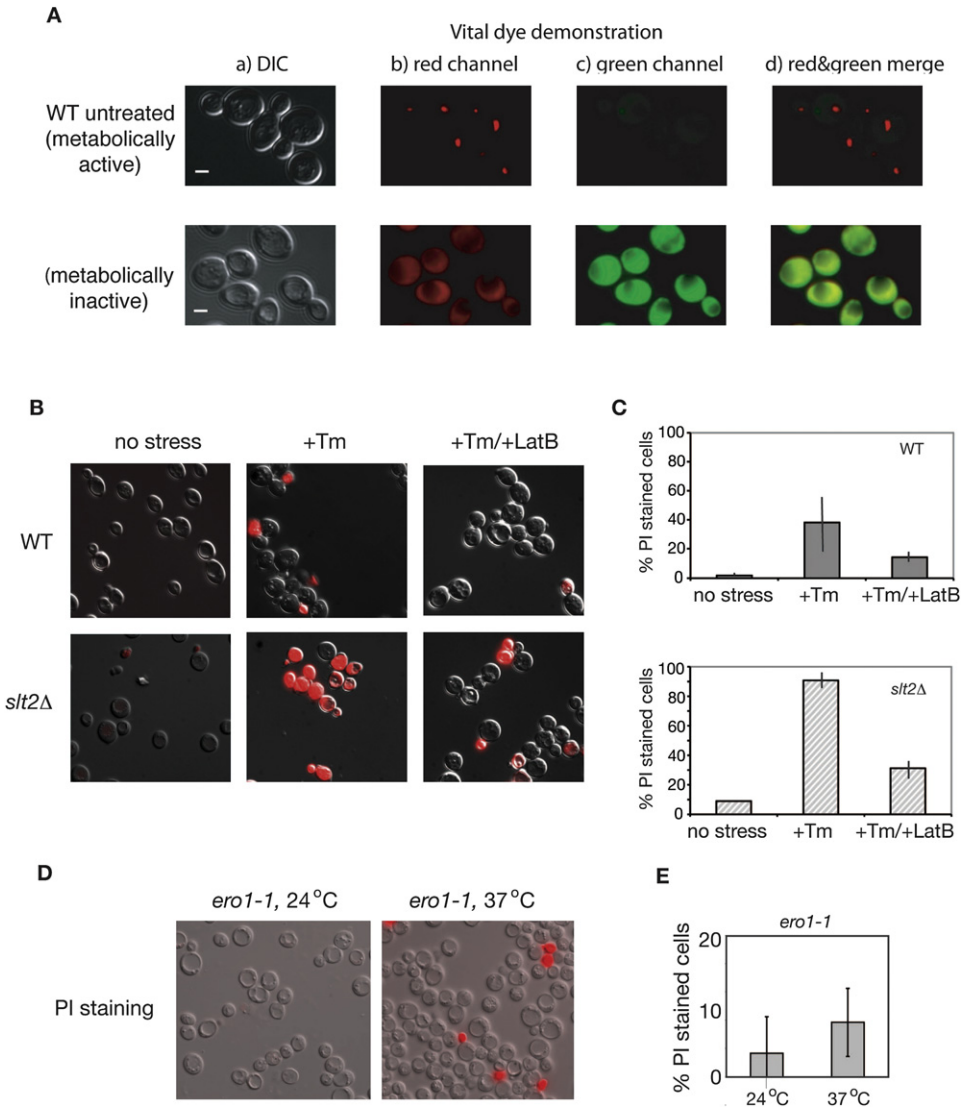

Figure S7. ER Stress Induces Increased Cell Death, Related to Figure 7

(A) The vital dye FUN-1 stains metabolically active yeast WT cells on cylindrical intravacuolar red rod-like structures (CIVSs) (Essary and Marshall, 2009), while cells rendered metabolically inactive by treatment with 10% bleach display diffuse green and red staining. (a) DIC, (b) red channel, (c) green channel, and (d) merge of red and green channels of normal (upper panels) or metabolically inactive (lower panels) cells are shown.

(B and C) Propidium iodide (PI) staining of untreated and Tm treated (1 μg/ml for 4 hr) wild-type (WT) or *slt2*Δ cells to enumerate dead cells. The fluorescent dye PI is membrane impermeable and thus excluded from viable cells, staining only dead cells. Following ER stress, dead cells (stained with PI) are noted in both cell populations, but are much more abundant in *slt2*Δ cells. The population of PI-stained WT cells consisted of small daughter cells attached to the mother cell. In contrast, both mother and daughter of *slt2*Δ cells were PI-stained. Furthermore, simultaneous treatment of cells with 400 μM LatB and 1 μg/ml Tm for 4 hr reduced the number of PI stained cells in both cell types. These results are consistent with the growth rescue of stressed *slt2*Δ cells by LatB (shown in Figure 7B). Strikingly, the population of PI-stained *slt2Δ* cells in the presence LatB was similar to ER stressed wild-type cells. Quantitation of PI stained cells (n = 100) is shown in (C).

(D and E) ER stress induction in *ero1-1* cells increases the number of cells stained with PI. When grown at permissive temperature (24°C), essentially no *ero1-1* cells were stained by PI. Temperature shift to the non-permissive temperature (37°C, 3 hr) resulted in a reproducible fraction of *ero1-1* stained by PI, although this fraction was smaller than for cells induced for ER stress by Tm treatment. Quantitation of stained cells is shown in (D). At least, 100 cells were counted for each data set, and standard deviation was calculated from three repeats of experiments.