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Nucleocytoplasmic shuttling of Ssd1 defines the destiny of its bound mRNAs

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SUMMARY

Mechanisms that control mRNA metabolism are critical for cell function, development and stress response. The *S. cerevisiae* mRNA-binding protein Ssd1 has been implicated in mRNA processing, aging, stress response and maintenance of cell integrity. Ssd1 is a substrate of the LATS/NDR tumor suppressor orthologue Cbk1 kinase. Previous data indicate that Ssd1 localizes to the cytoplasm, however biochemical interactions suggest that Ssd1 at least transiently localizes to the nucleus. We therefore explored whether nuclear localization is important for Ssd1 cytoplasmic functions. We identified a functional NLS in the N-terminal domain of Ssd1. An Ssd1 derived NLS-GFP fusion protein and several C-terminally truncated Ssd1 proteins, which presumably lack nuclear export sequences, accumulate in the nucleus. Alanine substitution of the Ssd1 NLS prevents Ssd1 nuclear entry, mRNA binding and disrupts Srl1 mRNA localization. Moreover, Ssd1-NLS mutations abolish Ssd1 toxicity in the absence of Cbk1 phosphorylation and cause Ssd1 to localize prominently to cytoplasmic puncta. These data indicate that nuclear shuttling is critical for Ssd1 mRNA binding and Ssd1-mRNA localization in the cytoplasm. Collectively these data support the model that Ssd1 functions analogously to hnRNPs, which bind mRNA co-transcriptionally, are exported to the cytoplasm and target mRNAs to sites of localized translation and P-bodies.

Keywords

Cbk1 kinase; RAM signaling network; morphogenesis; cell integrity; P-bodies

INTRODUCTION

mRNA binding proteins (mRNPs) are a functionally and structurally diverse group of proteins that play critical roles in gene transcription, mRNA processing, mRNA nuclear export, mRNA translation and mRNA decay (Goler-Baron *et al.*, 2008, Chartrand *et al.*, 2001, Oeffinger *et al.*, 2007). Many mRNA binding proteins first associate with their mRNA targets co-transcriptionally (Aguilera, 2005, Kelly & Corbett, 2009). These include mRNA processing proteins, mRNA transport proteins, reversible translational repressors and mRNA decay mediators (Giorgi & Moore, 2007, Besse & Ephrussi, 2008, Shen *et al.*, 2009, Shen *et al.*). The functions of many mRNPs are dependent on nucleo-cytoplasmic shuttling (Chartrand et al., 2001, Rodriguez *et al.*, 2004). Defects in mRNP nuclear import or export can disrupt gene expression and alter the fate of nascent mRNAs. mRNA-protein complexes

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are exported to the cytoplasm through the nuclear pores by means of adaptor proteins and mRNA export proteins, such as NXF1/Mex67 (Terry & Wente, 2009, Carmody & Wente, 2009, Erkmann & Kutay, 2004). In the cytoplasm, mRNA-mRNP complexes are passively or actively delivered to destinations where their mRNAs are translated, sequestered or degraded, depending on mRNP complex composition and the physiological state of the cell (Rodriguez et al., 2008).

Most mRNAs are delivered to ribosomes for translation, however some mRNA-mRNP complexes are actively transported to discrete cortical localizations where their associated mRNAs are stored or translated (Martin & Ephrussi, 2009, Rodriguez et al., 2008). Mechanisms that govern asymmetric mRNA localization and translation contribute to axis development, cell fate determination, asymmetric cell division, cell motility and neuronal development (Martin & Ephrussi, 2009, Smith, 2004, Johnstone & Lasko, 2001). For example, during *C. elegans* and Drosophila development, polar mRNP granules dictate axis formation and cell fate determination (Johnstone & Lasko, 2001, Anderson & Kedersha, 2009). Similarly, many mRNAs are transported to developing dendritic spines and synapses, where they contribute to neuronal development and signaling (Rodriguez et al., 2008). Some mRNAs are delivered or recruited to cytoplasmic mRNA granules, such as mRNA processing bodies (P-bodies) and stress granules, where they are transiently sequestered or degraded (Anderson & Kedersha, 2009, Balagopal & Parker, 2009, Rodriguez et al., 2008). The mechanisms of mRNA recruitment to P-bodies and stress granules are not fully understood, but are critical for adapting to cellular stress (Sheth & Parker, 2006, Anderson & Kedersha, 2008, Buchan et al., 2008).

The *S. cerevisiae* mRNA-binding protein Ssd1 provides insight to the mechanisms governing the cytoplasmic fate of mRNAs. *SSD1* is genetically linked to a variety of functions, including mRNA processing, translation, aging, cell integrity and stress response (Sutton *et al.*, 1991, Chiannilkulchai *et al.*, 1992, Turcq *et al.*, 1992, Stettler *et al.*, 1993, Uesono *et al.*, 1994, Tsuchiya *et al.*, 1996, Luukkonen & Seraphin, 1999, Moriya & Isono, 1999, Kaeberlein & Guarente, 2002, Wheeler *et al.*, 2003, Kaeberlein *et al.*, 2004, Tanaka *et al.*, 2007, Mir *et al.*, 2009, Ohyama *et al.*, 2010). Ssd1 function is directly linked to the RAM (regulation of Ace2 and polarized morphogenesis) signaling network, which regulates polarized growth, differential gene expression and maintenance of cell integrity (Kurischko *et al.*, 2011, Racki *et al.*, 2000, Bidlingmaier *et al.*, 2001, Weiss *et al.*, 2002, Nelson *et al.*, 2003, Bourens *et al.*, 2009). Notably, Ssd1 binds to and is phosphorylated by the RAM kinase and LATS/NDR tumor suppressor orthologue Cbk1 (Kurischko et al., 2011, Jansen *et al.*, 2009, Racki et al., 2000). Moreover, Ssd1 expression is toxic in cells harboring RAM mutations (Jorgensen *et al.*, 2002, Du & Novick, 2002, Kurischko *et al.*, 2005).

Recent data implicate Ssd1 and Cbk1 in the spatial regulation of mRNAs in the cytoplasm (Kurischko et al., 2011). Ssd1 specifically associates with a subset of mRNAs, including several mRNAs that encode *cbk1* dosage suppressors (Uesono *et al.*, 1997, Hogan *et al.*, 2008, Jansen et al., 2009, Kurischko et al., 2011). At least two Ssd1-associated mRNAs, the *cbk1* dosage suppressors *SRL1* and *UTH1*, localize asymmetrically to the bud tip during polarized growth (Hasegawa *et al.*, 2008, Shepard *et al.*, 2003). Significantly, Srl1 mRNA polarity is diminished by ~10 fold in *ssd1*Δ mutants, indicating a role for Ssd1 in asymmetric mRNA localization (Kurischko et al., 2011). Moreover, this Ssd1 function appears dependent on Cbk1 phosphorylation, since phosphomimetic Ssd1 enhances Srl1 mRNA polarity and phospho-deficient Ssd1 abolishes Srl1 mRNA polarity (Kurischko et al., 2011).

Ssd1 localization changes dramatically in response to cellular stress and Cbk1 inhibition (Kurischko et al., 2011). In most cells Ssd1 localizes diffusely to the cytoplasm, however

some Ssd1 can be observed at the bud and bud neck. Strikingly, glucose depletion, heat shock and salt stress cause Ssd1 to localize to P-bodies and stress granules (Kurischko et al., 2011, Jansen et al., 2009). Similarly, in the absence of Cbk1 phosphorylation, Ssd1 and Srl1 mRNA constitutively localize to P-bodies, suggesting that the toxicity of Ssd1 in *cbk1* mutants is due to the translational repression of Ssd1-associated mRNAs (Kurischko et al., 2011). In agreement, Cbk1 inhibition correlates with a decreased fraction of Ssd1-associated mRNAs on polysomes (Jansen et al., 2009). These data suggest that Ssd1 mediates P-body and stress granule-dependent translational repression of a subset of mRNAs.

Although Ssd1 influences mRNA localization in the cytoplasm (Kurischko et al., 2011), Ssd1 was not previously observed in the nucleus by microscopy. Nevertheless, several lines of evidence indicate that Ssd1 must have nuclear functions. Notably, Ssd1 binds the phosphorylated C-terminal domain of RNA polymerase II and associates with pre-spliced mRNAs, suggesting that Ssd1 binds its target mRNAs co-transcriptionally (Hogan et al., 2008, Phatnani *et al.*, 2004, Rodriguez-Gil *et al.*, Ohyama et al., 2010). Based on these data, we hypothesize that nucleo-cytoplasmic shuttling is important for Ssd1 function.

Here, we provide evidence that Ssd1 contains a functional nuclear localization sequence (NLS). Our data support the model that Ssd1 nucleo-cytoplasmic shuttling is critical for mRNA binding and the subsequent transport of Ssd1-mRNA complexes to sites of polarized growth or to sites of translational repression (P-bodies, stress granules), depending on the physiological state of the cell.

RESULTS

Ssd1 contains a functional nuclear localization sequence (NLS)

To test the hypothesis that nucleo-cytoplasmic shuttling is important for Ssd1 function, we surveyed Ssd1 for nuclear shuttling sequences. We identified a putative lysine-rich nuclear localization sequence (NLS; amino acids 417–427) in Ssd1 domain II (Fig. 1A). To test if the candidate Ssd1 NLS was capable of targeting GFP to the nucleus, we constructed a fusion protein consisting of GFP and the conserved domain II (designated $Ssd1^{332–450}$-GFP) and analyzed its localization in yeast. We observed that $Ssd1^{332–450}$-GFP accumulated in the nuclei of all cells, confirming the functionality of the Ssd1 NLS (Fig. 1B).

Several common laboratory strains contain the *ssd1-d* loss-of-function allele, which harbors a nonsense mutation that causes Ssd1 to be truncated at amino acid 697 ($Ssd1^{1–697}$) (Sutton et al., 1991, McDonald *et al.*, 2002). Since $Ssd1^{1–697}$ contains the NLS, it seemed possible that it and other NLS-containing Ssd1 mutants can localize to the nucleus. We therefore tested if $Ssd1^{1–697}$ and $Ssd1^{1–450}$, which are truncated after the NLS-containing domain, accumulate in the nucleus. We constructed $Ssd1^{1–697}$-GFP and $Ssd^{1–450}$-GFP strains by integrating GFP cassettes into the *SSD1* ORF. Although $Ssd1^{1–697}$-GFP and $Ssd^{1–450}$-GFP localized diffusely to the cytoplasm, each protein was readily detectable in the nuclei at all stages of the cell cycle, in contrast to wild type (full length) Ssd1, which was only apparent in the cytoplasm (Fig. 2). We confirmed the nuclear localization of truncated Ssd1 in cells expressing a plasmid based RFP-tagged nuclear marker, NLS-RFP (pRS425-NLS-mRFP). We observed similar results when truncated Ssd1 was modestly overexpressed via GPD promoter driven expression (Fig. 3A). These data indicate that the N-terminal NLS is functional and establish Ssd1 as a nucleo-cytoplasmic protein that preferentially localizes to the cytoplasm. Furthermore, because wild type Ssd1 is not detectable in the nucleus by these methods, these data suggest that C-terminal Ssd1 domains are important for nuclear export.

NLS mutations impair Ssd1 localization

If the putative Ssd1 NLS is responsible for nuclear import in vivo, then NLS mutations should prevent the nuclear accumulation of truncated Ssd1$^{1–450}$. Thus, we converted the NLS in Ssd1$^{1–450}$-GFP to alanines and monitored the localization of the mutated fusion protein (Ssd1$^{1–450}$-NLSΔ-GFP) in vivo. We expressed Ssd1$^{1–450}$-NLSΔ-GFPin yeast via low copy plasmids under the control of the constitutive GPD promoter. As expected, Ssd1$^{1–450}$-NLSΔ failed to accumulate in the nucleus, indicating that the NLS is essential for nuclear import of truncated Ssd1 (Fig. 3B). Notably, Ssd1$^{1–450}$-NLSΔ localized prominently to bright cytoplasmic puncta in all cells (n=37 cells). 3-D projections of the image data indicate that some of the cytoplasmic Ssd1$^{1–450}$-NLSΔ puncta are perinuclear (Suppl. Fig. S1, Supp Movie 1). The Ssd1$^{1–450}$-NLSΔ cytoplasmic puncta were similar to the P-body-associated Ssd1 puncta that were observed in stressed cells and in the absence of Cbk1 phosphorylation (Kurischko et al., 2011). Thus, we tested whether Ssd1$^{1–450}$-NLSΔ co-localized with the P-body protein Edc3. As previously observed for full length Ssd1 (Kurischko et al., 2011), most (~78%) of the Ssd1$^{1–450}$-NLSΔ puncta co-localized with Edc3 (n=38 puncta, 8 cells), even in the absence of stress (Fig. 3C). These data indicate that Ssd1 can be targeted to P-bodies in the absence of nuclear import. Furthermore, since Ssd1$^{1–450}$-NLSΔ lacks the mRNA-binding domain as defined by (Uesono et al., 1997)(Fig. 1A), these data also indicate that Ssd1 recruitment to P-bodies is independent of RNA binding.

To test if NLS mutations affect the cytoplasmic localizations of full length Ssd1, we introduced plasmids encoding GPD-driven Ssd1-NLSΔ-GFP into *ssd1Δ* cells. Ssd1-NLSΔ-GFP localized predominantly to 1–3 cytoplasmic puncta in all cells (n=71) (Fig. 4A). Moreover, the majority of the Ssd1-NLSΔ puncta (80%, n=15) co-localized with the P-body marker Edc3 (Fig. 4A). In contrast, similarly expressed wild type Ssd1 localizes to puncta (P-bodies) in less than 60% of cells (Kurischko et al., 2011). These data suggest that if Ssd1 cannot enter the nucleus, it predominantly localizes to P-bodies.

Ssd1 nuclear import is essential for Ssd1 function

It is well established that Ssd1 expression is lethal to *cbk1Δ* mutants, thus providing a simple assay for Ssd1 function. Previous data suggest that wild type Ssd1 is lethal in *cbk1Δ* cells because in the absence of Cbk1 phosphorylation, Ssd1 constitutively localizes to P-bodies and stress granules, leading to the translational repression of its associated mRNAs (Kurischko et al., 2011, Jansen et al., 2009). In support, Ssd1-9A, which lacks Cbk1 phosphorylation sites, constitutively localizes to P-bodies and is dominantly toxic (Kurischko et al., 2011). If nuclear entry is essential for Ssd1 function, then Ssd1-NLSΔ expression should not be toxic to *cbk1Δ* cells. Alternatively, if Ssd1 toxicity in *cbk1Δ* cells is exclusively due to cytoplasmic functions, then Ssd1-NLSΔ expression will be lethal to *cbk1*Δ cells. To distinguish between these possible outcomes, we introduced Ssd1-NLSΔ plasmids into *cbk1Δ ssd1Δ* mutant cells and assayed viability. Significantly, Ssd1-NLSΔ expression did not impair viability or growth of *cbk1Δ ssd1Δ* cells (data not shown and Fig. 4B). Moreover, *cbk1* deletion did not significantly affect Ssd1-NLSΔ localization (Fig. 4B). These data indicate that even though Ssd1-NLSΔ can localize to P-bodies, it likely does not repress mRNA translation in *cbk1Δ* cells. Taken together, these data indicate that Ssd1-NLSΔ is not fully functional and suggest that Ssd1-NLSΔ does not bind mRNA. Although unlikely, it is formally possible that modest alanine substitutions in the Ssd1 NLS disrupt Ssd1 protein conformation.

We recently established that Ssd1 influences the cytoplasmic localization of Srl1 mRNA, which encodes a cell wall mannoprotein (Kurischko et al., 2011). The polarized localization of Srl1 mRNA is significantly diminished (by ~10 fold) in *ssd1Δ* cells in comparison to

corresponding wild type cells and is restored (and modestly enhanced) by phosphomimetic Ssd1 (Kurischko et al., 2011). To determine if Ssd1 nuclear import is essential for Srl1 mRNA localization, we analyzed Srl1 mRNA in Ssd1-NLSΔ cells, using previously published methods (Kurischko et al., 2011, Haim-Vilmovsky & Gerst, 2009). We observed that Srl1 mRNA polarity was significantly diminished in Ssd1-NLSΔ cells (Suppl. Figure S2). Notably, Srl1 mRNA only localized exclusively to the bud in 13.9% of budded Ssd1-NLSΔ cells (n=226 cells), compared to >40% of wild type cells (Kurischko et al., 2011). These data indicate that Ssd1 nuclear import is critical for influencing the cytoplasmic localizations of at least one Ssd1-associated mRNA.

Ssd1 nuclear import is crucial for mRNA binding

Our data suggest that Ssd1 must enter the nucleus in order to bind mRNA, as shown for other mRNA-binding proteins (Kelly & Corbett, 2009, Aguilera, 2005). If this hypothesis is correct, then mutations that impair Ssd1 nuclear localization will prevent mRNA binding. To test if Ssd1-NLSΔ binds mRNA in vivo, we immunoprecipitated GFP-tagged Ssd1-NLSΔ from cells and assayed for associated mRNAs by spectroscopy and RT-PCR. We did not observe any detectable RNA associated with immunoprecipitated Ssd1-NLSΔ by UV spectroscopy, however wild type Ssd1 consistently precipitated RNA. Specifically, ~9.2 ug RNA associated with immunoprecipitated Ssd1-GFP from 525 ug of yeast extract. Since RT-PCR is a more sensitive method for detecting specific mRNAs than UV spectroscopy, we probed immunoprecipitated Ssd1 complexes for associated mRNAs (Srl1, Ccw12, Sim1 mRNAs) via RT-PCR, as described (Kurischko et al., 2011). We discovered that none of the probed mRNAs were detectable in immunoprecipitated Ssd1-NLSΔ complexes (Fig. 5, and data not shown). In contrast, wild type Ssd1 specifically co-precipitated with Srl1 mRNA and several other Ssd1-associated mRNAs (Fig. 5; and (Kurischko et al., 2011). As a control for specificity we also probed immunoprecipitated Ssd1 for Adh1 mRNA, which does not specifically interact with Ssd1. As expected, neither wild type Ssd1 nor Ssd1-NLSΔ precipitated Adh1 mRNA. Thus, Ssd1 nuclear import is critical for mRNA binding.

Ssd1 mRNA binding is essential for Ssd1 function but does not influence its nuclear export

The nuclear localizations of truncated $Ssd^{1–450}$-GFP and $Ssd1^{1–697}$ (Fig. 2) clearly establish that the Ssd1 mRNA binding domain and other C-terminal domains (III–VII) are not critical for nuclear import. However because full length Ssd1 does not accumulate in the nucleus, it seemed possible that mRNA binding facilitates efficient Ssd1 nuclear export. To test this hypothesis we deleted the Ssd1 RNA binding domain (amino acids 686–788) and assayed the activities and localization of the mutant Ssd1-RBDΔ in vivo. To confirm that Ssd1-RBDΔ does not bind mRNA, we immunoprecipitated Ssd1-RBDΔ from yeast and assayed for associated RNA, using the methods outlined above for Ssd1-NLSΔ. As expected, Ssd1-RBDΔ did not precipitate any RNA (Fig. 5, lane 4). In addition, Ssd1-RBDΔ expression could not suppress the diminished polarity of Srl1 mRNA in *ssd1Δ* cells, consistent with its inability to bind mRNA (Kurischko et al. 2011 and Suppl. Figure S2).

To test if Ssd1-RBDΔ is toxic to *cbk1Δ* cells, which presumably reflects the translational repression of Ssd1-associated mRNAs, we introduced plasmids into *cbk1Δ ssd1Δ* cells and assayed viability. We found that Ssd1-RBDΔ expression was not toxic to *cbk1Δ* cells, indicating that the RBD (amino acids 686–788) is critical for Ssd1 function (Kurischko et al., 2011) and see Fig. 6C). Thus as predicted, the Ssd1 RNA binding domain is essential for RNA binding and for Ssd1 function, in agreement with (Uesono et al., 1997).

How does the RBD domain influence Ssd1 localization? If mRNA binding facilitates Ssd1 nuclear export, then Ssd1-RBDΔ should accumulate in the nucleus. Thus, we introduced

Ssd1-RBDΔ-GFP plasmids into *ssd1Δ* cells that co-express Pap1-RFP or Edc3-RFP and monitored Ssd1-RBDΔ localization by fluorescence microscopy. When expressed via the constitutive GPD promoter, Ssd1-RBDΔ-GFP localized diffusely to the cytoplasm in all cells (n=83) and to bright cytoplasmic puncta in ~50% of cells (Fig. 6A, B), as described for GPD-driven wild type Ssd1 (Kurischko et al., 2011). Approximately 25% of the Ssd1-RBDΔ puncta co-localized with the P-body protein Edc3 (Fig. 6B, see arrowheads). Significantly, Ssd1-RBDΔ did not accumulate in the nucleus, suggesting that the mRNA binding domain, and hence mRNA binding, is not essential for Ssd1 nuclear export (Fig. 6A). These data indicate that mRNA binding is critical for Ssd1 function, but is not essential for efficient nuclear export.

Cbk1 influences the nuclear and cytoplasmic localizations of Ssd1$^{1–450}$

Cbk1 kinase clearly influences Ssd1 localization in the cytoplasm (Kurischko et al., 2011). Notably, Cbk1-mediated phosphorylations prevent Ssd1 from localizing to P-bodies (Kurischko et al., 2011). To test if Cbk1 regulates Ssd1 nuclear localization, we monitored Ssd1$^{1–450}$-GFP in *cbk1* mutant cells. Ssd1$^{1–450}$-GFP was integrated and expressed under control of the physiological *SSD1* promoter. As noted above, Ssd1$^{1–450}$-GFP localizes to nuclei in wild type cells (Fig. 2) and is not lethal to *cbk1Δ* cells (see Fig. 7A). Significantly, Ssd1$^{1–450}$ localized to nuclei in *cbk1Δ* cells, as in wild type cells. In addition, some Ssd1$^{1–450}$ localized to nuclear and cytoplasmic puncta (~1–4 puncta/cell) in *cbk1Δ* cells (Fig. 7A). About half of the cytoplasmic puncta colocalize with Edc3 (Fig. 7A, **arrowheads**). These data suggest that Cbk1 influences Ssd1 cytosolic and nuclear protein interactions.

As a second method to investigate the role of Cbk1 for nuclear Ssd1 localization, we monitored Ssd1$^{1–450}$-GFP localization upon Cbk1 inhibition in analog-sensitive *cbk1-as* cells (Weiss et al., 2002, Kurischko *et al.*, 2008). After 15 min of Cbk1 kinase inhibition (via 1NA-PP1 addition), some Ssd1$^{1–450}$-GFP redistributed to a prominent nuclear spot and to cytoplasmic puncta (Fig. 7B, C). Prior to Cbk1 kinase inhibition, ~15% of *cbk1-as* cells contained nuclear and/or cytoplasmic Ssd1$^{1–450}$-GFP puncta, compared to <2% in wild type cells. Upon 45 min of Cbk1 kinase inhibition, the percentage of cells with nuclear or cytoplasmic puncta increased to 60% (Fig. 7C). These data indicate that Cbk1 kinase is critical for maintaining diffuse Ssd1 nuclear localization, perhaps by stabilizing Ssd1 nuclear interactions or by preventing Ssd1 protein aggregation.

Ssd1 nuclear export is Crm1- and Mex67-independent

Our data indicate that Ssd1 must enter the nucleus in order to bind mRNA. Thus, the cytoplasmic functions of Ssd1-mRNA complexes require nuclear export. Published data suggest the export factors Crm1/Xpo1 and Mex67 as prime candidates for mediating Ssd1-mRNA nuclear export. Crm1/Xpo1 mediates the export of at least one Cbk1 substrate, Ace2, and several mRNP proteins, including Dbp5, Npl3 (Stade *et al.*, 1997, Watanabe *et al.*, 1999, Hutten & Kehlenbach, 2007, Weiss et al., 2002, Bourens *et al.*, 2008, Mazanka *et al.*, 2008, Sbia *et al.*, 2008). The Mex67-Mtr2/NXF1-p15 heterodimer complex is required for the nuclear export of most mRNAs (Stewart, 2007, Erkmann & Kutay, 2004, Cook *et al.*, 2007). We therefore monitored Ssd1-GFP localization in conditional *crm1* and *mex67* mutants to determine if Crm1 or Mex67 mediates Ssd1 nuclear export. Full length Ssd1 did not accumulate in the nuclei of temperature sensitive or leptomycin B-sensitive *crm1* mutants *(xpo1-1, xpo1-lpmS)* or *mex67-5* mutants at permissive or restrictive conditions, suggesting that Ssd1 export is Crm1 and Mex67-independent (**data not shown**).

Ssd1 C-terminal domains influence Ssd1 nuclear export

In principle, mutations that delete or disrupt nuclear export domains will cause more Ssd1 to accumulate in the nucleus than corresponding wild type Ssd1. Thus, we hypothesize that there are sequences within the C-terminal domains (III –VII) that function to keep most Ssd1 out of the nucleus. In support, wild type Ssd1 preferentially localizes to the cytoplasm, yet truncated $Ssd1^{1–450}$ and $Ssd1^{1–697}$ are readily detectable in the nucleus (Fig. 2). Note the absence of full length (wild type) Ssd1-GFP fluorescence in the nucleus compared to the surrounding diffuse cytoplasmic signal (Fig. 2). We calculated the nuclear-cytoplasmic fluorescence ratios to compare relative nuclear Ssd1 for each strain. The mean nuclear-cytoplasmic fluorescence ratios of GFP-tagged Ssd1, $Ssd1^{1–450}$ and $Ssd1^{1–697}$ are ~0.75, 1.6 and 1.25.

To identify Ssd1 nuclear export domains, we analyzed the localizations of several C-terminally truncated Ssd1 proteins. Ssd1 truncations were constructed by the targeted integration of a GFP cassette into the *SSD1* ORF after distinct conserved domains (Fig. 1A). Thus, all fusion proteins for these assays were expressed under the control of the physiological *SSD1* promoter. In contrast to full length Ssd1, truncated $Ssd1^{1–570}$, $Ssd1^{1–670}$ and $Ssd1^{1–1014}$ (which lack IV–VII, V–VII and VI–VII domains respectively) moderately accumulate in the nucleus (Fig. 8), suggesting that they are exported from the nucleus less efficiently than wild type Ssd1. Notably, $Ssd1^{1–1170}$, which lacks the C-terminal 80 amino acids of Ssd1 (domain VII), is less abundant in the nuclei than other C-terminal truncated Ssd1 ($Ssd1^{1–450}$, $Ssd1^{1–570}$, $Ssd1^{1–670}$ and $Ssd1^{1–1014}$) (Fig. 8A). Nevertheless, close inspection reveals that $Ssd1^{1–1170}$-GFP accumulates in the nucleus to a greater degree than wild type Ssd1-GFP (Fig. 8B). The nuclear to cytoplasmic ratios for all truncated Ssd1 were consistently greater than that of full length Ssd1 (Fig. 8B).

Collectively, these data suggest that domain VI (amino acids 1015–1170) and to a lesser degree domain VII are important for Ssd1 nuclear export. Our data support the model that Ssd1 domain VI contains sequences that facilitate binding to nuclear export factors. Ssd1 domain VII (amino acids 1171–1250) may influence nucleo-cytoplasmic shuttling by contributing to efficient nuclear export in conjunction with domain VI or by influencing the efficiency of nuclear import.

To determine if the C-terminally truncated Ssd1 proteins were functional, we introduced GFP-tagged $Ssd1^{1–570}$, $Ssd1^{1–670}$, $Ssd1^{1–1014}$ and $Ssd1^{1–1170}$ into *cbk1Δ* cells. None of the C-terminally truncated Ssd1 proteins were toxic to *cbk1Δ* cells, indicating that they are not fully functional in vivo (Fig. 9). These data likely indicate that the truncated proteins do not constitutively repress translation of Ssd1-associated mRNAs (see discussion). All C-terminal truncated proteins (from $Ssd1^{1–450}$ to $Ssd1^{1–1170}$) localize to nuclei in *cbk1Δ* cells to the same degree as in corresponding *CBK1* cells, indicating that Cbk1 is not essential for Ssd1 nuclear import (Fig. 9). However, in 20–30% of *cbk1Δ* cells, the truncated Ssd1 proteins localize to nuclear and cytoplasmic puncta. The percentage of *cbk1Δ* cells with nuclear and cytoplasmic puncta varies among the C-terminal truncations (see legend for Suppl. Fig. S3). Some, but not all, Ssd1 cytoplasmic puncta co-localize with the P-body protein Edc3 (Suppl. Fig. S3). These data support the model that Cbk1 influences nuclear and cytoplasmic Ssd1 interactions and support the conclusion that the Ssd1 N-terminal region (domains I – II) is sufficient for P-body recruitment, as described in (Kurischko et al., 2011).

Discussion

Our data provide strong evidence that Ssd1 is subject to nucleo-cytoplasmic shuttling that is critical for mRNA binding and all Ssd1-mRNA functions (see working model Fig. 10).

Significantly, we established that Ssd1 nuclear import is essential for mRNA binding and for dictating the cytoplasmic localizations of an Ssd1-associated mRNA. Our data support the model that Ssd1-mRNA interactions are established co-transcriptionally and that Ssd1 nuclear interactions are critical for defining the cytoplasmic fate of Ssd1-mRNA complexes.

Role of Cbk1 on Ssd1 function

We previously established that Cbk1 kinase regulates Ssd1 localization in the cytoplasm (Kurischko et al., 2011). During logarithmic growth, most Ssd1 localizes diffusely to the cytoplasm and some accumulates at sites of polarized growth. Upon Cbk1 inhibition, Ssd1 redistributes to cytoplasmic puncta that partly co-localize with P-bodies and stress granules, which are known to repress translation (Kurischko et al., 2011). These data support the model that Cbk1 negatively regulates Ssd1 function at P-bodies and therefore indirectly promotes the translation of Ssd1 associated mRNAs (Fig. 10). Thus, we surmise that in the absence of Cbk1, cells die because Ssd1 targets its associated mRNAs to P-bodies, where they are translationally repressed. In support, moderate overexpression of several genes encoding Ssd1-associated mRNAs rescues the lethality of conditional *cbk1* and dominant Ssd1-9A mutants (Kurischko et al., 2008, Kurischko et al., 2005, Kurischko et al., 2011).

In this study, we demonstrate that Cbk1 also influences the intra-nuclear localization of Ssd1. When Cbk1 is inhibited, most $Ssd1^{1–450}$ rapidly redistributes from a more diffuse nuclear localization to a punctate nuclear and cytoplasmic distribution. Likewise, *CBK1* deletion causes $Ssd1^{1–450}$ and other C-terminally truncated Ssd1 proteins to localize to nuclear and cytoplasmic puncta. Notably, only a fraction of the truncated Ssd1 cytoplasmic puncta co-localize with P-bodies (Suppl. Fig. S3). Thus, Cbk1 inhibition or deletion stimulates the appearance of at least two novel classes of Ssd1 puncta: nuclear/perinuclear puncta and P-body independent cytoplasmic puncta. These data suggest that Cbk1 promotes or stabilizes Ssd1 nuclear interactions and localization, perhaps indirectly by preventing Ssd1 from localizing to P-bodies or other puncta.

It is possible that some of the observed Ssd1 puncta are protein aggregates and that Cbk1 is important to prevent Ssd1 aggregation. In support, a recent study suggests that Ssd1 has the capacity to form prions (Alberti et al., 2009). Moreover, Ssd1 has potential prion-promoting sequences in the N-terminal Q-rich domain I and the Cbk1 phosphorylation domain (Alberti et al., 2009). Many newly identified prion-forming proteins are involved in gene expression and RNA regulation, similar to Ssd1 (Alberti et al., 2009). Ssd1 is also required for Hsp104-mediated disaggregation of protein aggregates (Mir et al., 2009). Collectively, these data may anticipate a role for Cbk1 and Ssd1 in regulating gene expression via modulation of protein aggregate/prion development.

Ssd1 nuclear interactions influence mRNA localization

Cbk1 mediated Ssd1 phosphorylation appears to stimulate the polarized localization and translation of an Ssd1-associated mRNA (Kurischko et al., 2011). Thus, Ssd1 belongs to a class of mRNA binding proteins that influence asymmetric mRNA localization (Czaplinski & Singer, 2006). At least two Ssd1-associated mRNAs, Srl1 and Uth1 mRNA, are delivered to the bud via the locasome complex, which is comprised of the mRNP She2, the adapter protein She3 and type V myosin (Sil & Herskowitz, 1996, Bohl *et al.*, 2000, Beach & Bloom, 2001, Oeffinger et al., 2007, Shepard et al., 2003, Paquin & Chartrand, 2008). In the absence of Ssd1, Srl1 mRNA polarity is diminished by ~10 fold and restored by phosphomimetic Ssd1-9D (Kurischko et al., 2011). Likewise, in the absence of the Ssd1 NLS or RBD, Srl1 mRNA polarity is significantly diminished (Suppl. Fig. S2). Thus, Cbk1 phosphorylation of Ssd1 prevents Ssd1-P-body association and promotes asymmetric mRNA localization and translation (Fig. 10). Furthermore, our data suggest that Ssd1 must

shuttle into and out of the nucleus in order to bind mRNAs (presumably during transcription) and deliver them to cytoplasmic locales where they will be translated.

It is tempting to speculate that Ssd1 must enter the nucleus to associate with proteins that are necessary for asymmetric delivery of mRNAs. Indeed She2, the best-studied mRNA-binding protein of its class, localizes to the nucleus, binds mRNAs co-transcriptionally and recruits other mRNP proteins, including Loc1p and the translation repressor Puf6p (Olivier *et al.*, 2005, Shen et al., 2009, Shen et al., Hasegawa et al., 2008, Paquin & Chartrand, 2008). Once in the cytoplasm, She2-mRNA complexes bind the adapter protein She3, which binds myosin V Myo4, and are transported along actin cables to the bud tip (Bohl et al., 2000, Beach & Bloom, 2001, Darzacq *et al.*, 2003, Olivier et al., 2005). Ssd1 may promote asymmetric Srl1 mRNA localization by enhancing the functions of She2 or other locasome-associated proteins.

How is Ssd1 nuclear export regulated?

Since Ssd1 nuclear import is essential for mRNA binding, Ssd1-mRNA complexes must be exported from the nucleus. Some mRNP proteins, such as *S. cerevisiae* She2 and *Drosophila* Staufen 2, both of which influence cytoplasmic mRNA localization, must bind mRNA in order to be exported from the nucleus (Du *et al.*, 2008, Macchi *et al.*, 2004). In contrast, Ssd1-RBDΔ, which contains a functional NLS but cannot bind mRNA, does not accumulate in the nucleus, suggesting that RNA binding is not a prerequisite for Ssd1 nuclear export.

Our data regarding the localization of truncated derivatives of Ssd1, suggest that Ssd1 domains VI and possibly VII play a prominent role in the efficient export of Ssd1 from the nucleus. Deletion of Ssd1 domains VI and VII leads to prominent nuclear enrichment, whereas deletion of VII alone (amino acids 1171–1250) leads to a much more modest nuclear accumulation (Fig. 8). Thus we hypothesize that Ssd1-mRNA nuclear export involves protein interactions at domains VI and VII. Intriguingly, Ssd1 domains VI and VII are highly conserved among fungi, suggesting that they are functionally important. Nevertheless, domains VI and VII do not contain known nuclear export sequences or other functional motifs. The identification of proteins associated with Ssd1 domains VI and VII is likely to provide further insight into the mechanism of Ssd1 nuclear export.

Ssd1-like mRNA functions in other organisms

Ssd1 is the first example of a yeast mRNP that shuttles into and out of the nucleus and has a role both in polarized mRNA localization and in translational repression. This complexity of Ssd1 function coupled with the diversity of its associated mRNAs likely account for the large variety of *SSD1* genetic interactions (Sutton et al., 1991, Chiannilkulchai et al., 1992, Turcq et al., 1992, Stettler et al., 1993, Uesono et al., 1994, Tsuchiya et al., 1996, Luukkonen & Seraphin, 1999, Moriya & Isono, 1999, Kaeberlein & Guarente, 2002, Wheeler et al., 2003, Kaeberlein et al., 2004, Tanaka et al., 2007, Mir et al., 2009).

Ssd1 may be functionally related to hnRNPs (heterogeneous nuclear ribonucleoproteins) in other organisms, which bind mRNA in the nucleus and influence cytoplasmic mRNA localization and translation (Krecic & Swanson, 1999, Percipalle *et al.*, 2009). These proteins are referred to as zip code binding proteins as they recognize specific 3′ or 5′ UTR sequences in their target mRNAs (Mili & Macara, 2009). Ssd1 also recognizes consensus sequences in the 5′ UTR of associated mRNAs (Hogan et al., 2008, Ohyama et al., 2010). It is possible that these sequences function as zip codes for Ssd1-dependent mRNA localizations.

Ssd1 may function analogously to both *Drosophila* hnRNP-C and FMRP, which competitively bind to the same region of APP (amyloid precursor protein) mRNA (Lee *et*

al., Zalfa *et al.*, 2006). hnRNP C promotes translation and FMRP represses translation and targets the mRNA complexes to P-bodies (Lee et al., 2010). In addition, Ssd1 may interact with cytoplasmic proteins that function analogously to neuronal NXF7 (nuclear export factor), which targets hnRNP A3 mRNA complexes to P-bodies or stress granules (Katahira et al., 2008).

Cbk1, Ssd1 and cancer

The functional relationship between Cbk1 and Ssd1 kinase may suggest a previously unanticipated mechanism for mammalian LATS/NDR tumor suppressor kinases in regulation of post-transcriptional gene expression. It is well established that the translational regulation of localized mRNA has a direct impact on cancer development and metastasis (Rodriguez et al., 2008). For example, the protein levels of the mRNA localization factors ZBP1 and IMP1 directly influence metastasis and tumor progression (Gu *et al.*, 2008, Lapidus *et al.*, 2007, Wang *et al.*, 2004, Kobel *et al.*, 2007, Dimitriadis *et al.*, 2007). LATS/NDR kinases are implicated in cellular transformation and growth control (Hergovich *et al.*, 2006, Hergovich & Hemmings, 2009). Despite their importance, the mechanisms by which they influence cancer progression are poorly understood (Hergovich & Hemmings, 2009). Our work suggests that a conserved function for LATS/NDR kinases is to regulate mRNA localization and metabolism via proteins such as Ssd1. Thus, further work on Cbk1 and Ssd1-dependent regulation of mRNA function may reveal insights into the LATS/NDR-mediated mechanisms of cancer development.

EXPERIMENTAL PROCEDURES

Yeast growth conditions and strain construction

Standard yeast genetic and culture methods were used as described (Guthrie & Fink, 1991, Kurischko et al., 2005). The strains used in this study are listed in Table I. Strains expressing C-terminal truncations of Ssd1 ($Ssd1^{1–450}$, $Ssd1^{1–570}$, $Ssd1^{1–670}$, $Ssd1^{1–697}$, $Ssd1^{1–1014}$ and $Ssd1^{1–1170}$) were constructed by integration of PCR-based GFP cassettes, as described (Longtine et al., 1998). The parental Pap1-cherryFP::HIS3 strain was constructed by integration of Pap1-cherryFP::HIS3 PCR cassette, amplified from strain MS7839 (gift of Dr. P. Melloy, Farleigh Dickinson University). The parental Nop56/Sik1-RFP::KANMX strain for FLY3074 was obtained from Dr. James Falvo (UCSF). The oligonucleotides used in this study are listed in Table III.

Plasmid construction

The plasmids and oligonucleotides used in this study are listed in Table II and III. *SSD1* was subcloned from YEp13-SSD1 (a gift from Dr. C. Boone, University of Toronto) into pUC19 for further manipulations. For SSD1-NLSΔ plasmids we obtained an *SSD1* fragment with each NLS amino acid codon (amino acids 417–427: KKEKEEKKRRK) mutated to alanine from Mr. Gene GmbH (Regensburg, Germany). pUC19-SSD1-NLSΔ plasmid (FLE1180) was constructed by replacing the 266 bp *Nsi*I-*Spe*I fragment of pUC19-SSD1 (FLE1069) with the corresponding *SSD1*-NLSΔ fragment. We subcloned the 1.6 kb *Nde*I-*Xba*I fragment of pUC19-SSD1-NLSΔ into the *Nde*I-*Xba*I sites of pENTRY-SSD1. We reinserted an internal 309 bp *Xba*I fragment that was lost during *Xba*I digest to yield pENTRY-SSD1-NLSΔ (FLE1212). pENTRY-$SSD1^{1–450}$-NLSΔ was constructed by PCR amplification of the N-terminal 1350 bp fragment of Ssd1 from pUC19-SSD1-NLSΔ (FLE1180) using oligos FLO508 and FLO527. $SSD1^{1–450}$-NLSΔ was subcloned into pDONR221 to create pENTRY-$SSD1^{1–450}$-NLSΔ (FLE1204). pENTRY-SSD1-RBDΔ vector (FLE1205) was constructed by deleting the 309 bp *Xba*I fragment from FLE1057, thereby creating an in-frame deletion of amino acids 686–788. All *SSD1* derivatives in entry vectors were subcloned into Gateway-compatible GFP and TAP plasmids (C-terminally tagged), as

described (Alberti et al., 2007). pDONR221 and all Gateway-compatible parent vectors were provided by Dr. Aaron Gitler (Univ. of Pennsylvania). All *SSD1* constructs were confirmed by sequencing.

The pENTRY vector (FLE1066) for the NLS-GFP reporter (FLE1067) was constructed by subcloning the PCR-amplified *SSD1* fragment corresponding to amino acids 332–450 (bp 996–1350), using oligos FLO507 and FLO508, into pDONR221. The RFP-tagged Edc3 plasmid was obtained from Dr. Roy Parker, Howard Hughes Medical Institute, Univ. of Arizona. Plasmid pRS425-NLS-mRFP (pKW1219) was obtained from Dr. Karsten Weis, Univ. of California, Berkeley (Madrid et al., 2006).

Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot analyses were conducted as described (Kurischko et al., 2011) using monoclonal anti-GFP and secondary anti-mouse AP-conjugated antibodies (Roche Applied Science and Promega). All yeast extracts were prepared in buffer containing the RNAse inhibitor RNAsin (Promega).

For protein-mRNA affinity precipitation experiments, GFP-tagged proteins were immunoprecipitated from 525 ug yeast extract that was pre-treated with 50 ug proteinase K at 70°C for 15 min. RNA was purified using the MasterPure kit (EPICENTRE Biotechnologies) and resuspended in 40 ul TE. 10 ul of each RNA sample was quantified by UV spectroscopy at 260 nm and 280 nm. The remaining RNA sample was used for RT-PCR procedures.

RT-PCR methods

RT-PCR with immunoprecipitated mRNAs was carried out as described (Peritz *et al.*, 2006, Kurischko et al., 2011). Yeast RNA was purified using the MasterPure kit (EPICENTRE Biotechnologies) and cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). The oligos used to amplify *ADH1* and *SRL1* are listed in Table III.

Fluorescence microscopy

All microscopy was carried out as described in (Kurischko et al., 2011). Wide-field fluorescence microscopy was carried out with a Leica DMR5 fluorescence microscope equipped with a 100× PL APO 1.46 NA oil objective and an ImagEM 16-bit cooled Hamamatsu EMCCD camera, as described (Nelson et al., 2003, Kurischko et al., 2008). Image-capture was controlled by Volocity software (Perkin Elmer). Spinning disk confocal microscopy was conducted with Leica Inverted DMI4000 microscope equipped with a 100× HC× PL APO 1.46 NA oil objective, a Yokogawa CSU-10 spinning disk confocal system and an ImagEM 16-bit cooled Hamamatsu EMCCD camera. Laser excitation was provided by a 488 nm (Spectra Physics) and a 561 nm laser (Cobolt Jive) controlled through LMM5 (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 was controlled by Metamorph software (MDS Analytical Technologies). Data analysis and 3D modeling was conducted with Volocity software (Perkin Elmer).

SRL1 mRNA localization was monitored in Ssd1-NLSΔ-TAP and Ssd1-RBDΔ-TAP cells, as described (Kurischko et al., 2011). Nuclear-cytoplasmic ratios of Ssd1-GFP were obtained using Metamorph software. Nuclei were defined by colocalization with NLS-RFP. The average GFP fluorescence signals in a region of interest (nucleus and cytoplasm) were measured. For cytoplasmic GFP measurements, vacuoles were avoided to prevent

underestimates. The background (external to cells) was subtracted from the nuclear and cytoplasmic fluorescence and the ratios were calculated for 16–26 cells per strain. The mean values with the corresponding standard deviations were calculated and plotted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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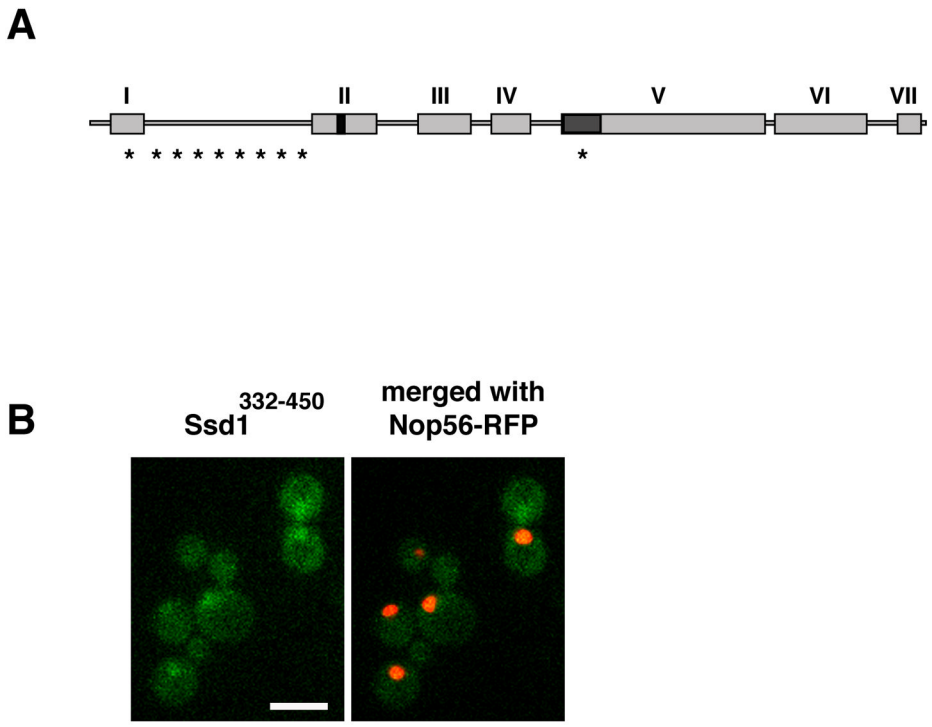

Figure 1. Ssd1 contains a functional NLS

A) Ssd1 consists of several domains that are highly conserved among yeast and fungi. Domain I (aa 31–78) contains a Q-rich stretch and is part of the RNA-pol II phospho-CTD binding domain (aa 1–160); domain II (aa 332–450) contains an NLS at aa 417–427; domain III (aa 490–570) and domain VII (aa 1170–1250) contain no obvious protein motifs; domain IV (aa 579–670) and domain VI (aa 1015–1150) have homology to bacterial VacB, an RNase II family member and domain V (aa 713–1014) has homology to E. coli RNB, an RNase II family member. The RNA binding domain (aa 686–788) overlaps with domain V. Asterisks denote putative Cbk1 phosphorylation sites.

B) Ssd1$^{332–450}$-GFP accumulates in the nucleus. A plasmid expressing the Ssd1 NLS containing domain (Ssd1$^{332–450}$) fused to GFP (FLE1067) was transformed into *ssd1Δ* cells containing the nucleolar marker Nop56-RFP (FLY3074). Images represent single optical sections captured via wide field fluorescence microscopy. Scale bar = 8 um.

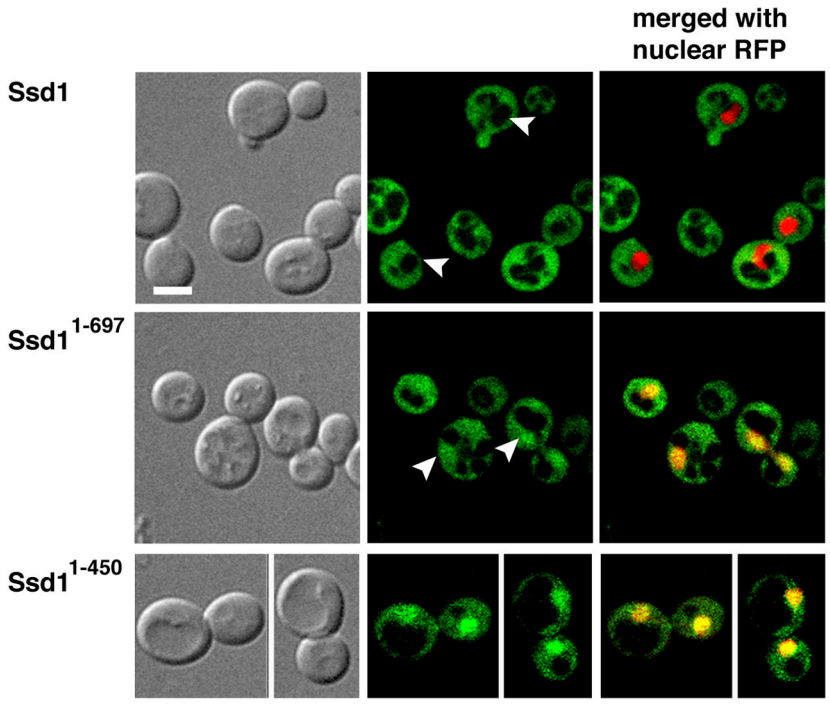

Figure 2. C-terminal truncated Ssd1 is detectable in the nucleus

The C-terminal truncated Ssd1$^{1–697}$ and Ssd1$^{1–450}$ accumulate in both the mother and daughter nuclei, compared to wild type Ssd1. Logarithmically growing cells expressing Ssd1-GFP (FLY1593), Ssd1$^{1–697}$-GFP (FLY3149) and Ssd1$^{1–450}$-GFP (FLY3168) co-expressing an RFP-tagged nuclear marker (NLS-RFP, pKW1219) were observed by spinning disk fluorescence microscopy. Arrowheads indicate nuclei. Nuclear/cytoplasmic fluorescence ratios are provided in Fig. 8B. Images represent single optical sections. Scale bar = 4 um.

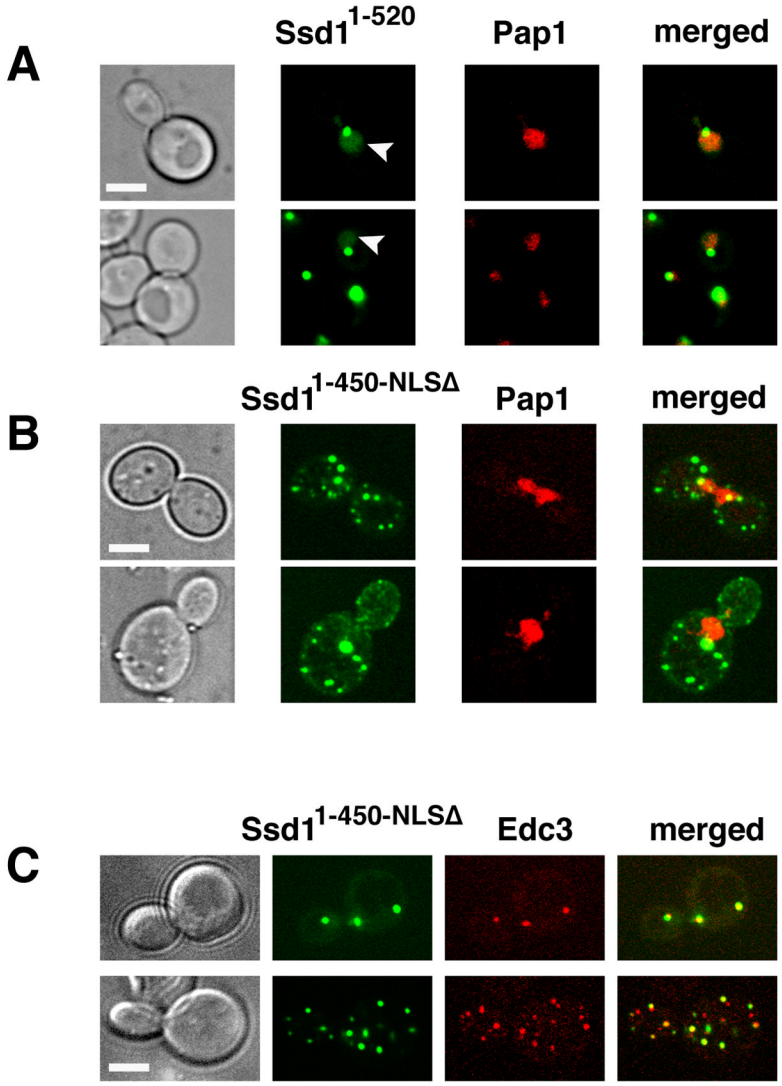

Figure 3. Ssd1$^{1–450}$-NLSΔ does not localize to nuclei

A) Truncated Ssd1$^{1–520}$-GFP, which contains the NLS domain, accumulates in the nucleus in all cells (n=34). Ssd1$^{1–520}$-GFP was expressed in *ssd1Δ* cells (FLY3365) via the GPD promoter on low copy plasmid (FLE1020). Ssd1$^{1–520}$-GFP nuclear localization was determined by co-localization with the nuclear marker Pap1-RFP (arrowhead). Ssd1$^{1–520}$-GFP localizes diffusely to the nucleus in 82.4% of the cells (n=34). Among these, 85.7% also contain 1–2 prominent nuclear/perinuclear puncta. The remaining cells (17.6%) contain single puncta localized near the nuclear periphery. Ssd1$^{1–520}$-GFP localizes to 1–3 cytoplasmic puncta in ~15% of all cells. B) A plasmid encoding Ssd1$^{1–450}$-NLSΔ-GFP (FLE1211) was introduced into *ssd1Δ* cells expressing the nuclear marker Pap1-RFP (FLY3365). Ssd1$^{1–450}$-NLSΔ fails to localize to nuclei and principally localizes to prominent cytoplasmic puncta, some of which are juxtanuclear. Ssd1$^{1–450}$-NLSΔ localizes to >5 cytoplasmic puncta in all (100%) cells. A 3-D model of the cell in the top panel is presented in Suppl. Fig. S1 and Suppl. Movie 1. C) The cytoplasmic Ssd1$^{1–450}$-NLSΔ-GFP puncta colocalize with the P-body marker Edc3-RFP (78.9%, n=38). All images were captures via by spinning disk fluorescence microscopy and represent a single optical section. Scale bars = 4 um.

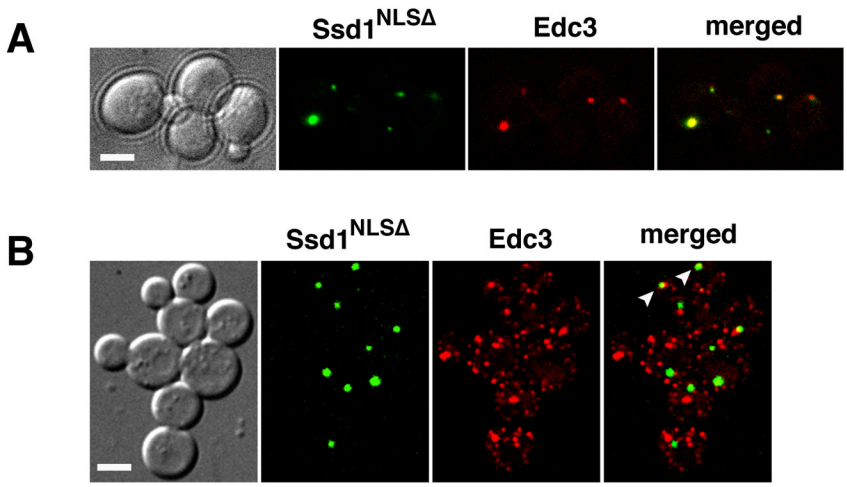

Figure 4. Ssd1-NLSΔ-GFP localizes to P-bodies and is not toxic to *cbk1Δ* cells

A) Ssd1-NLSΔ-GFP was expressed in *ssd1Δ* cells (FLY2184) via low copy plasmids (FLE1213). Ssd1-NLSΔ-GFP localizes to 1–3 cytoplasmic puncta per cell (n=71). ~80% of the cytoplasmic Ssd1-NLSΔ puncta colocalize with the P-body protein Edc3 (n=15). B) Ssd1-NLSΔ-GFP localizes to cytoplasmic puncta in *cbk1Δ* cells (FLY2293). Ssd1-NLSΔ is not toxic to *cbk1Δ* cells and localizes similarly in *cbk1Δ* as in *CBK1* cells. Arrowheads point to some Ssd1 puncta that colocalize with Edc3. All cells were monitored by spinning disk fluorescence microscopy and each image represents a single optical section. Scale bars = 4 um.

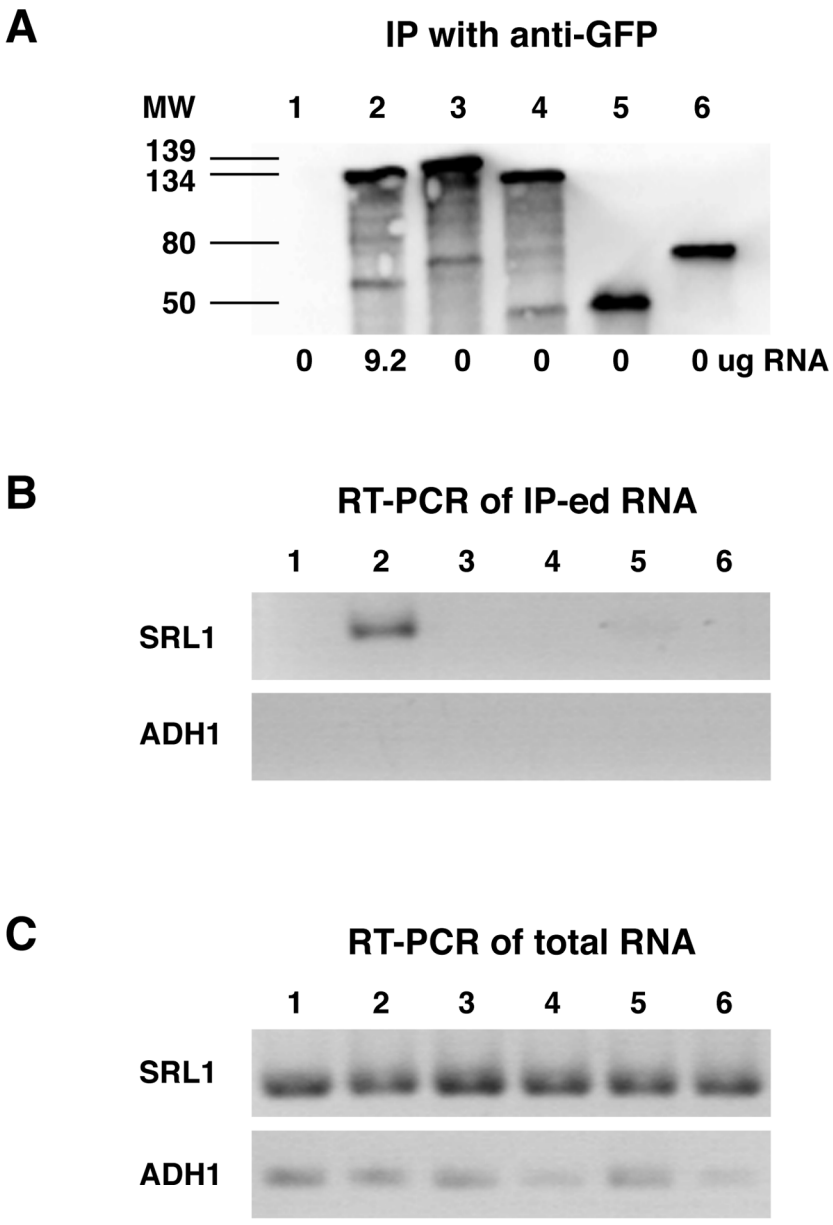

Figure 5. Ssd1-NLSΔ does not bind mRNA in vivo

GFP-tagged Ssd1 proteins were immunoprecipitated from 525 ug yeast lysate and assayed for associated mRNAs by UV spectroscopy and RT-PCR. A) Immunoblot of immunoprecipitated Ssd1-GFP probed with anti-GFP antibody. Lane 1: wild type cells (no GFP); lane 2: Ssd1-GFP; lane 3: Ssd1-NLSΔ; lane 4: Ssd1-RBDΔ; lane 5: $Ssd1^{1–450}$-NLSΔ; lane 6: Sec63-GFP. Sec63 does not bind RNA and thus serves as a negative control. The numbers below each lane represent the relative amount (ug) of total RNA associated with the immunoprecipitated proteins. Three independent experiments yielded the same results. B) Immunoprecipitated Ssd1 complexes (from A) were probed for *SRL1* and *ADH1* mRNA by RT-PCR. *ADH1* mRNA serves as a negative control since it does not bind Ssd1. C) Total cell extracts (starting material) were probed for *SRL1* and *ADH1* mRNA by RT-PCR. For these experiments, the strain FLY2184 was transformed with plasmids FLE1019, FLE1210, FLE1211, FLE1213 and pJK59.

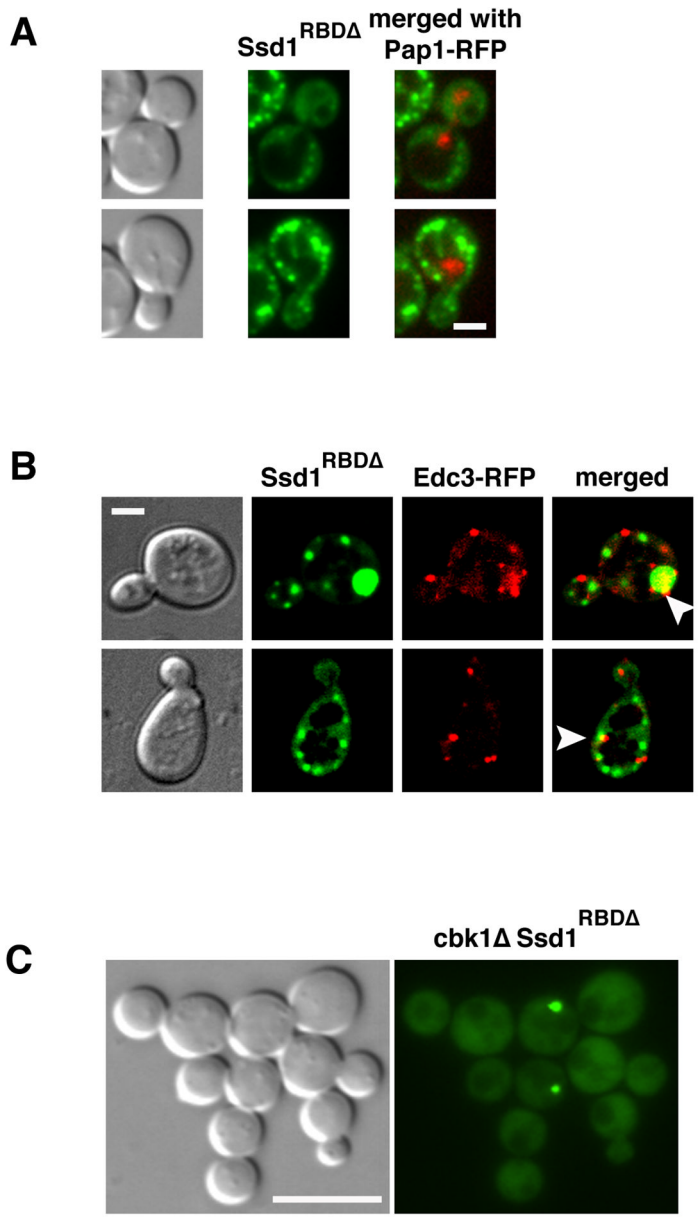

Figure 6. Ssd1-RBDΔ localizes to the cytoplasm

A) A plasmid encoding GFP-tagged Ssd1-RBDΔ (which lacks Ssd1 amino acids 686–788; FLE1210) was introduced into A) *ssd1Δ Pap1-RFP*, B*) ssd1Δ Edc3-RFP* and C) *cbk1Δ ssd1Δ* cells (FLY3365, FLY2184 + pRP1574, FLY2293). In *CBK1* cells, Ssd1-RBDΔ-GFP does not accumulate in the nucleus, but localizes diffusely to the cytoplasm (in all cells) and to multiple cytoplasmic puncta in ~50% of cells, as previously reported for GPD-driven Ssd1 (Kurischko et al., 2011). Some (~23%), but not all, Ssd1-RBDΔ-GFP puncta co-localize with Edc3 (see arrowheads). C) Ssd1-RBDΔ expression is not toxic to *cbk1Δ* cells, consistent with the observation that Ssd1-RBDΔ does not bind mRNA in vivo (see Fig. 5). All cell images represent single optical sections captured via spinning disk (A and B) and wide field (C) fluorescence microscopy. Scale bars = 2 um (A, B) and 4 um (C).

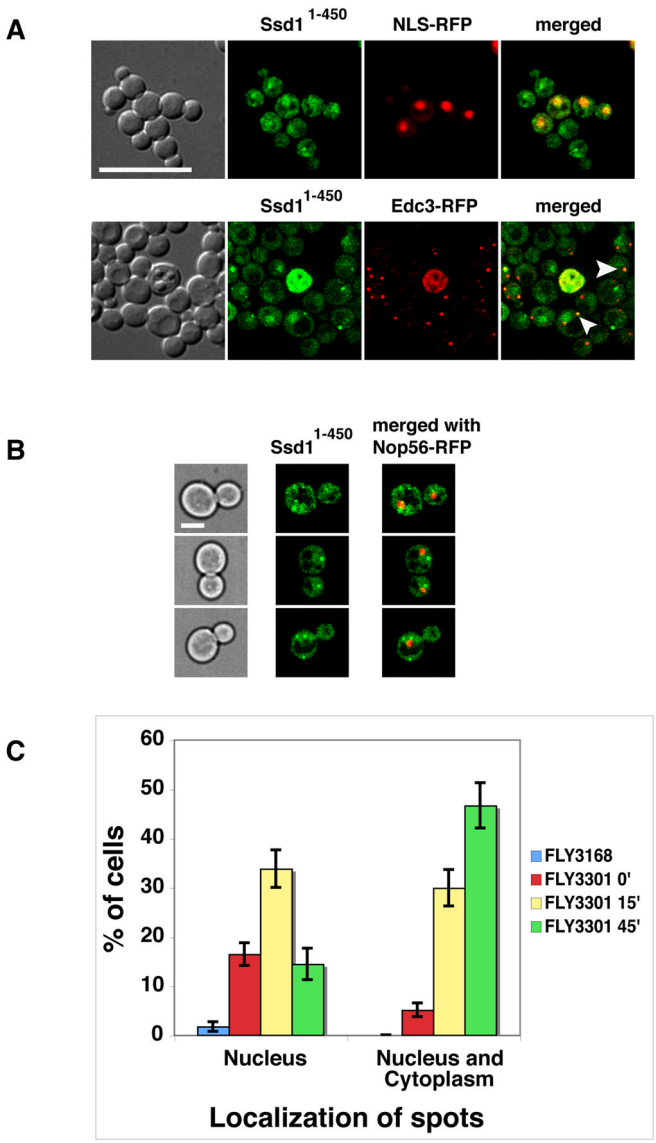

Figure 7. Nuclear and cytoplasmic Ssd1$^{1–450}$ is aberrant in *cbk1* mutants

A) The localization of physiologically expressed Ssd1$^{1–450}$-GFP was monitored in *cbk1*Δ cells (FLY3202) co-expressing an RFP-tagged nuclear marker (NLS-RFP, pKW1219) or P-body protein (Edc3-RFP, pRP1574). Ssd1$^{1–450}$-GFP accumulates in nuclei in *cbk1Δ* cells to the same extent as it does in *CBK1* cells (compare to Fig. 2). In addition, Ssd1$^{1–450}$ localizes to a few nuclear and cytoplasmic puncta in *cbk1Δ* cells. Approximately half of the Ssd1$^{1–450}$ cytoplasmic puncta colocalize with Edc3 (see arrowheads). B) Physiologically expressed Ssd1$^{1–450}$-GFP was monitored in analog-sensitive *cbk1-as* mutants that express the nucleolar marker Nop56-RFP (FLY3301). Micrographs were taken 25 min after addition of the Cbk1-as inhibitor 1NA-PP1. **C**) Graphical representation of the *cbk1-as* data. Upon Cbk1 inhibition, the number of cells with nuclear and cytoplasmic Ssd1$^{1–450}$-GFP puncta increases over time. In the absence of 1NA-PP1, *cbk1-as* cells exhibit enhanced level of nuclear puncta compared to corresponding *CBK1*+ cells (FLY3168). The cells were monitored by spinning disk fluorescence microscopy and all images represent single optical sections. Scale bars = 8 um (A) and 4 um (B).

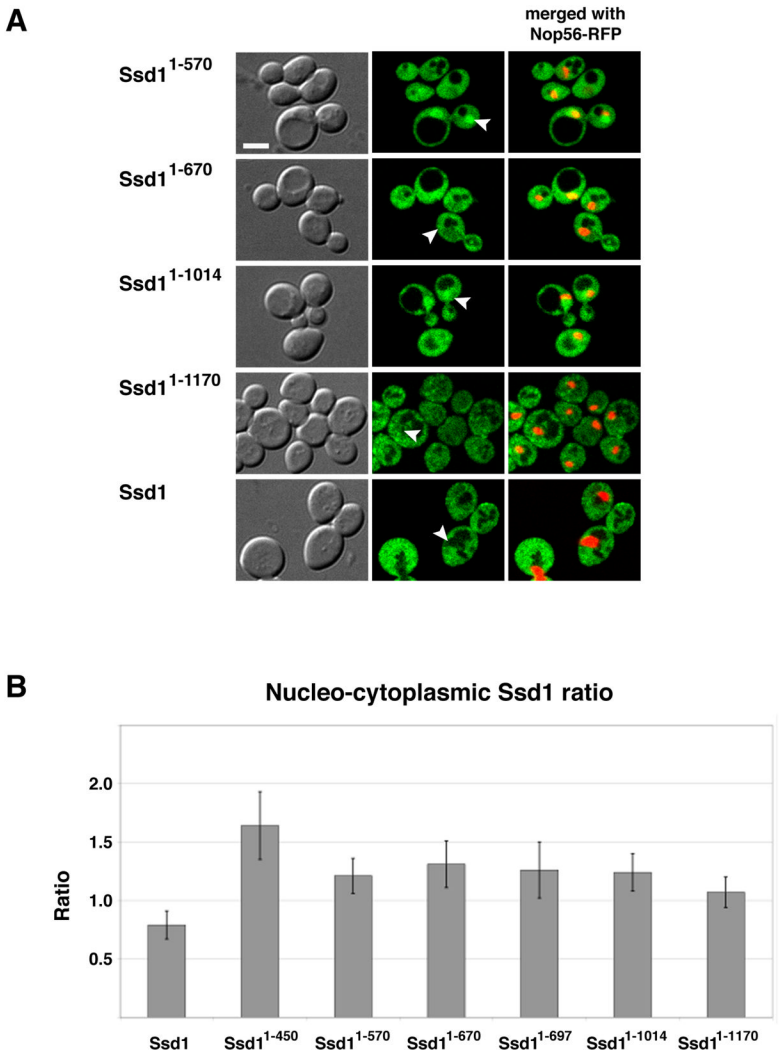

Figure 8. Nuclear localization of Ssd1 C-terminal truncations in wild type cells

A) Physiologically expressed Ssd1$^{1–570}$, Ssd1$^{1–670}$, Ssd1$^{1–1014}$, Ssd1$^{1–1170}$ and full length Ssd1-GFP were monitored in wild type cells co-expressing the nucleolar marker Nop56-RFP. Each image represents a single optical section captured via by spinning disk fluorescence microscopy. Arrowheads indicate nuclei. Scale bar = 4 um. **B)** Mean values and standard deviations of nuclear-cytoplasmic fluorescence ratios were plotted for full length and truncated Ssd1-GFP (n=16–26 cells per strain). Measurements and calculations were performed as described in Material and Methods. Nuclei were defined by NLS-RFP (pKW1219) fluorescence. All truncated Ssd1-GFP proteins accumulate in the nuclei to a greater degree than full length Ssd1 ($p<10^{-7}$). The raw data and p values for statistical significance are provided in Supplement Table 1. See Table I for the strains used in these experiments.

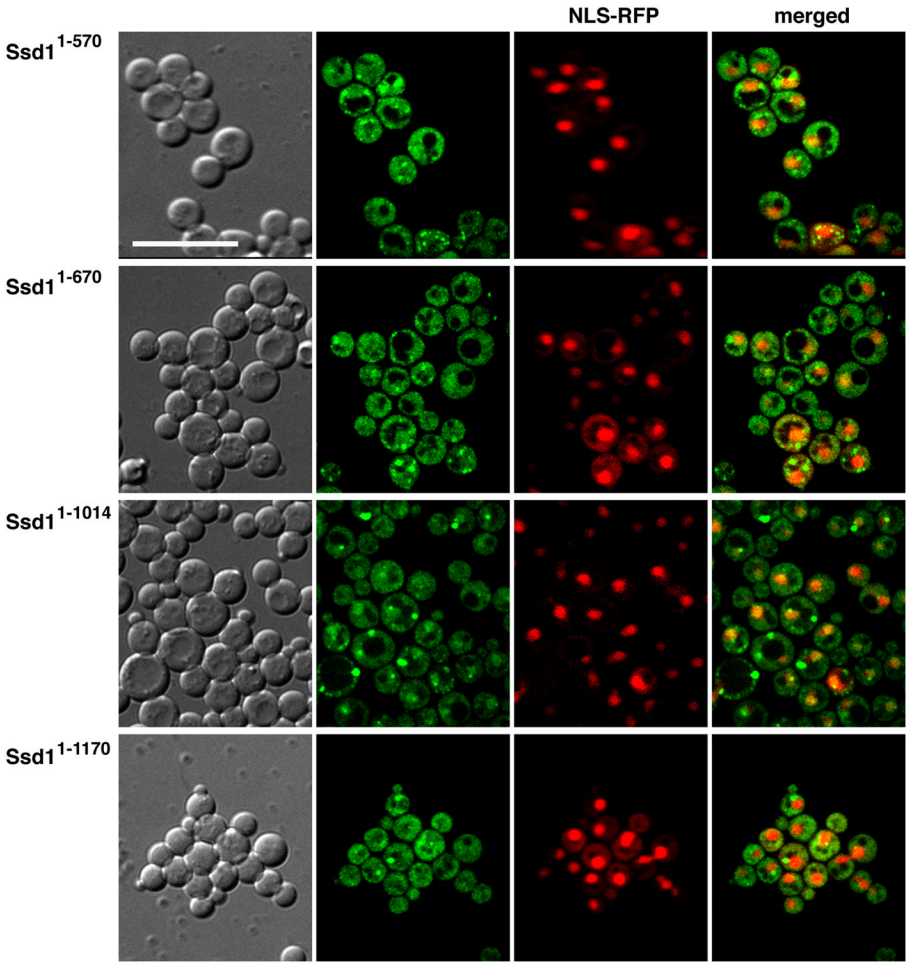

Figure 9. Expression of C-terminal truncations of Ssd1-GFP is not lethal in *cbk1Δ* cells

Physiologically expressed Ssd1$^{1–570}$, Ssd1$^{1–670}$, Ssd1$^{1–1014}$ and Ssd1$^{1–1170}$ were monitored in *cbk1Δ* cells containing the nuclear marker NLS-RFP (pKW1219). None of the truncated Ssd1 proteins is toxic, in contrast to full length Ssd1 (not shown). Truncated Ssd1 proteins localize diffusely to the nucleus and to a few prominent foci, many of which localize in or near the nucleus. Up to 50% of the cytoplasmic puncta co-localize with P-body protein Edc3 (see Supplemental Fig. S3). See Table I for the strains used in these experiments. The cells were monitored by spinning disk fluorescence microscopy and each image represents a single optical section. Scale bar = 8 um.

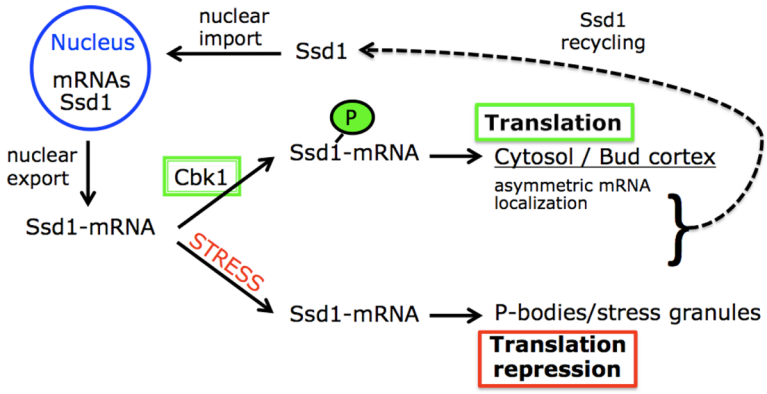

Figure 10. Working model of Ssd1 function
See Discussion for details.

Table I

Yeast strains

Strains	Relevant genotype
FLY1593	MATa SSD1-GFP::KANMX
FLY2184	MATα ssd1Δ::NATMX
FLY2293	MATa ssd1Δ::NATMX cbk1Δ::KANMX
FLY3074	MATα NOP56-RFP::KANMX ssd1Δ::NATMX
FLY3149	MATa SSD1$^{1-697}$-GFP::KANMX
FLY3168	MATa SSD1$^{1-450}$-GFP::KANMX
FLY3172	MATa SSD1$^{1-570}$-GFP::KANMX
FLY3176	MATa SSD1$^{1-670}$-GFP::KANMX
FLY3202	MATa SSD1$^{1-450}$-GFP::KANMX cbk1Δ::KANMX
FLY3206	MATa SSD1$^{1-570}$-GFP::KANMX cbk1Δ::KANMX
FLY3210	MATa SSD1$^{1-670}$-GFP::KANMX cbk1Δ::KANMX
FLY3219	MATα SSD1$^{1-450}$-GFP::KANMX NOP56-RFP::KANMX
FLY3222	MATa SSD1$^{1-570}$-GFP::KANMX NOP56-RFP::KANMX
FLY3225	MATa SSD1$^{1-670}$-GFP::KANMX NOP56-RFP::KANMX
FLY3290	MATa SSD1$^{1-1014}$-GFP::KANMX
FLY3292	MATa SSD1$^{1-1170}$-GFP::KANMX
FLY3301	MATa NOP56-RFP::KANMX SSD1$^{1-450}$-GFP::KANMX cbk1-as-HIS3::cbk1Δ::KANMX
FLY3308	MATα SSD1$^{1-1014}$-GFP::KANMX NOP56-RFP::KANMX
FLY3310	MATa SSD1$^{1-1170}$-GFP::KANMX NOP56-RFP::KANMX
FLY3313	MATα SSD1$^{1-1014}$-GFP::KANMX cbk1Δ::KANMX
FLY3316	MATa SSD1$^{1-1170}$-GFP::KANMX cbk1Δ::KANMX
FLY3365	MATa PAP1-RFP::HIS3 ssd1Δ::NATMX
FLY3433	MATa/α SSD1-GFP::KANMX/SSD1 PAP1-RFP::HIS3/PAP1
FLY3467	MATa SSD1$^{1-697}$-GFP::KANMX PAP1-RFP::HIS3
FLY3795	MATa/α SSD1-GFP::KANMX/SSD1 NOP56-RFP::KANMX/NOP56

Table II

Plasmids

Plasmids	Alias/relevant markers	Source
FLE1019	pAG415-GPDpr-SSD1-GFP	Kurischko et al., 2011
FLE1020	pAG415-GPDpr-SSD1$^{1–520}$-GFP	Kurischko et al., 2011
FLE1057	pENTRY-SSD1	Kurischko et al., 2011
FLE1066	pENTRY-SSD1$^{332–450}$	This work
FLE1067	pAG416-GPDpr-SSD1$^{332–450}$-GFP	This work
FLE1069	pUC19-SSD1	Kurischko et al., 2011
FLE1180	pUC19-SSD1$^{NLS\Delta}$	This work
FLE1204	pENTRY-SSD1$^{1–450\text{-}NLS\Delta}$	This work
FLE1205	pENTRY-SSD1$^{RBD\Delta}$	Kurischko et al., 2011
FLE1210	pAG415-GPDpr-SSD1$^{RBD\Delta}$-GFP	Kurischko et al., 2011
FLE1211	pAG415-GPDpr-SSD1$^{1–450\text{-}NLS\Delta}$-GFP	This work
FLE1212	pENTRY-SSD1$^{NLS\Delta}$	This work
FLE1213	pAG415-GPDpr-SSD1$^{NLS\Delta}$-GFP	This work
FLE1276	pAG415-GPDpr-SSD1$^{RBD\Delta}$-TAP	This work
FLE1277	pAG415-GPDpr-SSD1$^{NLS\Delta}$-TAP	This work
pJK59	pRS416-SEC63-GFP	Fehrenbacher et al., 2002
pKW1219	pRS425-NLS-mRFP	Madrid et al., 2006
pRP1574	pEDC3-chRFP-URA3	Roy Parker, Univ. of Arizona

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

Table III

Oligonucleotides

Oligos	Sequence
FLO499	5′-GAAAGTCTAGAATATAGGAGGAATTTTACGGACACTAATGAGGGTGGTCCCGGTGGTCGGATCCCCGGGTTAATTAA-3′
FLO504	5′-ATTCCTTTGAACAGTAGTGACGATTACCACAACGATGCATCTGTTGGTGGTCCCGGTGGTCGGATCCCCGGGTTAATTAA-3′
FLO505	5′-AGCGGTACATTAGGTTTGTTGAGACCATCCCAACAAGCTAATAGCGGTGGTCCCGGTGGTCGGATCCCCGGGTTAATTAA-3′
FLO506	5′-AACAATTTTCTTTCGAATGAATATTTGGATCAAAAAAATCCGCAAGGTGGTCCCGGTGGTCGGATCCCCGGGTTAATTAA-3′
FLO507	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAATAACGGAGGTGGACGTAAGTCCCTATT-3′
FLO508	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCAACAGATGCATCGTTGTGGTAATCGTCACTACTGTTCAAAGGAAT-3′
FLO520	5′-AGAAGATACGCTGATCATGTCGTTCATAGGCAATTAAAGGCCGTTGGTGGTCCCGGTGGTCGGATCCCCGGGTTAATTAA-3′
FLO521	5′-TATAGAAATTCCATTAAGAACAAATTCAGATCCACAGCCGCTGAGGGTGGTCCCGGTGGTCGGATCCCCGGGTTAATTAA-3′
FLO525	5′-GACCACAAAGTATTCAATCTGCCCTCCGCTTCGTAAAGGGTTACGATTTGCCAGATGAAGTTTTCGATGAAAATGAAAAGAGA CCATCAAAGAA
FLO526	5′-ATCCAGCTTGTAACACAAAATTCTCACCTAATGGTAAAATGTCGCTACACGCGAAAGAGCATATGTAGAGAGAATCTCGTAAAC GTTTTACTGCT
FLO527	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAACAAAATGTCTAAAAATAGCAACGTTAACA-3′
FLO534	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCACCTTCAACTTCAACATCGTCATT-3′
FLO635	5′-TCCAAAGCCAAAGGCCAACG-3′
FLO636	5′-CCGATCTTCCAGCCCTTAACG-3′
FLO639	5′-ATGCTTCAATCCGTTGT-3′
FLO640	5′-CGTATTTGGGGTGACTG-3′