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

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The mRNA-binding protein Ssd1 is a substrate for the *Saccharomyces cerevisiae* LATS/NDR orthologue Cbk1, which controls polarized growth, cell separation, 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.

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 (Hergovich et al., 2006, 2008). Mammalian LATS and NDR kinases function as tumor suppressors and are implicated in a variety of cancers, including B cell lymphomas, prostate, pancreatic, and ovarian cancers. Despite their importance, the mechanisms by which LATS/NDR kinases influence cancer progression are poorly understood (Hergovich and Hemmings, 2009).

Data regarding fungal LATS/NDR kinase orthologues offer insight to the molecular mechanisms of mammalian LATS/NDR function. In yeast, two distinct LATS/NDR kinase orthologues control several aspects of cell growth and proliferation. One class of yeast LATS/NDR kinases, *Saccharomyces cerevisiae* Dbf2/Dbf20 and *Schizosaccharomyces pombe* Sid2, are key components of a conserved signaling network known as the *S. cerevisiae* mitotic exit network (MEN) and *S. pombe* septum initiation network (SIN) that coordinate cytokinesis and mitotic exit (Johnston et al., 1990; Komarnitsky et al., 1998; Hou et al., 2000; Luca et al., 2001; Balasubramanian et al., 2004). The other yeast LATS/NDR orthologues, *S. cerevisiae* Cbk1 and *S. pombe* Orb6, belong to a MEN/SIN-related signaling network that regulates polarized morphogenesis and maintenance of cell integrity (Verde et al., 1998; Racki et al., 2000; Bidlingmaier et al., 2001; Hou et al., 2003; Nelson et al., 2003).

S. cerevisiae Cbk1 is a late-acting kinase in the RAM signaling network, which is comprised of two kinases and four associated proteins (Jorgensen et al., 2002; Nelson et al., 2003). Each RAM protein is essential for viability and localizes to sites of polarized growth (Racki et al., 2000; Bidlingmaier et al., 2001; Du and Novick, 2002; Nelson et al., 2003). Current data suggest that Cbk1 and RAM regulate several functions, including polarized growth, mating efficiency, cell separation, and maintenance of cell integrity (Weiss et al., 2002; Nelson et al., 2003; Kurischko et al., 2005, 2008; Bourens et al., 2009; Jansen et al., 2009; Panozzo et al., 2010). Recent data suggest that Cbk1 also influences polarized secretion and Golgi function (Kurischko et al., 2008). Cbk1 controls cell separation via the daughter cell–specific transcription factor Ace2, which induces the expression of septum degradation enzymes at the end of mitosis (Colman-Lerner et al., 2001; Weiss et al., 2002; Bourens et al., 2008;

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Abbreviations used in this paper: GPD, glyceraldehyde-3-phosphate dehydrogenase; P-bodies, processing bodies.

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Mazanka et al., 2008; Sbia et al., 2008). The mechanism of Cbk1 function in other processes is less understood; however, it is increasingly apparent that Cbk1 has a role in cell wall biogenesis. In support, the lethality of *cbk1Δ* and other RAM mutants is suppressed by moderate overexpression of certain cell wall biosynthesis proteins, including the mannoproteins Sim1, Srl1, and Ccw12 (Kurischko et al., 2005, 2008).

Perhaps the most important function for Cbk1, with respect to cell viability, is to regulate the mRNA-binding protein Ssd1. In support, *CBK1* and all *RAM* genes are dispensable for viability in cells harboring *ssd1Δ* or the polymorphic *ssd1-d* mutation (Du and Novick, 2002; Jorgensen et al., 2002; Nelson et al., 2003; Kurischko et al., 2005). Moreover, Cbk1 phosphorylates Ssd1 in vitro and associates with Ssd1 via two-hybrid assays (Racki et al., 2000; Jansen et al., 2009). These data support the model that Cbk1 negatively regulates Ssd1 and suggest that the failure to inhibit Ssd1 is detrimental to cell integrity.

The molecular functions of Ssd1 are 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; Kaeberlein and Guarente, 2002; Wheeler et al., 2003; Kaeberlein et al., 2004; Wojda et al., 2007; Jansen et al., 2009; Mir 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).

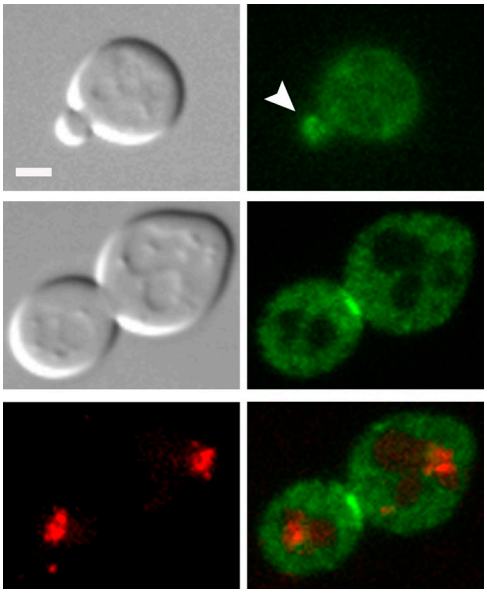

Figure 1. **Ssd1 localizes to sites of polarized growth.** Logarithmically growing cells expressing Ssd1-GFP and Pap1-RFP (a nuclear marker) (FLY3433) were observed by fluorescence microscopy. Ssd1-GFP was enriched in small buds (13–40% of cells, n = 250 cells from four experiments) and bud necks of large budded cells (4–9% of cells, n = 282 cells from four experiments). Ssd1 was not detectable in the nucleus by these methods. Bar, 2 µm.

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. 2 A). We rarely (<1% cells) observed Ssd1-GFP at

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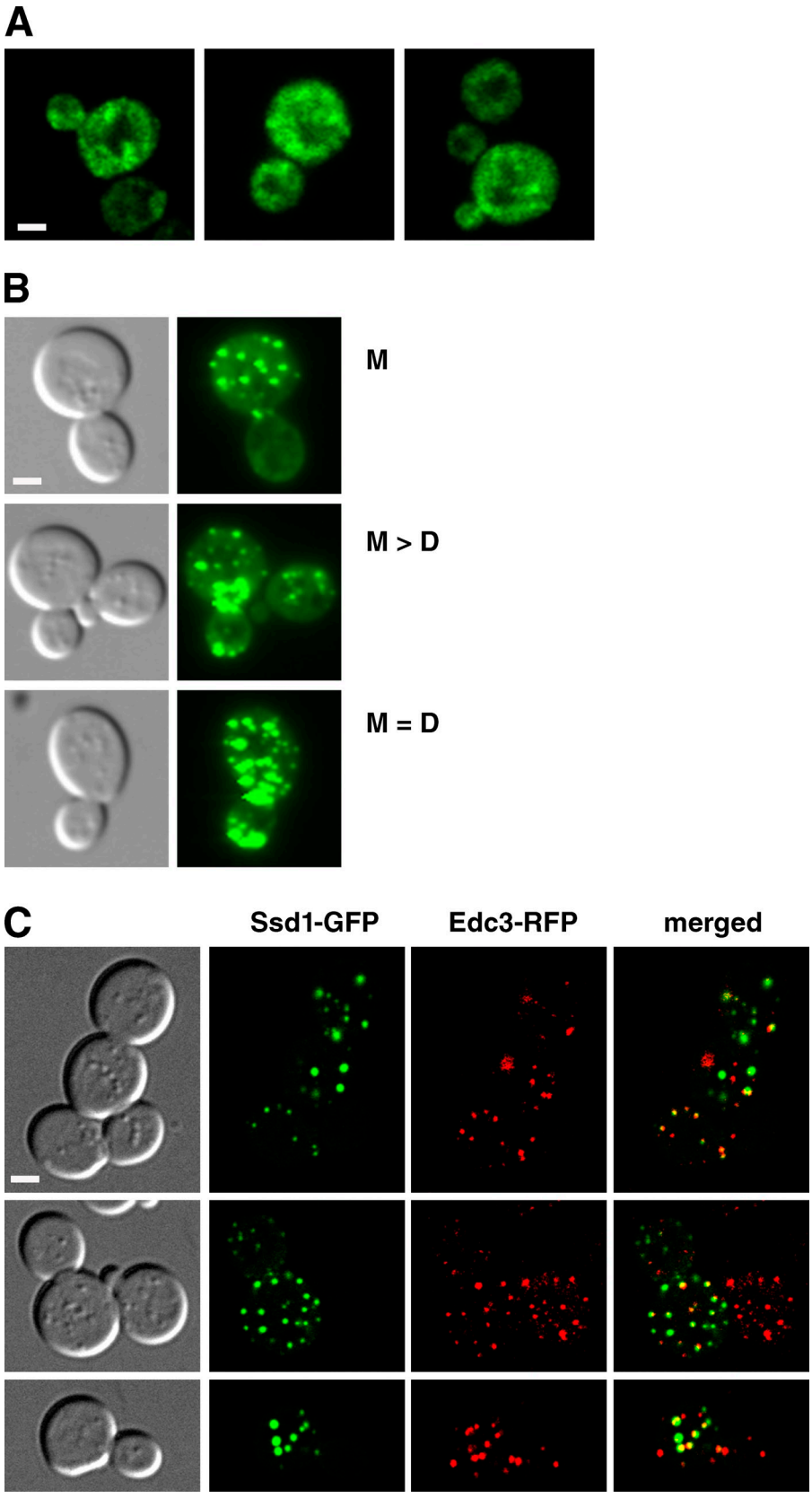

Figure 2. **Ssd1 localizes to P-bodies.** (A) Ssd1-GFP localizes to cytoplasmic puncta upon Cbk1 kinase inhibition. *cbk1-as* cells expressing Ssd1-GFP (FLY3249) were treated with 1NA-PP1 for 45 min and analyzed by fluorescence microscopy. Approximately 70% of 1NA-PP1–treated *cbk1-as* cells contain 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 (FLE1019) 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 colocalize 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.

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 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

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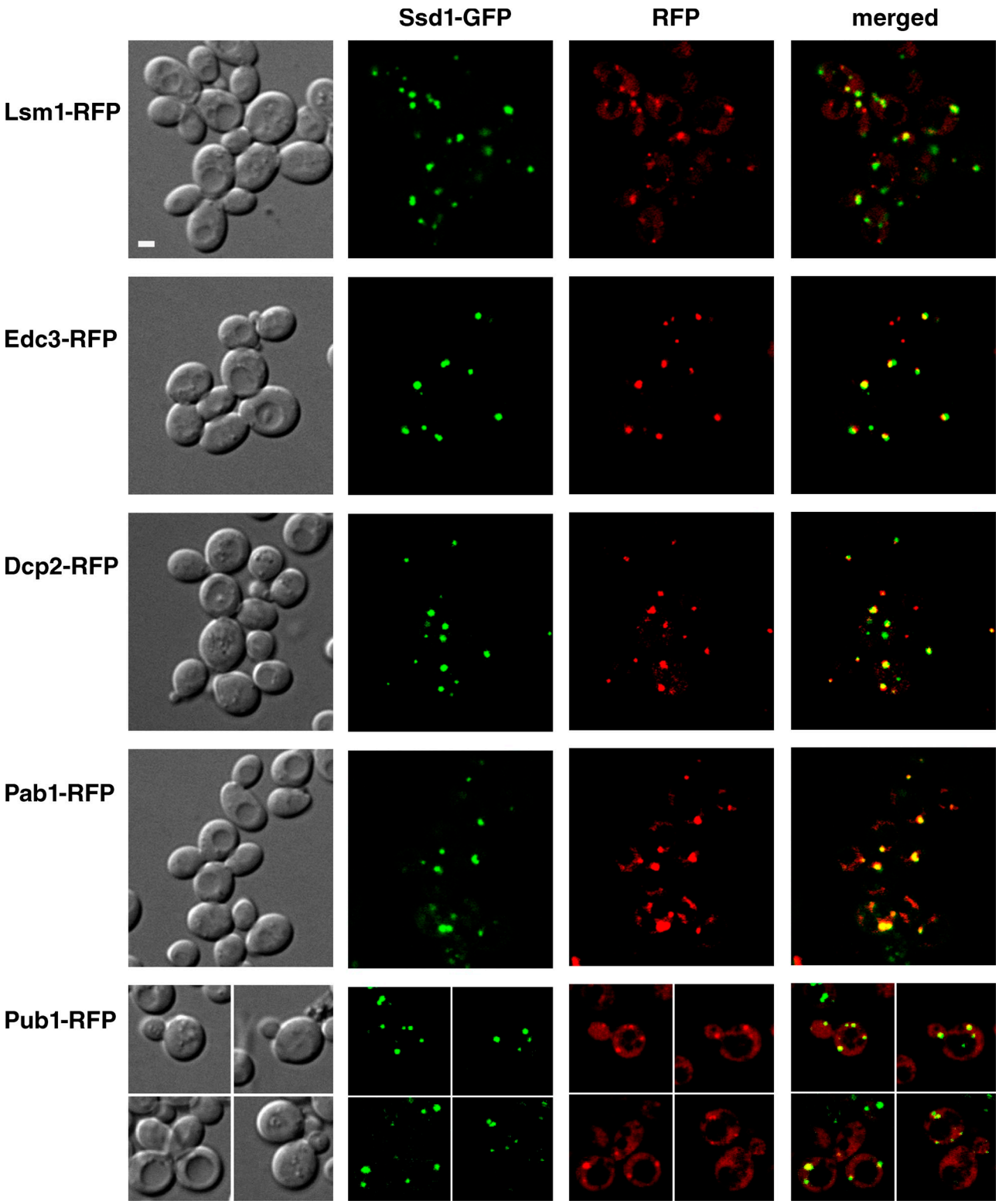

Figure 3. **Ssd1 localizes to P-bodies and stress granules in response to cellular stress.** ssd1Δ cells (FLY2184) containing Ssd1-GFP and RFP-tagged P-body proteins Edc3, Lsm1, Dcp2, or stress granule proteins Pab1 and Pub1 were transferred to glucose-depleted medium for 10 min and analyzed by spinning 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.

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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 Dcp2 (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.

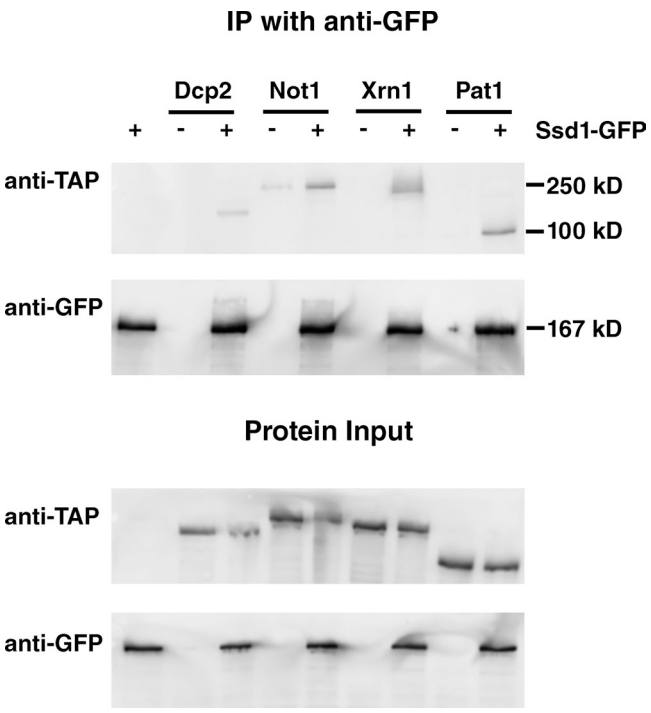

Figure 4. **Ssd1 coprecipitates with P-body and stress granule proteins.** Ssd1-GFP was immunoprecipitated from extracts of logarithmically growing yeast expressing TAP-tagged P-body or stress granule proteins and Ssd1-GFP. Immunoblots were probed with anti-GFP or anti-TAP antibodies. Input = immunoblots of total cell extract. Lanes 2, 4, 6, and 8 are from cells expressing TAP-tagged proteins only (Dcp2, Not1, Xrn1, Pat1). Lanes 1, 3, 5, 7, and 9 also contain pGPD-Ssd1-GFP (FLE1019). Dcp2, Not1, Xrn1, and Pat1 coprecipitate with Ssd1-GFP. Note that a trace of Not1-TAP nonspecifically precipitates in the absence of Ssd1-GFP (lane 4); however, much more coprecipitates with Ssd1-GFP. Control experiments (not depicted) confirm that Ssd1 coprecipitates with Cbk1, as anticipated by previously published two-hybrid assays (Racki et al., 2000).

To biochemically corroborate data from the colocalization experiments, we conducted coprecipitation 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 $Ssd1^{1\text{-}450}$, which lack the mRNA-binding domain, as defined by Uesono et al. (1994). Upon stress induction (hypertonic stress, heat shock, and glucose depletion), $Ssd1^{1\text{-}450}$ prominently localized to P-bodies within several minutes of stress induction (unpublished data). These data indicate that the

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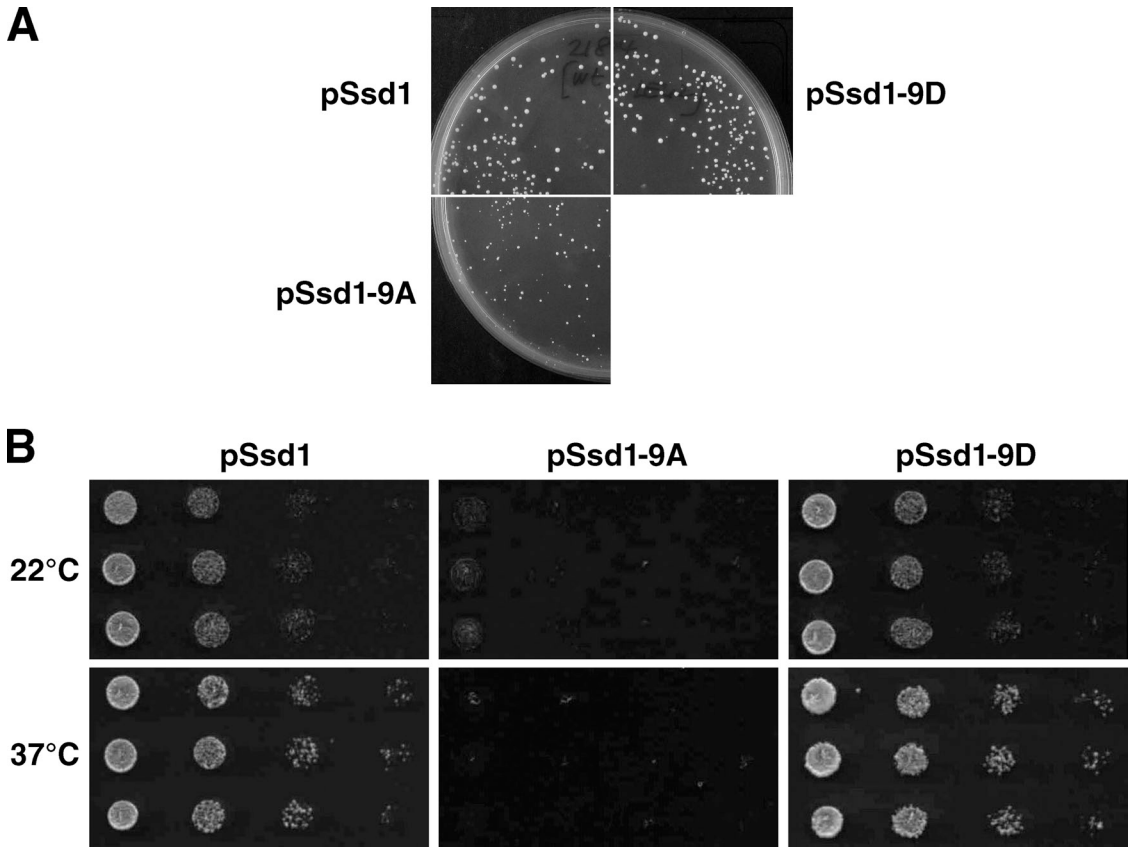

Figure 5. **In vivo expression of Ssd1-9A and Ssd1-9D.** (A) Transformants of *ssd1Δ* (FLY2184) cells carrying low copy plasmids encoding wild-type Ssd1, Ssd1-9A, or Ssd1-9D (FLE1079, FLE1080, FLE1081) under the control of the physiological *SSD1* promoter. (B) 10-fold dilution series of three separate 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.

N-terminal 450–amino acid region of Ssd1 is sufficient for P-body recruitment and that establishment and maintenance of Ssd1–P-body interactions are independent of mRNA binding.

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 phosphomimetic 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. 6 A). 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. 6 C). Furthermore, Ssd1-RBDΔ is not toxic to *cbk1Δ* cells, in contrast to wild-type Ssd1, indicating that the RNA-binding domain is essential for Ssd1 function (unpublished data). Ssd1-9A-RBDΔ expression also disrupted polarized Srl1 mRNA localization, similarly to *ssd1Δ* (see Fig. S4 B). Thus, the Ssd1 mRNA-binding domain is essential for Ssd1 function, in agreement with Uesono et al. (1997).

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

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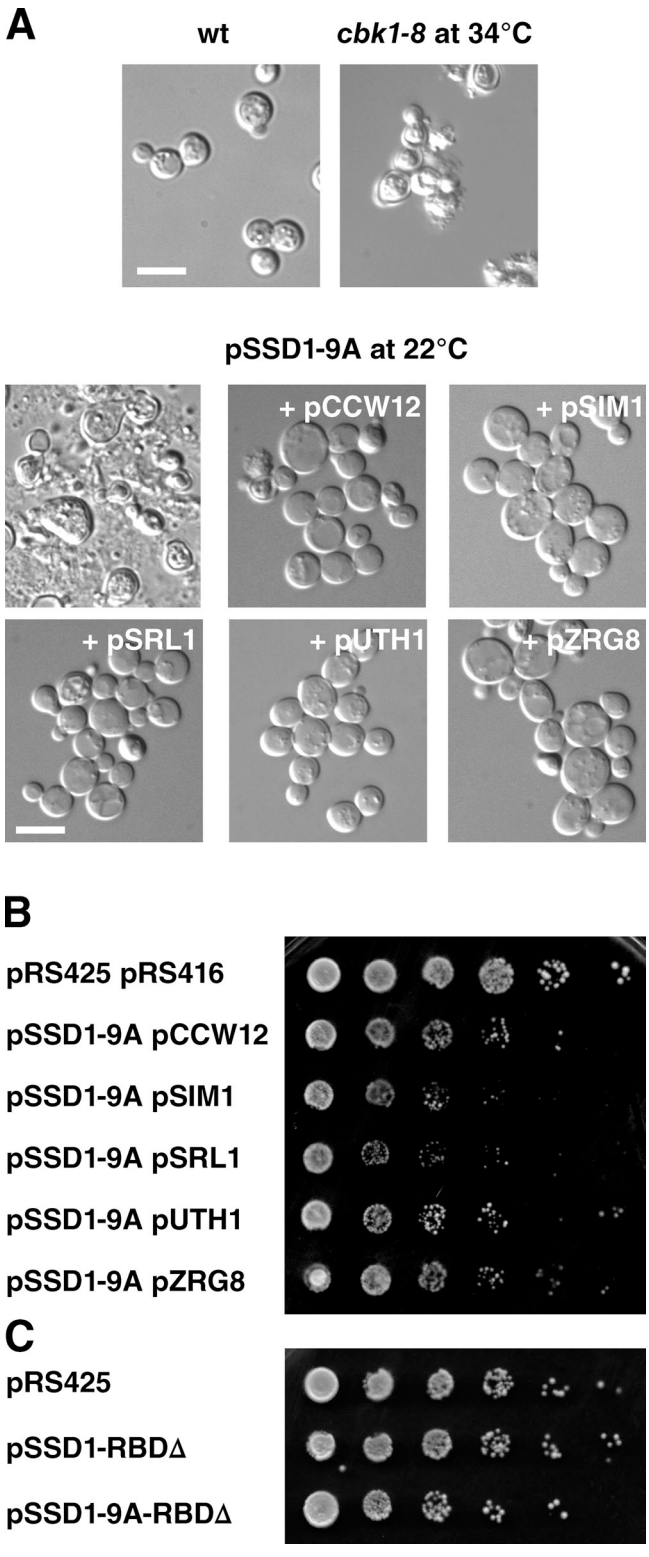

Figure 6. **Ssd1-9A causes lysis phenotypes that are suppressed by *cbk1* dosage suppressors.** (A) Top panels: wild-type cells and *cbk1-8* mutants at 34°C. The lysis phenotypes of *cbk1-8* cells resemble those of Ssd1-9A–expressing cells at 22°C. Bottom panels: *ssd1Δ* cells carrying high copy plasmids of *CCW12*, *SIM1*, *SRL1*, *UTH1*, or *ZRG8* were transformed with pRS316-Ssd1-9A. Cells were maintained at 22°C. Bars, 8 µm. (B) 10-fold dilution series of *ssd1Δ* cells (FLY2184) containing pRS316-Ssd1-9A and *cbk1* dosage suppressor plasmids (p*CCW12*, *pSIM1*, p*SRL1*, p*UTH1*, and p*ZRG8*). Negative control cells contain empty vectors pRS416 and pRS425. (C) Ssd1-9A toxicity is dependent on the mRNA-binding domain.

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 (p*CCW12*, *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. 7 A). 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).

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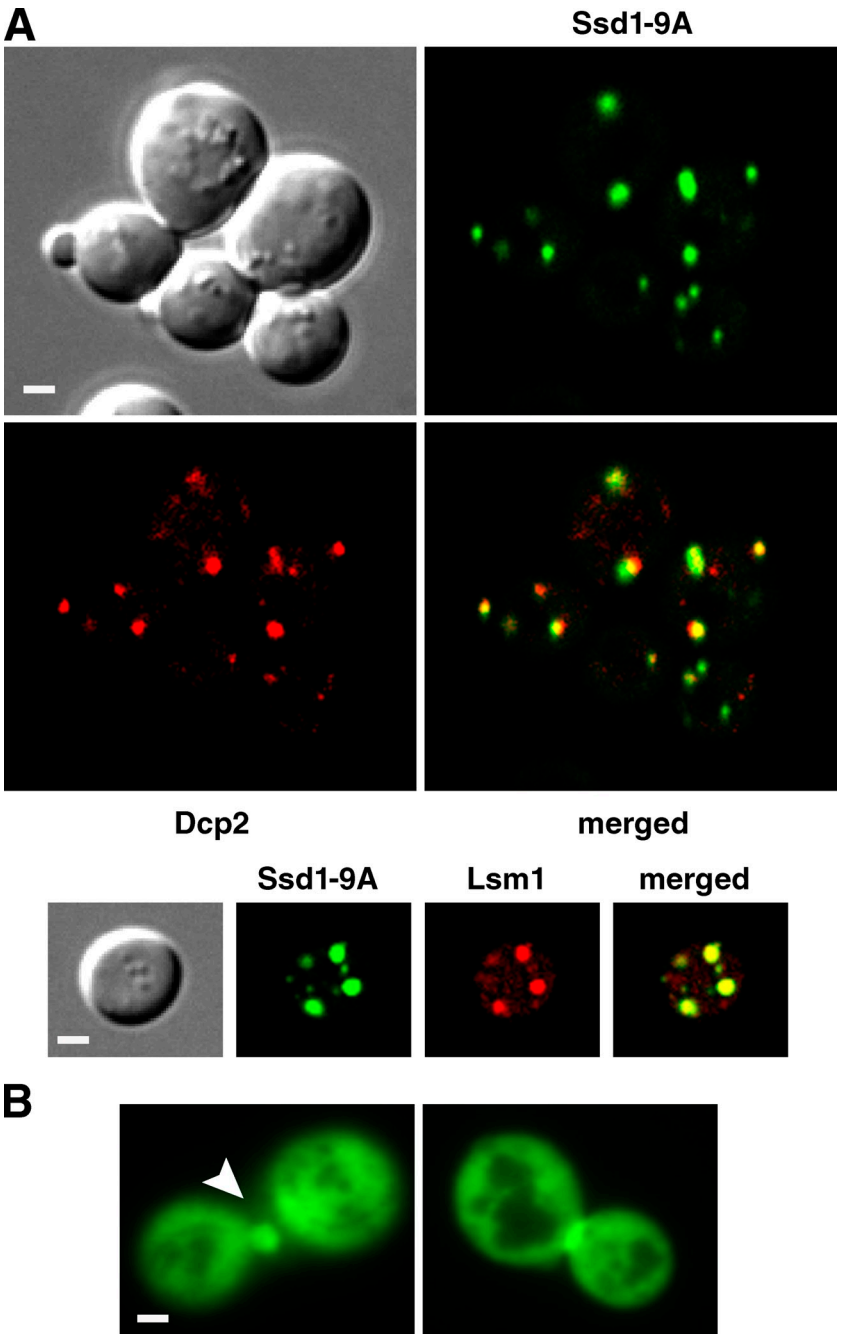

Figure 7. **pGAL-Ssd1-9A-GFP localizes to P-bodies in otherwise unstressed cells and Ssd1-9D localizes to the cytoplasm, bud cortex, and bud neck.** (A) *ssd1Δ* cells were transformed with plasmids encoding galactose-inducible Ssd1-9A-GFP and Dcp2-RFP or Lsm1-RFP (P-body markers). Cells were observed by spinning disk confocal microscopy after 1–2 h of galactose induction. Ssd1-9A-GFP colocalizes with Dcp2-RFP and Lsm1-RFP in every cell that has detectable Ssd1-9A-GFP. All Ssd1-9A-GFP puncta colocalize with P-body proteins (n = 15 cells). Over the same time course, galactose induction did not cause wild-type Ssd1-GFP or phosphomimetic Ssd1-9D-GFP (from GAL expression vectors) to associate with cytoplasmic puncta (not depicted). Galactose addition did not cause P-bodies to aggregate in *ssd1Δ* or wild-type cells (not depicted). Each image represents a single optical section. (B) *ssd1Δ* cells expressing Ssd1-9D-GFP (from plasmid FLE1090) were observed by fluorescence microscopy. The arrowhead points to enriched Ssd1 localization in small buds. Ssd1-9D was rarely detectable (<1% cells) in P-bodies of log phase cells, in contrast to GPD-driven wild-type Ssd1 (Fig. 2 B). Ssd1-9D-GFP (expressed via the GPD promoter) localizes to the cortex in ∼54% (n = 77) of small budded cells and the bud neck in ∼31% (n = 51) of large budded cells. We obtain similar results when Ssd1-9D-GFP is expressed under the control of its physiological promoter. Bars 2 µm.

(Fig. 5 and unpublished data). Moreover, the morphology and viability of cells expressing Ssd1-9D plasmids were indistinguishable from corresponding control cells containing empty vectors. These data support the model that Cbk1 negatively regulates Ssd1 by phosphorylation, and thereby prevents the dominant toxic/lethal effects of hypo-phosphorylated Ssd1.

To determine if Cbk1 phosphorylation directly influences Ssd1 localization, we analyzed Ssd1-9D-GFP localization in asynchronous cells. Like wild-type Ssd1, most Ssd1-9D localized diffusely to the cytoplasm. Ssd1-9D also localized prominently to the bud cortexes and bud necks of some small and large budded cells, similar to wild-type Ssd1 (Fig. 7 B). Significantly, and in contrast to wild-type Ssd1 or Ssd1-9A, Ssd1-9D rarely localized to bright cytoplasmic foci (P-bodies) when 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

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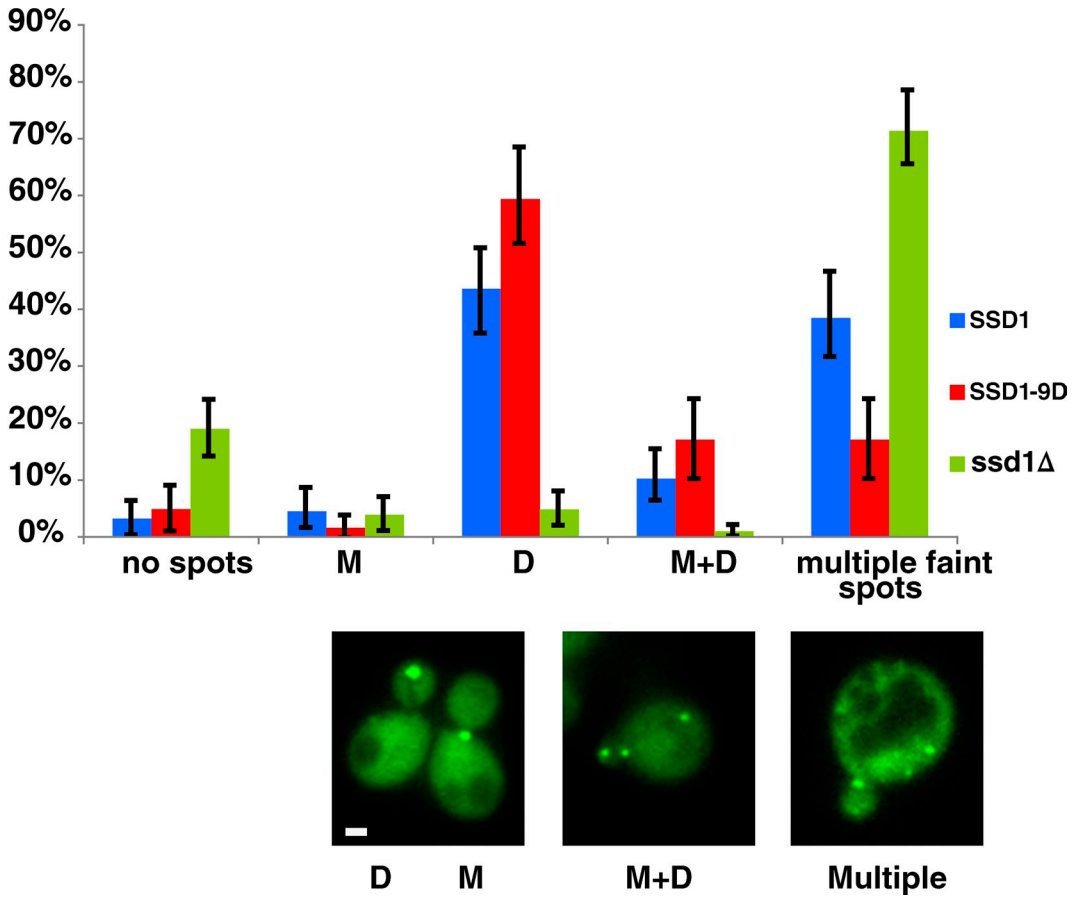

Figure 8. **Asymmetric *SRL1* mRNA localization is significantly diminished in *ssd1Δ* cells and rescued by phosphomimetic Ssd1-9D.** *SRL1* mRNA was GFP tagged and analyzed from budded cells, as described in Materials and methods. *SRL1* mRNA localization was analyzed and quantified from *ssd1Δ* cells (FLY3196) containing Ssd1 plasmids or empty vector. Cells expressing wild-type Ssd1 (*SSD1*; FLE1083), Ssd1-9D (SSD1-9D; FLE1087), and empty vector (*ssd1Δ;* pRS415) exhibited five general patterns of *SRL1* mRNA localization, as depicted in the graph and representative images. These include cells with no *SRL1* mRNA spots (no spots), multiple faint spots, and 1–3 bright spots restricted to the mother (M), bud (D), or both mother and bud (M + D). Data from three independent experiments were quantified. Each experiment yielded the same results and indicated that *SRL1* mRNA was significantly less polarized in *ssd1Δ* cells (fewer cells with *SRL1* mRNA restricted to the bud "D") than in 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

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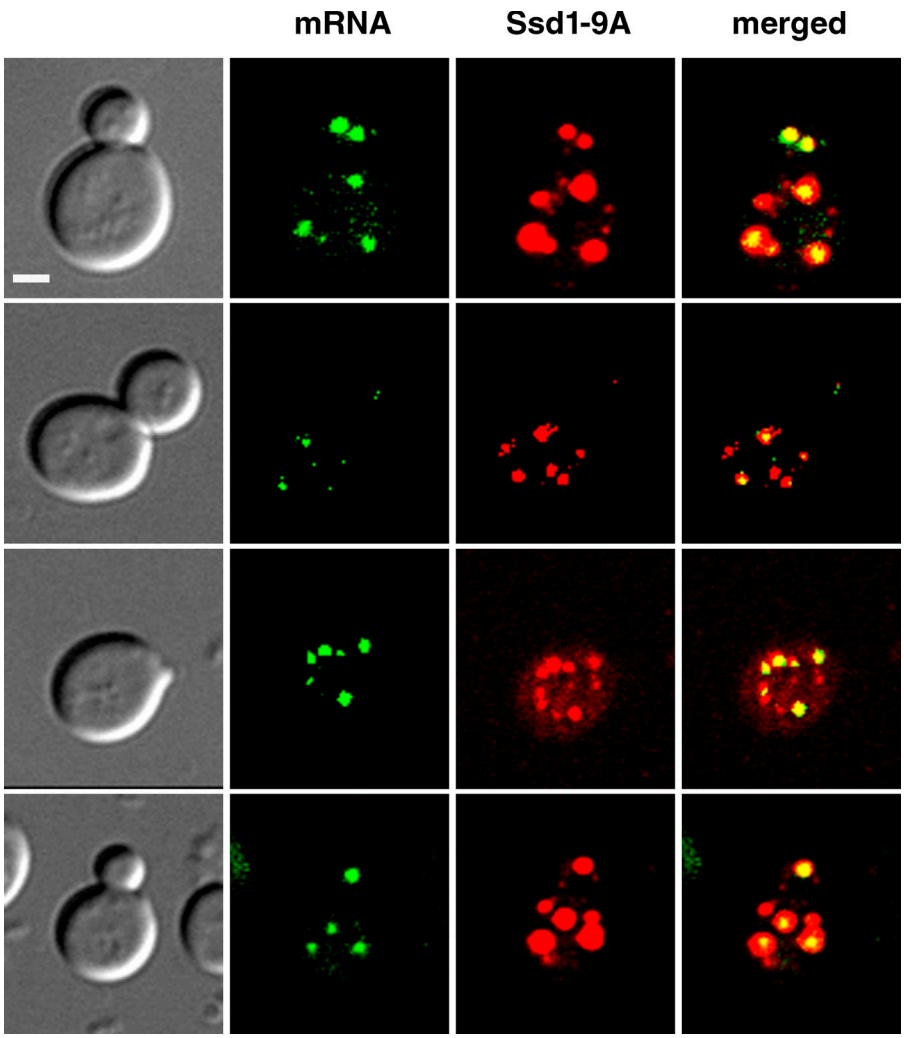

Figure 9. ***SRL1* mRNA colocalizes with Ssd1-9A.** Cells expressing GAL-inducible Ssd1-9A-RFP and *SRL1* mRNA-GFP (FLY3196 with plasmid FLE1244) were transferred to galactose medium for 1–2 h. *SRL1* mRNA and Ssd1-9A localization were subsequently observed by spinning disk confocal microscopy. In all cells with detectable Ssd1-9A-RFP and *SRL1* mRNA-GFP puncta, all *SRL1* mRNA spots colocalized with Ssd1-9A puncta (100%, n = 45 cells). Parallel experiments established that nearly all Ssd1-9A puncta colocalize with P-bodies (see Fig. 7). The images represent single optical sections. Bar, 2 µm.

the diminished *SRL1* mRNA polarity in *ssd1Δ* cells to a greater degree (~1.4-fold) than wild-type Ssd1 (Fig. 8). Moreover, Ssd1-9D expression reduced the percentage of cells with dispersed *SRL1* mRNA puncta by approximately two- to threefold, in comparison to corresponding *ssd1Δ* cells and *SSD1*-expressing cells. These data indicate that Cbk1-phosphorylated Ssd1 promotes and enhances the polarity of *SRL1* mRNA in asynchronously growing cells.

Dephosphorylated Ssd1 traps associated mRNAs in P-bodies

Because Ssd1-9A constitutively localizes to P-bodies, we predicted that Ssd1-9A expression also brings its associated mRNAs to P-bodies. Thus, we monitored *SRL1* mRNA localization in cells expressing galactose-inducible RFP-tagged Ssd1-9A. Significantly, within 2 h of Ssd1-9A induction, *SRL1* mRNA dispersed to multiple cytoplasmic puncta, all of which colocalized with Ssd1-9A (Fig. 9). We observed the same punctate *SRL1* mRNA localization in cells expressing HA-tagged Ssd1-9A (Fig. S4). In contrast, *SRL1* mRNA did not disperse or redistribute to P-bodies when wild-type Ssd1 or Ssd1-9D expression was induced for the same duration (unpublished data). Corresponding immunoblots confirmed that Ssd1-9A and wild-type 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 *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

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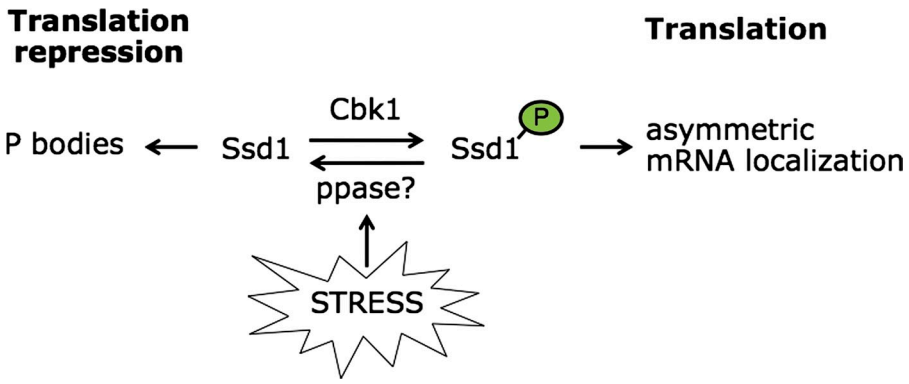

Figure 10. **Model of Cbk1 and Ssd1 function.** See Discussion for description.

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). Polarity 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 untranslating 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 mRNPs. 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

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no experimental evidence supporting the notion that Ssd1 promotes mRNA degradation in vitro or in vivo. Recombinant Ssd1 has no RNase activity in vitro (Uesono 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. S4 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 Brr1 (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* mutations (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.

Table I. **Yeast strains**

Strains	Relevant genotype
BY4741	MATα *SSD1 CBK1*
FLY2184	MATα *ssd1Δ:NATMX*
FLY2293	MATa *cbk1Δ::KANMX ssd1Δ::NATMX*
FLY2884	MATa/α *cbk1-8::HIS3:: cbk1Δ::KANMX/ cbk1-8::HIS3:: cbk1Δ::KANMX*
FLY3137	MATa/α *SSD1-GFP::KANMX/SSD1-GFP:: KANMX*
FLY3196	MATa *SRL1-mTAG ssd1Δ::NATMX [pCP-MS2-GFP]*
FLY3249	MATα *SSD1-GFP::KANMX* cbk1-as-HIS3:: cbk1Δ::KANMX
FLY3433	MATa/α *SSD1-GFP::KANMX/SSD1 PAP1-RFP::HIS3/PAP1*

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, resuspended 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,

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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$^{1\text{-}520}$-GFP	This paper
FLE1079	pRS316-SSD1	This paper
FLE1080	pRS316-SSD1-9A	This paper
FLE1081	pRS316-SSD1-9D	This paper
FLE1083	pRS415-SSD1	This paper
FLE1087	pRS415-SSD1-9D	This paper
FLE1090	pAG415-GPD-SSD1-9D-GFP	This paper
FLE1160	YGPM12a13 (UTH1)	Thermo Fisher Scientific
FLE1205	pENTRY-SSD1$^{RBD\Delta}$	This paper
FLE1206	pENTRY-SSD1-9A$^{RBD\Delta}$	This paper
FLE1207	pAG416-GAL-SSD1-9A-GFP	This paper
FLE1208	pAG416-GAL-SSD1-9A-HA	This paper
FLE1209	pAG415-GPD-SSD1-9A$^{RBD\Delta}$-GFP	This paper
FLE1210	pAG415-GPD-SSD1$^{RBD\Delta}$-GFP	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$^{RBD\Delta}$-TAP	This paper
pRP1084	pRS416-LSM1-RFP	Roy Parker, University of Arizona
pRP1084	pRS416-LSM1-RFP	Roy Parker, University of Arizona
pRP1085	pRS415-LSM-RFP	Roy Parker, University of Arizona
pRP1155	DCP2-RFP-LEU2	Roy Parker, University of Arizona
pRP1186	DCP2-RFP-URA3	Roy Parker, University of Arizona
pRP1574	EDC3-chRFP-URA3	Roy Parker, University of Arizona
pRP1661	PUB1-mCherry-URA3	Roy Parker, University of Arizona
	pLOX-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, 164, 228, 295, and 319, and T to A at position 261 (SSD1-9A); S to D at positions 40, 42, 122, 126, 160, 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 Gitler (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-RBDΔ and Ssd1-9A-RBDΔ plasmids were constructed by deleting the internal XbaI fragment of *SSD1*. *UTH1* was isolated from the plasmid YGPM12a13 (Thermo Fisher Scientific) by excising the NaeI–BglII fragment and subcloning it into the NaeI–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

Co-immunoprecipitation 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 Hepes, 1% NP-40, 60 mM 2-glycerophosphate, 1 mM EDTA, 2 mM DTT, 10 mM NaF, and protease inhibitors) and lysed in a Mini-Beadbeater (Biospec Products). 2 mg of protein extract was precleared with protein G–Sepharose (Invitrogen), incubated with 2 μg anti-GFP antibody (Roche), and precipitated with ∼50 μl 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 anti–mouse 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 immunoblots, 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).

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Table III. **Oligonucleotides**

Oligos	Sequence	Purpose	Gene
FLO1	5′-CCATGTTTAACCGTCCGTGCATTAAATCCATTCATGAAGAGG GTAGGTGGTCCCGGTGGTCGGATCCCCGGGTTAATTAA-3′	Tagging	SSD1
FLO2	5′-AGTGAAAAACAAGAAAAACAGCAATGACGATATTGG TAGAAGAGAGAATTCGAGCTCGTTTAAAC-3′	Tagging	SSD1
FLO203	5′-CTTTCAGCGCAAAGATTTGGCCCAATTATTCCATCTTTA TACACTCGGATCCCCGGGTTAATTAA-3′	Deletion	SSD1
FLO527	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGG AGATAACAAAATGTCTAAAAATAGCAACGTTAACA-3′	Gateway cloning	SSD1
FLO536	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTACCCT CTTCATGAATGGATTTAA-3′	Gateway cloning	SSD1
FLO560	5′-GTACAACACCACTTCGATTACTAATTCGACCAGTTG GTGAACGCTGCAGGTCGACAACCC-3′	mRNA tagging	SRL1
FLO561	5′-AAACAAAAAGAACTTAAAAGGACACGTTTGAACTCA TAATGCATAGGCCACTAGTGGATC-3′	mRNA tagging	SRL1
FLO588	5′-ATGAAATTCTCAACTGCC-3′	RT-PCR	SIM1
FLO589	5′-GGTGAAGTGGAAGAAGTACT-3′	RT-PCR	SIM1
FLO590	5′-ATGCAATTTTCTACTGTCGC-3′	RT-PCR	CCW12
FLO591	5′-ACAACAACAAAGCAGCG-3′	RT-PCR	CCW12
FLO618	5′-ATGAAACTGTCCGCCAC-3′	RT-PCR	SUN4
FLO619	5′-TGTAACTGCAACGGCCC-3′	RT-PCR	SUN4
FLO620	5′-ATGGGGAAACAGTCAAA-3′	RT-PCR	DSE2
FLO621	5′-CGACGGAATTAGCTAAA-3′	RT-PCR	DSE2
FLO622	5′-ATGCGTCTCTCTAACCTAATT-3′	RT-PCR	SCW4
FLO623	5′-TGAAGCGGCACCATTAA-3′	RT-PCR	SCW4
FLO624	5′-GGAACTTAAACTGATGT-3′	RT-PCR	CTS1
FLO625	5′-CAACCGATGCCTTTGAT-3′	RT-PCR	CTS1
FLO627	5′-TACGCCGACACCACTACCT-3′	qRT-PCR	SRL1
FLO628	5′-GTGGACGTGATAGTCGAGGTGGAG-3′	qRT-PCR	SRL1
FLO629	5′-GGTATCATGGTCGGTATGGGTCA-3′	qRT-PCR	ACT1
FLO630	5′-TGTTCTTCTGGGGAACTTCTCA-3′	qRT-PCR	ACT1
FLO631	5′-TGCGGACCCTTTTGCCAC-3′	qRT-PCR	CCW12
FLO632	5′-ATCCGGCGGTAGCGCAT-3′	qRT-PCR	CCW12
FLO633	5′-TTGGTGCTGGCTCCACTGG-3′	qRT-PCR	SIM1
FLO634	5′-ACACCCGTCAGCACCGC-3′	qRT-PCR	SIM1
FLO635	5′-TCCAAAGCCAAAGGCCAACG-3′	qRT-PCR	ADH1
FLO636	5′-CCGATCTTCCAGCCCTTAACG-3′	qRT-PCR	ADH1
FLO639	5′-ATGCTTCAATCCGTTGT-3′	RT-PCR	SRL1
FLO640	5′-CGTATTTGGGGTGACTG-3′	RT-PCR	SRL1

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; elongation 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 DMI4000; Leica) equipped with a 100x HCX 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 (Cobolt Jive) controlled through the LMM5 module (Spectral Applied Research). The emissions were 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 Volocity (PerkinElmer) or MetaMorph software.

Online supplemental material

Fig. S1 shows that physiologically expressed Ssd1 colocalizes with P-bodies in glucose-depleted cells. Fig. S2 shows that *UTH1* is a dosage suppressor of *cbk1-8* mutants. Fig. S3 shows that Ssd1 precipitates *SRL1*, *SIM1*, *CCW12*, *SUN4*, *DSE2*, *SCW4*, and *CTS1* mRNAs and does not affect steady-state mRNA levels. Fig. S4 shows that Ssd1-9A but not Ssd1-9A-RBDΔ expression causes SRL1 mRNA to localize to P-bodies. Fig. S5 shows confirmation of *SRL1* mRNA-tag expression in wild-type and *ssd1Δ* cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201011061/DC1.

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