The yeast Cbk1 kinase regulates mRNA localization via the mRNA-binding protein Ssd1

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Introduction

LATS/NDR kinases are an evolutionarily conserved subfamily of AGC (protein kinase A/G/C) kinases that are implicated in cellular transformation and growth control, and cell integrity. We discovered that most Ssd1 localizes diffusely within the cytoplasm, but some transiently accumulates at sites of polarized growth. Cbk1 inhibition and cellular stress cause Ssd1 to redistribute to mRNA processing bodies (P-bodies) and stress granules, which are known to repress translation. Ssd1 recruitment to P-bodies is independent of mRNA binding and is promoted by the removal of Cbk1 phosphorylation sites. SSD1 deletion severely impairs the asymmetric localization of the Ssd1-associated mRNA, SRL1. Expression of phosphomimetic Ssd1 promotes polarized localization of SRL1 mRNA, whereas phosphorylation-deficient Ssd1 causes constitutive localization of SRL1 mRNA to P-bodies and causes cellular lysis. These data support the model that Cbk1-mediated phosphorylation of Ssd1 promotes the cortical localization of Ssd1–mRNA complexes, whereas Cbk1 inhibition, cellular stress, and Ssd1 dephosphorylation promote Ssd1–mRNA interactions with P-bodies and stress granules, leading to translational repression.

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et al., 2009); however, the molecular function of Ssd1 is not well understood; however, Ssd1 is genetically linked to multiple cellular functions, including maintenance of cell integrity, mRNA processing, stress signaling, and cellular aging (Evans and Stark, 1997; Uesono et al., 1997; Moriya and Isono, 1999; Ibeas et al., 2001; Jorgensen et al., 2002; Kaebel and Guariento, 2002; Wheeler et al., 2003; Kaebel and Guariento, 2004; Wojda et al., 2007; Jansen et al., 2009; Moriya and Isono, 1999; Ibeas et al., 2001; Jansen et al., 2009). Recent data also link Ssd1 to ribosome assembly/function (Li et al., 2009). Insight to Ssd1 functions comes from the identification of its associated mRNAs. Ssd1 was shown to specifically associate with a subset of mRNAs, including several that encode cell wall mannoproteins and cbk1 dosage suppressors (Hogan et al., 2008; Jansen et al., 2009). Intriguingly, some Ssd1-associated mRNAs, including SRL1, were shown to localize asymmetrically to the bud tip during polarized growth (Shepard et al., 2003; Hasegawa et al., 2008). Thus, Ssd1 may modulate the expression and/or mRNA localization of cell integrity proteins.

To elucidate the role of Cbk1 kinase on Ssd1 localization and function, we analyzed the effects of phospho-deficient and phosphomimetic Ssd1 mutants in vivo. Here, we provide evidence that Cbk1-mediated phosphorylations promote the cortical localization and function of Ssd1–mRNA complexes while repressing the growth inhibitory functions of Ssd1 at mRNA processing bodies (P-bodies) and stress granules. Further, our data indicate that Cbk1 inhibition and cellular stress promote Ssd1–mRNA interactions with P-bodies and stress granules, which sequester mRNAs from translation machinery, thus supporting the model that hypo-phosphorylated Ssd1 mediates the translational repression of its associated mRNAs. These data reveal a new role for LATS/NDR tumor suppressor kinases in mRNA regulation.

Results

Ssd1 localizes to bud cortex and bud necks

Current data suggest that Cbk1 kinase and Ssd1 are functionally linked to cell growth and maintenance of cell wall integrity (Jorgensen et al., 2002; Kurischko et al., 2005, 2008; Jansen et al., 2009); however, the molecular function of Ssd1 is poorly understood. The majority of Cbk1 kinase localizes to the bud cortex during bud growth and to the bud neck during cytokinesis, consistent with a role in polarized growth (Racki et al., 2000; Colman-Lerner et al., 2001; Weiss et al., 2002; Nelson et al., 2003). In contrast, Ssd1 was reported to localize uniformly throughout the cytoplasm (Uesono et al., 1997; Jansen et al., 2009).

Given the cooperative roles of Cbk1 and Ssd1 in polarized growth and cell wall biogenesis, we postulated that some Ssd1 must localize similarly to Cbk1. Thus, we reinvestigated the subcellular distribution of Ssd1 using cells expressing integrated Ssd1-GFP under the control of its physiological promoter. We observed a complex pattern of Ssd1–GFP localization in logarithmically growing cells, indicating that Ssd1 is spatially regulated. Most Ssd1 localized diffusely to the cytoplasm, as previously reported (Uesono et al., 1997; Jansen et al., 2009). In addition, Ssd1 concentrated to the bud cortex in some (13–40%, depending on the culture) small budded cells (Fig. 1). Ssd1 also localized to the bud neck in 4–9% of large budded cells (Fig. 1). These data indicate that a fraction of Ssd1 localizes similarly to Cbk1, which is consistent with a Cbk1-dependent function for Ssd1 during polarized growth.

Cbk1 influences Ssd1 localization

To determine if Cbk1 kinase influences Ssd1 localization we monitored Ssd1–GFP in cells expressing an analogue-sensitive cbk1-as allele, which encodes a derivative of Cbk1 that is specifically inhibited by the drug 1NA-PP1 (Weiss et al., 2002). When cbk1-as cells were treated with 1NA-PP1 for 45 min, some Ssd1–GFP redistributed from the predominantly diffuse cytoplasmic localization to randomly distributed cytoplasmic puncta (Fig. 2A). We rarely (<1% cells) observed Ssd1–GFP at
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affected in mock-treated cbk1-as cells or in 1NA-PP1–treated wild type (unpublished data). These data suggest that Ssd1 localization is modulated by Cbk1-dependent phosphorylation

the bud cortex or bud neck after Cbk1 inhibition, suggesting that the establishment or maintenance of Ssd1 at sites of polarized growth is Cbk1 dependent. Ssd1 localization was not

Figure 2. Ssd1 localizes to P-bodies. (A) Ssd1-GFP localizes to cytoplasmic puncta upon Cbk1 kinase inhibition. cbk1-as cells expressing Ssd1-GFP (FLY3249) were treated with 1NA-PP1 for 45 min and analyzed by fluorescence microscopy. Approximately 70% of 1NA-PP1–treated cbk1-as cells contain cytoplasmic puncta (n = 144). The images represent single optical sections. (B) Moderately overexpressed Ssd1 localizes to cytoplasmic puncta (P-bodies) preferentially in mother cells. ssd1Δ cells (FLY2184) containing pGPD-Ssd1-GFP (FLY1019) were observed by fluorescence microscopy. Approximately 30–60% of budded cells contained Ssd1-GFP cytoplasmic puncta (as determined by analyzing three independent cultures, n > 250). Of these, 55% contained Ssd1 puncta exclusively in the mother (M), 23% contained Ssd1 mostly in the mother (M > D) and 22% contained Ssd1 equally distributed between mother and bud (M = D): n = 152. This asymmetrical Ssd1 puncta localization mirrors the localization of Cbk1 kinase to the growing bud (Weiss et al., 2002) and may reflect the higher efficiency of Cbk1-dependent Ssd1 phosphorylation in buds. (C) Ssd1-associated cytoplasmic puncta co-localize with the P-body protein Edc3 (>63% Ssd1 puncta colocalize with Edc3). Cells in Fig. 2 B were transformed with plasmid encoding Edc3-RFP (pRP1574). The images in B and C are single optical sections captured via spinning disk confocal microscopy. Bars, 2 µm.
Figure 3. Ssd1 localizes to P-bodies and stress granules in response to cellular stress. ssd1Δ cells (FLY2184) containing Ssd1-GFP and RFP-tagged P-body proteins Edc3, Lsm1, Dcp2, or stress granule proteins Pab1 and Pub1 were transferred to glucose-depleted medium for 10 min and analyzed by spinning disk confocal microscopy. GFP- and RFP-tagged proteins were encoded by plasmids (FLE1019 for Ssd1-GFP; see Table II for P-body and stress granule plasmids). Ssd1 colocalizes with P-body proteins in all (100%) glucose-depleted cells that have visible Ssd1 puncta (n = 75 Lsm1-RFP cells; n = 40 Edc3-RFP cells; n = 100 Dcp2-RFP cells; n = 112 Pab1-RFP cells). Furthermore, most Ssd1 puncta (~70–90%) colocalize with P-body and stress granule proteins. Percentage of Ssd1 puncta that colocalize with P-body/stress granule proteins: 91% for Lsm1, 89% for Edc3, 73% for Dcp2, 84% for Pab1, and 88% for Pub1 (n = 10–15 cells). Parallel experiments indicate that 1 M NaCl treatment also causes Ssd1 to colocalize with P-bodies in cytoplasmic puncta (not depicted). All images represent single optical sections. Bar, 2 µm.
and support the hypothesis that Cbk1 kinase promotes the cortical localization of Ssd1 and/or represses the association of Ssd1 with cytoplasmic puncta.

**Modest Ssd1 overexpression promotes an association with P-bodies in mother cells**

We also monitored Ssd1 localization after its modest overexpression. We introduced low copy plasmids of **SSD1-GFP** under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter and monitored Ssd1 localization in asynchronous ssd1Δ cells. Modest Ssd1-GFP overexpression did not cause any obvious growth or cell integrity defects. However, up to 60% of the cells containing GPD-driven Ssd1-GFP plasmids contained prominent cytoplasmic foci that were usually excluded from buds (Fig. 2 B). Considering that most Cbk1 kinase localizes to buds during polarized growth (Racki et al., 2000; Colman-Lerner et al., 2001; Weiss et al., 2002; Nelson et al., 2003), these data are consistent with the model that moderate Ssd1 overexpression overwhelms regulatory Cbk1 phosphorylations in mother cells where Cbk1 concentrations are low, but not in buds where Cbk1 concentrations are higher.

**Ssd1 associates with P-bodies and stress granules**

The Ssd1 cytoplasmic puncta caused by modest Ssd1 overexpression or by Cbk1 inhibition were reminiscent of cytoplasmic mRNA P-bodies and stress granules, which sequester untranslated mRNAs and contribute to translational repression during cellular stress (Parker and Sheth, 2007; Anderson and Kedersha, 2008, 2009; Buchan et al., 2008). In support, Ssd1 was reported to partly colocalize with a P-body protein during stationary phase (Jansen et al., 2009). To determine if Ssd1 associates with P-bodies or stress granules during logarithmic growth, we monitored Ssd1 localization in cells expressing GPD-driven Ssd1-GFP and RFP-tagged P-body and stress granule proteins. We observed that ~60% of the Ssd1-GFP cytoplasmic puncta colocalized with the core P-body protein Edc3 in logarithmically growing cells (Fig. 2 C). These data support the model that Ssd1 at least transiently associates with mRNA-sequestering protein complexes that mediate translational repression.

**Cellular stresses, such as glucose depletion, hypertonic stress, and heat shock trigger P-body aggregation and stress granule formation** (Parker and Sheth, 2007; Anderson and Kedersha, 2008, 2009; Buchan et al., 2008). To test if cellular stress influences Ssd1 localization, we monitored Ssd1-GFP localization after glucose depletion. Ssd1 localized prominently to cytoplasmic puncta within 10 min of glucose depletion (Fig. 3; Fig. S1). Most of the Ssd1 cytoplasmic puncta (~73–90%) colocalized with the P-body proteins Edc3, Lsm1, and Dep2 (Fig. 3; Fig. S1). Ssd1 also colocalized with the stress granule proteins Pab1 and Pub1 upon glucose depletion. We observed similar results in salt-stressed cells (unpublished data). Collectively, these data suggest that Cbk1 kinase inhibition and cellular stress promote Ssd1 recruitment to both P-bodies and stress granules, which in turn may lead to the translational repression of Ssd1-associated mRNAs.

To biochemically corroborate data from the colocalization experiments, we conducted co-precipitation experiments with cells expressing Ssd1-GFP and representative TAP-tagged P-body and stress granule proteins. We discovered that Ssd1 specifically coprecipitated with the P-body or P-body–associated proteins Dcp2, Not1, and Xrn1 (Fig. 4). Ssd1 also coprecipitated with Pat1, a topoisomerase II–associated deadenylation-dependent mRNA-decapping factor necessary for both P-body and stress granule assembly (Buchan et al., 2008). These data confirm our colocalization experiments and suggest that a key function for Ssd1 involves P-body and stress granule interactions.

**mRNA binding is not necessary for Ssd1-P-body association**

Because P-bodies and stress granules are comprised of mRNA–protein complexes, Ssd1 recruitment or binding to P-bodies might occur via mRNA interactions. To determine if Ssd1 mRNA binding is a prerequisite for associating with P-bodies, we analyzed the localization of truncated derivatives of Ssd1, such as Ssd1495C, which lack the mRNA-binding domain, as defined by Uesono et al. (1994). Upon stress induction (hypertonic stress, heat shock, and glucose depletion), Ssd11-495A prominently localized to P-bodies within several minutes of stress induction (unpublished data). These data indicate that the...
were very fragile and propagated poorly. Microscopic analysis revealed that Ssd1-9A expression caused severe cell morphology and lysis defects that were remarkably similar to the phenotypes of conditional cbk1 mutants (Fig. 6A). Ssd1-9A expression was also lethal in cbk1Δ ssd1Δ double mutant cells, as is wild-type Ssd1 (unpublished data). These data support the model that Cbk1 negatively regulates Ssd1 and indicate that hypo-phosphorylated Ssd1 causes dominant and severe growth and cell integrity defects.

To test if the toxicity of Ssd1-9A expression is dependent on mRNA binding, we constructed a plasmid expressing Ssd1-9A protein that lacks the mRNA-binding domain (RBD 686–788), as defined by Uesono et al. (1997). Ssd1-9A-RBDΔ expression was not toxic to ssd1Δ or wild-type cells and did not cause any obvious growth defects (Fig. 6C). Furthermore, Ssd1-RBDΔ is not toxic to cbk1Δ ssd1Δ cells, in contrast to wild-type Ssd1, indicating that the RNA-binding domain is essential for Ssd1 function (unpublished data). These data support the model that Cbk1 negatively regulates Ssd1 and indicate that hypo-phosphorylated Ssd1 causes dominant and severe growth and cell integrity defects.

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Phosphorylation-deficient Ssd1 is toxic and causes cellular lysis

Ssd1 contains multiple Cbk1/LATS kinase consensus sequences for phosphorylation in its N-terminal region, as defined by Hao et al. (2008) and Mazanka et al. (2008). Significantly, in vivo expression of an Ssd1 mutant protein lacking multiple Cbk1 phosphorylation sites was reported to be toxic to yeast (Jansen et al., 2009); however, the nature of its toxicity and the effect of Cbk1-dependent phosphorylations on Ssd1 localization were not established. To explore the physiological significance of Cbk1-mediated phosphorylations on Ssd1 function and localization, we analyzed the phenotypes of Ssd1 phosphorylation site mutants. We converted nine putative Cbk1 phosphorylation sites to nonphosphorylatable alanines to yield Ssd1-9A. We also converted putative Cbk1 phosphorylation sites to phosphomimetic aspartate residues to yield phosphorymimetic Ssd1-9D. We introduced low copy plasmids encoding wild-type or mutant Ssd1 into wild-type, ssd1Δ, and cbk1Δ ssd1Δ yeast strains and analyzed cellular phenotypes.

We discovered that Ssd1-9A expression was dominantly toxic to wild-type and ssd1Δ cells at 22°C and lethal at 37°C (Fig. 5). The Ssd1-9A transformants of wild-type and ssd1Δ cells were very fragile and propagated poorly. Microscopic analysis revealed that Ssd1-9A expression caused severe cell morphology and lysis defects that were remarkably similar to the phenotypes of conditional cbk1 mutants (Fig. 6A). Ssd1-9A expression was also lethal in cbk1Δ ssd1Δ double mutant cells, as is wild-type Ssd1 (unpublished data). These data support the model that Cbk1 negatively regulates Ssd1 and indicate that hypo-phosphorylated Ssd1 causes dominant and severe growth and cell integrity defects.

Ssd1-9A toxicity is diminished by cbk1 dosage suppressors

If Cbk1 phosphorylation prevents the toxicity of Ssd1, then the lysis phenotypes of cbk1 loss-of-function mutants and cells

Figure 5. In vivo expression of Ssd1-9A and Ssd1-9D. (A) Transformants of ssd1Δ (FLY2184) cells carrying low copy plasmids encoding wild-type Ssd1, Ssd1-9A, or Ssd1-9D (FLE1079, FLE1080, FLE1081) under the control of the physiological SSD1 promoter. (B) 10-fold dilution series of three separate transformants (primary colonies) for each plasmid were spotted onto plates and incubated at 22 or 37°C. Note the poor growth for the Ssd1-9A cells at 22°C and the lethality at 37°C.
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expressing Ssd1-9A must occur via similar mechanisms. If this hypothesis is correct, then some cbk1 dosage suppressors may abrogate or diminish the toxicity of Ssd1-9A expression. We previously established that high copy plasmids of the cell wall biogenesis proteins, Ccw12, Sim1, Srl1, and Uth1 suppress the lethality of cbk1 loss-of-function mutants (Kurischko et al., 2005 and Fig. S2). Thus, we introduced Ssd1-9A plasmids into cells containing cbk1 dosage suppressor plasmids (pCCW12, pSIM1, pSRL1, pUTH1, pZRG8) and assayed cell morphology and cell integrity by microscopy. We discovered that the cellular lysis and aberrant morphology phenotypes of Ssd1-9A cells were partially rescued by these cbk1 dosage suppressors at 22°C, 34°C (Fig. 6, A and B), and 37°C (not depicted). These data support the hypothesis that the toxicity of Ssd1-9A expression and the cell integrity defects of cbk1 loss-of-function mutants occur via the same or similar mechanisms.

Ssd1-9A constitutively localizes to P-bodies

Because Ssd1 localizes to P-bodies in response to Cbk1 inhibition, we hypothesized that hypo-phosphorylated Ssd1-9A constitutively localizes to P-bodies and stress granules. Thus, we analyzed the localization of GFP-tagged Ssd1-9A in ssd1Δ cells. Because constitutive Ssd1-9A expression (via GPD promoter) was toxic for cell growth, we conducted experiments with cells containing galactose-inducible Ssd1-9A plasmids. When cells were grown in repressing conditions (glucose-containing medium), Ssd1-9A–GFP was not detectable by fluorescence microscopy and did not cause any obvious phenotype (unpublished data). Upon 2 h galactose induction, Ssd1-9A localized prominently to cytoplasmic puncta in nearly all cells (Fig. 7A). Most of the Ssd1-9A cytoplasmic puncta colocalized with P-body markers. We never observed phospho-deficient Ssd1-9A at bud tips or bud necks. These results suggest that the lethality of ssd1-9A and conditional cbk1 mutants correlates with enhanced or constitutive Ssd1 localization to P-bodies and stress granules. Furthermore, these data support the model that hypo-phosphorylated Ssd1 is toxic to cells via P-body–dependent translational repression of Ssd1-associated mRNAs. In agreement, Ssd1 is no longer toxic when it cannot bind mRNAs (Fig. 6 C and Fig. S4 B).

Cbk1 promotes the cortical functions of Ssd1

To determine the role of Cbk1 phosphorylation on Ssd1 function, we analyzed the phenotypes of cells expressing phosphomimetic Ssd1-9D. In contrast to phospho-deficient Ssd1-9A, phosphomimetic Ssd1-9D was not toxic to wild-type, ssd1Δ, or cbk1Δ ssd1Δ cells when expressed on low copy plasmids under the control of its endogenous promoter or the GPD promoter.

Plasmids expressing Ssd1-RBDΔ (missing mRNA-binding domain, RBD 686–788) and Ssd1-9A-RBDΔ were introduced into ssd1Δ cells (FLE1210 and FLE1209). In contrast to Ssd1-9A expression, Ssd1-RBD and Ssd1-9A-RBDΔ expression is not toxic. Ssd1 expression was confirmed by immunoblots (not depicted).
expressed via the constitutive GPD promoter. These data support the model that Cbk1-dependent phosphorylations prevent Ssd1 from associating with P-body aggregates, but allow Ssd1 to localize to sites of polarized growth.

Phosphorylated Ssd1 regulates the polarized distribution of associated mRNAs Ssd1 binds a subset of mRNAs, many of which encode cell wall biogenesis proteins (Hogan et al., 2008; Jansen et al., 2009). Intriguingly, several Ssd1-associated mRNAs are encoded by cbk1 dosage suppressors, such as SRL1, SIM1, CCW12, and UTH1 (Kurischko et al., 2005; see Fig. S2). Moreover, two of these mRNAs (SRL1 and UTH1) are known to polarize to the bud cortex in an actin- and myosin V–dependent fashion.
In ssd1Δ cells containing empty vector, SRL1 mRNA polarity was diminished by ~10-fold compared with SSD1 cells (Fig. 8). Moreover, the percentage of ssd1Δ cells with multiple faint SRL1 mRNA puncta or no spots was ~30% and several-fold greater, respectively, than corresponding SSD1 and SSD1-9D cells. 95% confidence intervals are represented by the solid black lines. Non-overlapping confidence intervals indicate statistically significant differences at significance level 0.05. Each image represents a single optical section. Bar, 2 µm.

(Shepard et al., 2003). In light of the polarized localizations of wild-type Ssd1 and phosphomimetic Ssd1-9D in some budded cells, it seemed plausible that Ssd1 influences asymmetric mRNA localization during polarized growth.

To determine whether Ssd1 influences mRNA localization, we monitored SRL1 mRNA localization in vivo using a GFP-tagging strategy described previously (Haim et al., 2007; Haim-Vilmovsky and Gerst, 2009). We compared the pattern of SRL1 mRNA localization in asynchronously growing ssd1Δ cells containing Ssd1 plasmids or empty vector. In ~50% of the cells expressing wild-type SSD1, SRL1 mRNA polarized to 1–3 prominent spots in the bud (Fig. 8; Shepard et al., 2003). We also observed that SRL1 mRNA localized to multiple faint spots randomly distributed throughout the cytoplasm in ~40–50% of the cells. The overall distribution of SRL1 mRNA localization was similar in small, medium, and large budded cells, indicating that the different patterns of SRL1 mRNA localization were not obviously enhanced during cell cycle progression (unpublished data). We obtained similar results with wild-type cells (unpublished data).

In ssd1Δ cells containing empty vector, SRL1 mRNA polarity was diminished by ~10-fold compared with SSD1 cells (Fig. 8). Moreover, the percentage of ssd1Δ cells with multiple faint SRL1 mRNA puncta or no spots was ~30% and several-fold greater, respectively, than corresponding SSD1 cells (Fig. 8). These data indicate that Ssd1 is critical for establishing or maintaining asymmetric SRL1 mRNA localization during polarized growth.

Ssd1-9D promotes the polarized localization of SRL1 mRNA

If Cbk1-mediated phosphorylations simply inactivate Ssd1, then constitutively phosphorylated Ssd1 should phenocopy ssd1 loss-of-function alleles, such as ssd1Δ. However, in light of the cortical localization of wild-type Ssd1 and Ssd1-9D, we thought it to be likely that Cbk1 stimulates some cortical Ssd1 functions and protein interactions. To test if Ssd1-9D can rescue the SRL1 mRNA localization defects in ssd1Δ cells, we monitored SRL1 mRNA localization in ssd1Δ cells expressing Ssd1-9D plasmids. Significantly, Ssd1-9D expression restored
Ssd1 were expressed to similar levels in these experiments (unpublished data). These results support the model that in the absence of Cbk1 phosphorylations, hypo-phosphorylated Ssd1 sequesters its associated mRNAs in P-bodies and stress granules, where the mRNAs are translationally repressed. These data further suggest that enhanced Ssd1–mRNA interactions with P-bodies and stress granules are the primary cause of Ssd1 toxicity in \( \text{cbk1} \) mutants.

**Discussion**

Collectively, our data support the model that the essential function of Cbk1 is to modulate Ssd1 and mRNA metabolism during polarized growth (Fig. 10). For this model, Cbk1 phosphorylates Ssd1 to promote the polarized localization of mRNAs involved in bud growth and cell wall biogenesis. Thus, Cbk1-phosphorylated Ssd1 may function to promote protein synthesis at cortical sites of growth. In contrast, Cbk1 inhibition or cellular stress lead to elevated levels of dephosphorylated Ssd1 and thereby promote Ssd1 association with P-bodies and stress granules. We suggest that Ssd1 associations with P-bodies and stress granules lead to the translational repression of Ssd1-associated mRNAs. Thus, the cell lysis phenotypes of
ssd1-9A and conditional cbk1 mutant cells are likely caused by the reduced expression of cell wall proteins that are encoded by Ssd1-associated mRNAs. In support, the polysome fraction of several Ssd1-associated mRNAs is dramatically decreased upon Cbk1 inhibition (Jansen et al., 2009). Furthermore, the lethality of ssd1-9A and cbk1 mutants is rescued by moderate overexpression of several Ssd1-associated mRNAs encoding cell wall proteins (Srl1, Ccw12, Sim1, and Uth1).

### Ssd1 cortical function: a role for Ssd1 in asymmetric mRNA localization?

Several lines of evidence suggest that Cbk1-phosphorylated Ssd1 contributes to asymmetric mRNA localization. First, both phosphomimetic Ssd1-9D and wild-type Ssd1 localize to bud cortex and bud neck. Moreover, two Ssd1-associated mRNAs, SRL1 and UTH1, localize asymmetrically to the bud tip (Shepard et al., 2003). Both mRNAs encode cell wall biosynthesis proteins and suppress cbk1 and ssd1-9A mutants when moderately overexpressed (Kurischko et al., 2005; Fig. 6 and Fig. S2). Most notably, the polarized localization of SRL1 mRNA is dramatically reduced in ssd1Δ cells, and is restored and modestly enhanced by Ssd1-9D expression (Fig. 8). Based on the physical interactions between Ssd1 and SRL1 mRNA and the observation that Ssd1 transiently localizes to the bud cortex, we hypothesize that Ssd1 directly modulates the delivery, retention, and translation of at least some of its associated mRNAs to the sites of polarized growth. Thus, Cbk1 and Ssd1 may help ensure that its associated mRNAs are translated where they are needed.

Cbk1-phosphorylated Ssd1 may influence SRL1 mRNA localization directly or indirectly via promoting or stabilizing mRNA interactions with either mRNA localization machinery or with cortical proteins. In S. cerevisiae, 24 mRNAs, including SRL1 mRNA, localize to the bud tips via myosin V (Myo4)-mediated transport along actin cables (Shepard et al., 2003; Martin and Ephrussi, 2009). Palindrome landmark proteins such as Sec3 were also shown to facilitate asymmetric mRNA and cortical ER interactions (Aronov et al., 2007). Although SSD1 and the mRNA polarity machinery are not essential for viability in yeast, the directed localization of mRNAs provides a mechanism for regulating gene expression with precise temporal and spatial control. The spatial distribution of mRNAs is more economical than the post-translational protein delivery with regard to localized protein expression because mRNAs can be translated many times (Martin and Ephrussi, 2009).

### Ssd1 and P-body interactions

The association of Ssd1 with P-bodies and stress granules suggests a role for Ssd1 in translation repression. P-bodies are conserved RNA protein granules that serve as storehouses for nontranslated mRNAs and sometimes target mRNAs for degradation (Bruno and Wilkinson, 2006; Sheth and Parker, 2006; Buchan et al., 2008; Gallo et al., 2008; Nissan and Parker, 2008). They consist of translation repressors, mRNA decapping proteins, and a 5′–3′ exonuclease. P-bodies are particularly important for translational repression during cellular stress, when repression of many mRNAs is crucial to halt growth and enhance cell survival (Bruno and Wilkinson, 2006; Sheth and Parker, 2006; Buchan et al., 2008; Gallo et al., 2008; Nissan and Parker, 2008). P-bodies are critical for the formation of stress granules, which are comprised of aggregates of untranslated mRNAs, a subset of translation initiation factors, the 40S ribosome subunit, and poly(A)-binding proteins, such as Pab1 and Pub1 (Anderson and Kedersha, 2008, 2009; Buchan et al., 2008). Stress granules form in response to translation initiation defects and cellular stress and dynamically interact with P-bodies, suggesting an exchange of mRNP. Both P-body– and stress granule–mediated mRNA sequestration provides a rapid and reversible mechanism for translational repression. Our data regarding Ssd1 and P-body/stress granule interactions support the model that in the absence of Cbk1-mediated phosphorylations, Ssd1-associated mRNAs are transitionally repressed via P-body and/or stress granule sequestration. In agreement, SRL1 mRNA colocalizes with Ssd1-9A (Fig. 9), which constitutively localizes to P-bodies. Moreover, Cbk1 kinase inhibition reduces the levels of several Ssd1-associated mRNAs in polysome fractions (Jansen et al., 2009).

Our results also support the model that some cellular stresses (glucose depletion, hypertonic stress, heat shock) promote the P-body and stress granule association of Ssd1 and subsequent translational repression of cell growth proteins. The properties of the phospho-deficient ssd1-9A mutant suggest the existence of a stress-induced phosphatase that promotes Ssd1–P-body/stress granule complexes by removing Cbk1 phosphorylations (Fig. 10). Alternatively or additionally, there may be mechanisms that inhibit Cbk1 activity or disrupt protein interactions in response to stress.

In principle, unphosphorylated Ssd1 could repress the expression of cell wall proteins by promoting mRNA degradation. Indeed, the mRNA-binding domain of Ssd1 shares some homology with RNase II (Uesono et al., 1997). However, there is...
no experimental evidence supporting the notion that Ssd1 promotes mRNA degradation in vitro or in vivo. Recombinant Ssd1 has no RNase activity in vitro (Useno et al., 1997) and microarray and RT-PCR experiments reveal no changes in the steady-state levels of Ssd1-associated mRNAs (Li et al., 2009 and Fig. S3). Moreover, SRL1 mRNA is clearly detectable in ssd1Δ or ssd1-9A cells by microscopic and RT-PCR methodology (Fig. 9; Figs. 4 and S5). Intriguingly, recent data indicate that Ssd1 is required to stabilize a population of CLN2 mRNA after heat shock (Ohyama et al., 2010). Taken together, Ssd1 may be required to prevent the degradation of some mRNAs during heat shock and other cellular stresses.

Other Cbk1 targets for asymmetric protein expression

Cbk1 may influence asymmetric protein expression via both Ssd1-dependent and Ssd1-independent mechanisms. Indeed, it is well established that Cbk1 regulates daughter cell–specific transcription of cell separation proteins via Ace2 transcription factor (Colman-Lerner et al., 2001; Weiss et al., 2002). Cbk1 phosphorylates Ace2 on its N-terminal nuclear export sequence (NES), thereby interfering with its Crm1-dependent nuclear export while promoting Ace2 transcriptional activity in the daughter cell nucleus (Jansen et al., 2006).

Intriguingly, the mRNAs of several Ace2-regulated genes, such as CTS1 and DSE2, were shown to bind Ssd1 (Hogan et al., 2008; Jansen et al., 2009; Fig. S3). Moreover, CTS1 mRNA was shown to be transcribed only in the daughter cell and to colocalize with the cortical ER (Bourens et al., 2008). Thus, it is likely that Cbk1-phosphorylated Ssd1 also mediates the spatial distribution and expression of these and other Ssd1-associated mRNAs, as we have demonstrated for SRL1 mRNA.

Cbk1 may also regulate the asymmetric expression of proteins via polarized secretion mediators. In support, Cbk1 influences secretion and Golgi-mediated glycosylation (Kurischko et al., 2008). Moreover, the polarized secretion machinery is critical for the asymmetric localization of several mRNAs (Aronov et al., 2007). Thus, Cbk1 may regulate polarized growth and cell wall biosynthesis via the integrated coordination of multiple processes, from daughter cell–specific transcription to polarized mRNA localization and secretion.

Recent data suggest that the role of Cbk1 in mRNA metabolism extends beyond Ssd1. Notably, Cbk1 and Ssd1 are genetically linked to other mRNA-associated proteins, such as the PUF family RNA-binding protein Mpt5 and the spliceosome-associated Br1 (Kaeberlein and Guarente, 2002; Bourens et al., 2009). MPT5 and BRR1 were identified as suppressors of the mating defect of cbk1 mutants and MPT5 deletion rescues the lethality of cbk1Δ mutants (Bourens et al., 2009). ssd1 mpt5 double mutants also display enhanced phenotypes, suggesting that they function in parallel mRNA processes (Kaeberlein and Guarente, 2002). Intriguingly, Mpt5 contains multiple consensus sites for Cbk1 phosphorylation (Mazanka et al., 2008; Bourens et al., 2009), suggesting that Cbk1 directly regulates cell growth and development via multiple mRNA-associated processes.

Collectively, our data suggest unanticipated functions for LATS/NDR kinases in regulating cell growth and cancer development via post-transcriptional mRNA-dependent mechanisms. Our experiments also provide a link between LATS/NDR kinases and stress signaling. Because alterations in stress responses are a major hallmark of transformation, it is possible that an important tumor suppressor function of LATS/NDR kinases is to repress cell growth during cellular stress, perhaps via similar mechanisms as Cbk1. Finally, data regarding Ssd1-dependent mechanisms for asymmetric mRNA localization may also reveal a possible mechanism for LATS/NDR kinase in mediating polarized morphogenesis. For example, mutations in one of the Drosophila and Caenorhabditis elegans Cbk1 orthologues cause cellular morphogenesis defects that are particularly evident in neurons (Hergovich et al., 2006; Jan and Jan, 2010). Because neuronal morphogenesis requires the polarized delivery of mRNAs to growth cones and dendritic spines, it is tempting to speculate that the neuronal morphogenesis defects of LATS/NDR mutants could be the direct consequence of defective mRNA localization mechanisms. Further work on this important class of kinases and their substrates will clarify their specific mechanisms in controlling cell morphogenesis and proliferation.

Materials and methods

Yeast growth conditions and strain construction

Standard yeast genetics and culture methods were used as described previously (Guthrie and Fink, 1991; Kurischko et al., 2005). The strains used in this paper are listed in Table I. The Ssd1-GFP strains were constructed by integration of PCR-based cassettes, as described previously (Longtine et al., 1998). All TAP-tagged yeast strains were obtained from Invitrogen. SRL1 mRNA in vivo imaging was performed as described previously (Haim et al., 2007). In brief, at the 3’ UTR of SRL1 the cassette containing 12 MS2-CP binding sites, together with the S. pombe his5+ gene as a selective marker, was integrated. The selective marker was deleted and the strain was transformed with the MS2-CP-GFP-containing plasmid to visualize the tagged mRNA. Plasmids for mRNA tagging were provided by Dr. Jeffrey Gerst (Weizmann Institute of Science, Rehovot, Israel).

Where designated, cells were exposed to different stresses as described previously (Buchan et al., 2008; Nissan and Parker, 2008). For glucose depletion, the cells were spun down, washed with glucose-free medium, resuspended in the same medium, and incubated for 10–15 min. For hypertonic stress induction, logarithmically growing cells were pelleted, re-suspended in medium containing 1 M NaCl, and incubated for 5–10 min.

Plasmid construction and molecular biology

The plasmids and oligonucleotides used in this paper are listed in Table II and Table III. SSD1 was subcloned from YEp13-SSD1 (a gift from C. Boone,
Table II. Plasmids

| Plasmids             | Alias/relevant markers                  | Source                          |
|----------------------|-----------------------------------------|---------------------------------|
| FLE710               | YEp13-SIM1                              | Kurischko et al., 2005          |
| FLE711               | YEp13-CCW12                             | Kurischko et al., 2005          |
| FLE716               | YEp13-SRL1                              | Kurischko et al., 2005          |
| FLE718               | YEp13-ZRG8                              | Kurischko et al., 2005          |
| FLE980               | pGP564                                   | Thermo Fisher Scientific        |
| FLE1019              | pAG415-GPD-SSD1-GFP                      | This paper                     |
| FLE1020              | pAG415-GPD-SSD1-1×GFP                    | This paper                     |
| FLE1079              | pRS316-SSD1                             | This paper                     |
| FLE1080              | pRS316-SSD1-19A                         | This paper                     |
| FLE1081              | pRS316-SSD1-19D                         | This paper                     |
| FLE1083              | pRS415-SSD1                             | This paper                     |
| FLE1087              | pRS415-SSD1-19D                         | This paper                     |
| FLE1090              | pAG415-GPD-SSD1-9D-GFP                   | This paper                     |
| FLE1160              | YGPM12a13 (UTH1)                         | Thermo Fisher Scientific        |
| FLE1205              | pENTRYSSD1B5a                           | This paper                     |
| FLE1206              | pENTRY-SSD1-9A                         | This paper                     |
| FLE1207              | pAG416-GAL-SSD1-9A-GFP                   | This paper                     |
| FLE1208              | pAG416-GAL-SSD1-9A-HA                    | This paper                     |
| FLE1209              | pAG415-GPD-SSD1-9A-HA                    | This paper                     |
| FLE1210              | pAG415-GPD-SSD1-9A                      | This paper                     |
| FLE1244              | pAG415-GAL-SSD1-9A-dsRed                | This paper                     |
| FLE1271              | YGPM11e20 (CBK1)                        | Thermo Fisher Scientific        |
| FLE1272              | pGP564-UTH1                             | This paper                     |
| FLE1278              | pAG415-GPD-SSD1-9A-RBD5A-TAP             | This paper                     |
| pR1084               | pRS416-LSM1-RFP                         | Roy Parker, University of Arizona |
| pR1084               | pRS416-LSM1-RFP                         | Roy Parker, University of Arizona |
| pR1085               | pRS415-LSM-RFP                          | Roy Parker, University of Arizona |
| pR1155               | DCP2-RFP-LEU2                           | Roy Parker, University of Arizona |
| pR1186               | DCP2-RFP-URA3                           | Roy Parker, University of Arizona |
| pR1574               | EDC3-chRFP-URA3                         | Roy Parker, University of Arizona |
| pR1661               | PUB1-mCherry-URA3                       | Jeffrey Gerst, Weizmann Institute of Science |
|                     | plOIX-HIS5-MS2L                          | Jeffrey Gerst, Weizmann Institute of Science |
|                     | pCP-MS2-GFP[3x]                         | Jeffrey Gerst, Weizmann Institute of Science |
|                     | pSH47                                   | Jeffrey Gerst, Weizmann Institute of Science |
|                     | PAB1-RFP-URA3                           | Charles Cole, Dartmouth Medical School |

Unless otherwise designated, all genes are expressed under the control of their physiological promoters.

University of Toronto, Ontario, Canada) into pUC19 for further manipulations. SSD1-9A and SSD1-9D mutations were synthesized by Geneart AG and correspond to the following amino acids substitutions: S to A at positions 42, 104, 126, 152, 228, 259, and 319, and T to A at positions 261 (SSD1-9A); S to D at positions 40, 42, 126, 164, 228, 259, and 319 (SSD1-9D). SSD1-9A and SSD1-9D plasmids were constructed by subcloning the N-terminal HpaI–SpeI fragment of mutated SSD1 into pUC19-SSD1. From there, the entire SSD1 (promoter region and ORF with 3′ UTR) was cloned into pRS316 and pRS415 to create FLE1079, FLE1080, FLE1081, FLE1083, and FLE1087 plasmids. Wild-type and mutant SSD1 were subcloned into Gateway-compatible GFP, dsRED (RFP), or HA plasmids, as described previously (Alberti et al., 2007). All Gateway-compatible vectors were provided by Dr. Aaron Giller (University of Pennsylvania, Philadelphia, PA). The wild-type SSD1 ORF without the Stop codon was amplified by PCR using the corresponding oligos and cloned into the pDONR221 vector. The corresponding SSD1-9A and -9D plasmids were constructed by restriction enzyme–mediated cloning. Ssd1-RBD3 and Ssd1-9A/RBD3 plasmids were constructed by deleting the internal XbaI fragment of SSD1. UTH1 was isolated from the plasmid YGPM12a13 (Thermo Fisher Scientific) by excising the Nael–BglII fragment and subcloning it into the Nael–BamHI sites of pGP564, creating FLE1272.

Plasmids encoding RFP-tagged P-body and stress granule proteins were obtained from Dr. Roy Parker (Howard Hughes Medical Institute, University of Arizona, Tucson, AZ) and Dr. Charles Cole (Dartmouth Medical School, Hanover, NH).

Immunoblots and immunoprecipitation

Coimmunoprecipitation and immunoblot analyses were conducted as described previously (Kurischko et al., 2005, 2008) using monoclonal anti-HA antibody (Roche), monoclonal anti-GFP antibody (Roche), or polyclonal anti-TAP antibody (Thermo Fisher Scientific). For coIP, cells were resuspended in lysis buffer (150 mM NaCl, 50 mM Heps, 1% NP-40, 60 mM 2-glycerophosphate, 1 mM EDTA, 2 mM DTT, 10 mM NaF, and protease inhibitors) and lysed by a Mini-Beadbeater (Biospec Products). 2 μg of protein extract was precleared with protein G–Sepharose for 1 h at 4°C. The protein-bound beads were washed with lysis buffer and resuspended in 50 μl SDS sample buffer and loaded on 7.5% SDS-PAGE (15 μl per lane). Immunoblots were probed with primary antibodies and secondary AP-conjugated antibody and anti–rabbit antibodies (Promega) and processed for ECF, as per manufacturer’s protocol (Invitrogen). Immunoblots were digitized and, where designated, quantified with a STORM PhosphorImager (GE Healthcare). For whole-cell immunoblots, cells were TCA precipitated and processed for ECF, as per manufacturer’s protocol (Invitrogen). Immunoblots were digitized and, where designated, quantified with a STORM PhosphorImager (GE Healthcare). Co-immunoprecipitation and immunoblot analyses were conducted as described previously (Kurischko et al., 2008).

RT-PCR

RT-PCR experiments with immunoprecipitated complexes were performed as described previously (Peritz et al., 2006). We used the MasterPure kit (Epicentre Biotechnologies) for yeast RNA purification. The cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis kit (Roche).
### Table III. Oligonucleotides

| Oligos | Sequence | Purpose | Gene |
|-------|----------|---------|------|
| FLO1  | 5’-CCATGTTAACCGTCGATCAAATCACAGACGTTAGGACCTGTT | Tagging | SSD1 |
| FLO2  | 5’-AGATGGGGAAGAGTTTTGGGAGGACGTTAGGACCTGTT | Tagging | SSD1 |
| FLO203 | 5’-CTTCAGCCGCGAGTGCTTGAT | Deletion | SSD1 |
| FLO527 | 5’-GGGGAGACGTTTGACAAAAAACGGGCGGACGG | Gateway cloning | SSD1 |
| FLO536 | 5’-GGGGAGACTTTGACAAAAAACGGGCGGACGG | Gateway cloning | SSD1 |
| FLO560 | 5’-GTACACACCCACTTGGAGCTCAGGGGACGG | mRNA tagging | SRL1 |
| FLO561 | 5’-AAACAAATAAAGCTTTGACGGGACGG | mRNA tagging | SRL1 |
| FLO588 | 5’-ATGAAATTCACGCTTGC | qRT-PCR | SIM1 |
| FLO589 | 5’-GGTGAAGTGGATTTAA | qRT-PCR | SIM1 |
| FLO590 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CCW12 |
| FLO591 | 5’-ACACCAAAAGGAGGACGGACGTTAGGACCTGTT | qRT-PCR | CCW12 |
| FLO618 | 5’-ATGGAGAACCACTTGC | qRT-PCR | SUN4 |
| FLO619 | 5’-ATGGAGAACCACTTGC | qRT-PCR | SUN4 |
| FLO620 | 5’-ATGGAGAACCACTTGC | qRT-PCR | DSE2 |
| FLO621 | 5’-ATGGAGAACCACTTGC | qRT-PCR | DSE2 |
| FLO622 | 5’-ATGGAGAACCACTTGC | qRT-PCR | SCW4 |
| FLO623 | 5’-ATGGAGAACCACTTGC | qRT-PCR | SCW4 |
| FLO624 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO625 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO627 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO630 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO631 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO632 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO633 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO634 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO635 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO636 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO639 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |
| FLO640 | 5’-ATGGAGAACCACTTGC | qRT-PCR | CTS1 |

The oligos used to amplify fragments of SUN4, CTS1, DSE2, SCW4, SRL1, CCW12, and SIM1 are listed in Table III. qRT-PCR was performed using the Lightcycler 1.5 system (Roche). The initial cDNA denaturation was for 10 min at 95°C and was followed by 50 cycles: denaturation at 95°C for 1 s; annealing at 55°C for 5 s; and extension at 72°C for 8 s. The oligos for ACT1, ADH1, CCW12, SIM1, and SRL1 are listed in Table III.

**Fluorescence microscopy**

Routine fluorescence microscopy was performed with a fluorescence microscope (model DMR5; Leica) equipped with a 100x Plan Apochromat 1.46 NA oil objective and a 16-bit cooled EMCCD camera (ImagEM; Hamamatsu Photonics), as described previously (Kurischko et al., 2008). Most colocalization experiments were conducted on a spinning disk confocal system controlled by MetaMorph software (MDS Analytical Technologies). The spinning disk microscope was an inverted microscope (model DMR5; Leica) equipped with a 100x Plan Apochromat 1.46 NA oil objective, a spinning disk confocal system (CSU-10; Yokogawa) and a 16-bit cooled EMCCD camera (ImagEM; Hamamatsu Photonics). Laser excitation was provided by a 488-nm (Spectra Physics) and a 561-nm laser (CoherentJive). Oligonucleotide tagged expression was controlled through the LMM5 module (Spectral Applied Research). The emission was provided by a 488-nm (Spectra Physics) and a 561-nm laser (Cobolt Jive). The excitation wavelength for GFP was 488 nm and for RFP was 561 nm. The emission was collected at 503–552 nm for GFP and 583–650 nm for RFP.

Z stacks were taken for a total thickness of 1.8–3.4 µm at a step size of 0.2 µm. Image capture and analysis was controlled via Velocity (PerkinElmer) or MetaMorph software.

**Online supplemental material**

Fig. S1 shows that physiologically expressed Ssd1 colocalizes with P-body in glucose-depleted cells. Fig. S2 shows that UTH1 is a dosage suppressor of cdk1-b mutants. Fig. S3 shows that Ssd1 precipitates SRL1, SRL1, CCW12, SUN4, DSE2, SCW4, and CTS1 mRNAs and does not affect steady-state mRNA levels. Fig. S4 shows that Ssd1-9A but not Ssd1-9ARBD. expression causes SRL1 mRNA to localize to P-bodies. Fig. S5 shows that mRNA-tagging expression in wild-type and ssd1Δ cells.

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