Altered nuclear tRNA metabolism in La-deleted *Schizosaccharomyces pombe* is accompanied by a nutritional stress response involving Atf1p and Pcr1p that is suppressible by Xpo-t/Los1p

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**ABSTRACT** Deletion of the *sla1* gene, which encodes a homologue of the human RNA-binding protein La in *Schizosaccharomyces pombe*, causes irregularities in tRNA processing, with altered distribution of pre-tRNA intermediates. We show, using mRNA profiling, that cells lacking *sla1* have increased mRNAs from amino acid metabolism (AAM) genes and, furthermore, exhibit slow growth in Edinburgh minimal medium. A subset of these AAM genes is under control of the AP-1–like, stress-responsive transcription factors Atf1p and Pcr1p. Although *S. pombe* growth is resistant to rapamycin, *sla1*-Δ cells are sensitive, consistent with deficiency of leucine uptake, hypersensitivity to NH4, and genetic links to the target of rapamycin (TOR) pathway. Considering that perturbed intranuclear pre-tRNA metabolism and apparent deficiency in tRNA nuclear export in *sla1*-Δ cells may trigger the AAM response, we show that modest overexpression of *S. pombe los1* (also known as Xpo-t), encoding the nuclear exportin for tRNA, suppresses the reduction in pre-tRNA levels, AAM gene up-regulation, and slow growth of *sla1*-Δ cells. The conclusion that emerges is that *sla1* regulates AAM mRNA production in *S. pombe* through its effects on nuclear tRNA processing and probably nuclear export. Finally, the results are discussed in the context of stress response programs in *Saccharomyces cerevisiae*.

**INTRODUCTION**

The protein La is a multifunctional RNA-binding protein (Maraia, 2001; Wolin and Cedervall, 2002; Bayfield et al., 2010) that serves as a chaperone for precursor-tRNAs (pre-tRNAs) during the intranuclear phase of their maturation, which includes folding, 5′ and 3′ RNA cleavages, multiple modifications, and CCA addition to the processed 3′ end (Maraia and Lamichhane, 2011). La is ubiquitous in eukaryotes and essential in mammals (Park et al., 2006) but nonessential in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, although its deletion causes aberrancies relative to the normal pattern of pre-tRNA intermediates (reviewed in Maraia and Lamichhane, 2011). La is ubiquitous in eukaryotes and essential in mammals (Park et al., 2006) but nonessential in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, although its deletion causes aberrancies relative to the normal pattern of pre-tRNA intermediates (reviewed in Maraia and Lamichhane, 2011). La is ubiquitous in eukaryotes and essential in mammals (Park et al., 2006) but nonessential in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, although its deletion causes aberrancies relative to the normal pattern of pre-tRNA intermediates (reviewed in Maraia and Lamichhane, 2011). La is ubiquitous in eukaryotes and essential in mammals (Park et al., 2006) but nonessential in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, although its deletion causes aberrancies relative to the normal pattern of pre-tRNA intermediates (reviewed in Maraia and Lamichhane, 2011). La is ubiquitous in eukaryotes and essential in mammals (Park et al., 2006) but nonessential in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, although its deletion causes aberrancies relative to the normal pattern of pre-tRNA intermediates (reviewed in Maraia and Lamichhane, 2011). La is ubiquitous in eukaryotes and essential in mammals (Park et al., 2006) but nonessential in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, although its deletion causes aberrancies relative to the normal pattern of pre-tRNA intermediates (reviewed in Maraia and Lamichhane, 2011). La is ubiquitous in eukaryotes and essential in mammals (Park et al., 2006) but nonessential in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, although its deletion causes aberrancies relative to the normal pattern of pre-tRNA intermediates (reviewed in Maraia and Lamichhane, 2011). La is ubiquitous in eukaryotes and essential in mammals (Park et al., 2006) but nonessential in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, although its deletion causes aberrancies relative to the normal pattern of pre-tRNA intermediates (reviewed in Maraia and Lamichhane, 2011). La is ubiquitous in eukaryotes and essential in mammals (Park et al., 2006) but nonessential in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, although its deletion causes aberrancies relative to the normal pattern of pre-tRNA intermediates (reviewed in Maraia and Lamichhane, 2011).
distribution of pre-tRNA intermediates that can be rescued by human La (Van Horn et al., 1997; Intine et al., 2000, 2002; Huang et al., 2006; Bayfield et al., 2007; Bayfield and Maria, 2009).

In addition to function in translation, tRNAs also serve widely as metabolic sensors (Soll, 1993; Banerjee et al., 2010; Phizicky and Hopper, 2010). Pathways for biogenesis and intracellular transport of tRNAs have been linked to growth, nutrition, and stress responses (Phizicky and Hopper, 2010). In S. cerevisiae, accumulation of aberrant pre-tRNAs that cannot be processed or defects in their nuclear export stimulate Gcn4p (Qiu et al., 2000), a master transcription activator of amino acid metabolism (AAM) and other genes (Natarajan et al., 2001). However, whereas Gcn4p induction due to some stress pathways depends on the kinase Gcn2p (Hinnebusch, 2005), the GCN4-mediated response to aberrant pre-tRNA metabolism, termed nuclear surveillance, is independent of GCN2 (Qiu et al., 2000). S. cerevisiae and S. pombe La proteins can offset the nuclear surveillance response and 3′ end-mediated decay of aberrant pre-tRNAs (Anderson et al., 1998; Huang et al., 2006; Copela et al., 2008; Ozanick et al., 2009; reviewed in Maria and Lamichhane, 2011).

The DNA damage response program also links pre-tRNA metabolism to Gcn4p (Weinert and Hopper, 2007). In this case, intron-containing pre-tRNAs accumulate in the nucleus due to altered shuttling of Los1p, the major nuclear exporter of tRNA (Ghavidel et al., 2007). This pathway is distinguished by the fact that, in contrast to tRNA splicing in vertebrates, which is nuclear (Melton et al., 1980; Lund and Dahlberg, 1998; Paushkin et al., 2004), tRNA splicing in S. cerevisiae occurs in the cytoplasm (Yoshihisa et al., 2003, 2007). Significantly, nuclear export of intron-containing pre-tRNAs appears to prevent their signaling activity (reviewed in Phizicky and Hopper, 2010; Pierce et al., 2010). Subcellular location of tRNA splicing has not been determined for S. pombe, in which potential relationships between Los1p/Xpo-t, nuclear accumulation of intron-containing pre-tRNAs, and links to stress are also unknown.

S. cerevisiae Gcn4p is related to c-jun, a component of mammalian AP-1 bZIP transcription factor (TF; Vogt et al., 1987). Whereas Gcn4p is a single polypeptide and AP-1 is a heterodimer encoded by c-jun and c-fos (Curran and Franza, 1988), they recognize similar DNA sequences and can functionally replace each other in vivo (Struhl, 1988; Oliviero et al., 1992). S. pombe has no Gcn4p, although it has AP-1-like activity (Jones et al., 1988). The bZIP protein ATF resembles Gcn4p (Kim and Struhl, 1995). S. pombe Atf1p (a.k.a., mts1/gad7) and Pcr1p form a heterodimer with AP-1/Gcn4p-like activity (Takeda et al., 1995; Kanoh et al., 1996). Although S. pombe lacking atf1+ or pcr1+ share stress phenotypes, they also show distinct deficiencies (Kanoh et al., 1996) in meiosis, mating, and sporulation (Wahls and Smith, 1994; Kon et al., 1997; Yamada et al., 2004), suggesting activity as a heterodimer or independent of each other.

There has been no reported mRNA profiling or growth phenotypes for La-deleted S. cerevisiae or S. pombe (for synthetic interactions see Yoo and Wolin, 1997; Pannone et al., 1998; Copela et al., 2006). Here we report that sla-1Δ cells exhibit slow growth in Edinburgh minimal media (EMM), up-regulation of AAM genes, and other stress phenotypes mediated via atf1+ and pcr1+. A major component of growth inhibition in EMM is due to hypersensitivity to NH4Cl. Consistent with involvement of atf1+ and pcr1+ in nitrogen metabolism and mating, sla-1Δ cells also up-regulate nitrogen and mating genes. Leucine auxotrophic sla-1Δ cells are deficient in leucine uptake and hypersensitive to rapamycin, supported by genetic links to the TOR pathway (Weisman et al., 1997; Valenzuela et al., 2001; Cherkasova and Hinnebusch, 2003; Fingar and Blenis, 2004; Wallischleger et al., 2006). We tested the idea that aberrant nuclear pre-tRNA metabolism in sla-1Δ cells may contribute to the stress responses. Ectopic expression of los1+, which encodes a major tRNA exporter, suppresses the decrease in pre-tRNAs in sla-1Δ cells, slow growth, and up-regulation of AAM mRNAs. Thus a genetic response to altered nuclear pre-tRNA metabolism in S. pombe cells lacking sla1+ leads to nutritional sensitivity, growth inhibition, and induction of AAM mRNAs that is offset by Los1p/Xpo-t.

RESULTS
Deletion of sla1+ causes stress-response phenotypes: up-regulation of AAM genes, slow growth in Edinburgh minimal media, and heat sensitivity

Microarray analysis was done on RNA from our wild-type (WT) strain (yAS99, leu1-32 ura4-Δ; Table 1) and its isogenic sla1-Δ strain (yAS113, sla1-Δ leu1-32 ura4-Δ) grown in the standard rich media used for S. pombe—yeast extract with supplements (YES) media (Supplemental Figure S1). This revealed that sla1-Δ cells have elevated levels of a set of mRNAs that significantly overlap (P = 3e−12) with genes in an AAM module previously defined (Tanay et al., 2005). Additional microarray analysis showed that most of the same AAM and other mRNAs were found significantly elevated in the sla1-Δ cells when grown in EMM (Supplemental Figure S1). S. pombe AAM genes are enriched for an upstream DNA sequence, TGACT, which is similar to the binding sites for budding yeast Gcn4p (see supporting Figure 6 in Tanay et al., 2005).

We examined some of the S. pombe AAM mRNAs by Northern analysis: C132.04 (glutamate dehydrogenase, gdh2; involved in aspartate, proline, nitrogen, and glutamate metabolism), ppc1+ (involved in oxidative stress response), C1105 (lysine biosynthesis), and C56E4.03 (amino acid aminotransferase). By comparing to rRNA, which provides a loading control, this confirmed the up-regulation in sla1-Δ detected by microarray and showed that ectopic expression of sla1+ from a plasmid reversed it (Figure 1A).

Considering up-regulation of AAM genes, it might be expected that sla1-Δ cells may display a growth advantage in conditions that cause amino acid starvation, such as in 3-aminoimidazole (3AT; Struhl and Davis, 1977). However, the slow growth of sla1-Δ relative to wild type was unaffected by 3AT (data not shown). Moreover, although WT and sla1-Δ cells grew comparably in rich (YES) media, sla1-Δ exhibited slow growth in EMM, the standard minimal media for S. pombe, which was relieved by ectopic sla1+ on a plasmid (10-fold dilutions; Figure 1B). EMM is defined media that contains dextrose, amino acids, vitamins, and other supplements that do not cause starvation-induced stress responses such as mating or sporulation (Forsburg, 2003). We also deleted sla1+ in other genetic backgrounds, and they revealed slow growth in EMM (but not YES) relative to their isogenic sla1+ parent strains (Figure 1, B vs. D, Supplemental Data, and Supplemental Figure S2). Strain-specific growth variability in EMM in each strain was worsened by sla1+ deletion (Supplemental Figure S2).

Human La (hLa) also suppressed the growth deficiency of sla1-Δ in EMM (Figure 1B). hLaSSΔ5M, which lacks a short basic motif that inhibits pre-tRNA processing in S. pombe (Intine et al., 2000), reproducibly suppressed sla1-Δ slow growth a bit more than hLa (data not shown). Because human La can suppress the pre-tRNA processing defects of sla1-Δ cells and functionally reverse pre-tRNA processing and nuclear trafficking–related phenotypes (Intine et al., 2000, 2002; Huang et al., 2006; Bayfield et al., 2007; Bayfield and Maria, 2009), this suggested that the slow growth of sla1-Δ is due to defective pre-tRNA metabolism.
Sla1p acts in part via atf1+ and pcr1+ to down-regulate expression of AAM genes and promote growth in EMM and at elevated temperature

As noted, S. pombe and S. cerevisiae AAM genes share similar upstream DNA, and Atf1p and Pcr1p are candidate Gcn4p homologues in S. pombe. We deleted sla1- in existing atf1-Δ, pcr1-Δ, and parent strains (Jia et al., 2004) and examined AAM mRNA levels by Northern analysis (Figure 1C, each loaded at 1x and 2x amounts). Using rpl8+ mRNA as a loading control with sequential probens of the same blots, we found that atf1+ or pcr1+ deletion in sla1+– replicaets cells decreased C132.04 and ppr1+ mRNA expression comparably relative to the WT parental strain (SPJ83, lanes 1–6), whereas deletion of both atf1+ and pcr1+ did not further decrease these mRNAs (lanes 7 and 8). Thus Atf1p/Pcr1p appears to drive expression of these genes in rich (YES) media (Figure 1C). Similar results were found in EMM, consistent with our microarray and Northern analyses (data not shown).

Although atf1+ or pcr1+ deletion also decreased C132.04 and ppr1+ mRNAs in sla1-Δ cells, the negative effects on these mRNAs were greater for atf1+ than pcr1+ (Figure 1C, lanes 9–14). Quantification (data not shown) revealed that deletion of atf1+ or pcr1+ in sla1- decreased these mRNAs ~1.7-fold more than their deletion in the WT (SPJ83). Whereas C1105 mRNA is negatively affected by sla1+, it appears to be unaffected by atf1+ or pcr1+ deletion (Figure 1C). Ispo6+ (induced during sexual differentiation or nitrogen starvation) was elevated in sla1-Δ cells relative to WT (Figure 1C; compare lanes 1 and 2 with 9 and 10), confirming the microarray data, and sensitive to atf1+ deletion in sla1- but less so to pcr1+ deletion (Figure 1C, lanes 9–14). We conclude that up-regulation of a subset of AAM genes in sla1-Δ cells depends on Atf1p and Pcr1p, in some cases to different degrees, whereas others are up-regulated independent of Atf1p and Pcr1p, suggesting that other transcription factors are involved.

We next asked whether deletion of atf1+ or pcr1+ suppresses the slow growth of sla1-Δ (Figure 1D). Deletion of atf1+ or pcr1+ from sla1-Δ or its isogenic WT strain improves growth in EMM (Figure 1D), consistent with roles for atf1+ and pcr1+ in general growth inhibition on EMM. Deletion of sla1-Δ had little effect on atf1+ or pcr1+ growth, consistent with the idea that atf1+ and pcr1+ antagonize growth derepression by sla1+.

Whereas sla1-Δ cells grow normally in YES, they exhibit slow growth at 37°C, and this inhibition is suppressed by deletion of either atf1+ or pcr1+ (Figure 1D).

slat+ cells are defective in leucine uptake and hypersensitive to NH4Cl and rapamycin

We analyzed different ingredients of YES and EMM for effects on sla1+– cell growth (data not shown) and found that the NH4Cl in EMM was inhibitory. Replacing NH4+ with proline alleviated growth inhibition of sla1+– cells (Figure 2A), which is intriguing since proline is believed to constitute a relatively poor nitrogen source (Weisman et al., 2005, 2007). Addition of NH4+ to YES also resulted in very significant growth inhibition of sla1+ (Figure 2A). Thus growth of sla1+– cells is highly sensitive to NH4Cl.

Further analysis suggested impaired leucine uptake by our sla1+– cells, which carry metabolic markers leu1- and ura4+. Providing leu1+ (on pRep3X plasmid) suppressed sla1+ slow growth in EMM (Figure 2B), whereas providing excess leucine in the media did not (data not shown). By contrast, providing ura4+ suppressed the growth phenotype to a far less extent than did leu1+ on the otherwise identical plasmid (Figure 2C).

The data suggested deficient leucine uptake by sla1+– cells, worthy of more direct examination. Leucine uptake is regulated by the TOR pathway (Weisman et al., 2005). Therefore, as a control, we

![Table 1: Yeast strains.](https://example.com/table1.png)
created a tor1Δ mutant in the same genetic background that was expected to be deficient in leucine uptake (Weisman et al., 2005). We measured uptake in EMM containing either NH₄⁺ or proline (Sychrova et al., 1989; Karagiannis et al., 1999; Matsumoto et al., 2002; Weisman et al., 2005). Figure 2D (top) shows that sla1Δ cells are quite defective in leucine uptake in EMM (containing NH₄⁺), even relative to the tor1Δ mutant. The rate of leucine uptake by sla1Δ cells appeared to be less compromised in proline than in NH₄⁺ (Figure 2D; compare top and bottom). Note that whereas decreased leucine uptake characterizes sla1Δ cells, other limitations and/or parameters may contribute to their slow growth.

Rapamycin-mediated inhibition of TOR is manifested by growth inhibition of S. cerevisiae and mammalian cells, including of tu

themselves found that pRep4X-sla1Δ in sla1Δ cells produces fourfold higher Sla1p levels than produced from the chromosomal sla1Δ locus (R. V. Intine and R. J. Maria, unpublished results). When sla1Δ was grown in EMM lacking thiamine, minimal complementation of rapamycin sensitivity by ectopic sla1Δ was observed relative to WT (Figure 3A, 3). Thiamin significantly improved complementation of rapamycin sensitivity by ectopic sla1Δ (Figure 3A, 5). The data suggest that S. pombe growth inhibition by rapamycin is sensitive to Sla1p levels.

Different laboratory strains of S. pombe vary in genetic polymorphisms (Iben et al., 2011) and sensitivity to NH₄⁺ versus proline (e.g., see Figure 1, B vs. D, and Supplementary Figure S2). To examine rapamycin sensitivity in another genetic background, we deleted sla1Δ from SPG17 (Table 1), a laboratory “wild-type” strain (Grewal and Klar, 1997; Irvine et al., 2009; Smith et al., 2010). Figure 3B shows that the sla1Δ-deleted SPG17, designated CY1604, is sensitive to rapamycin relative to its parent SPG17 and to CY1627, in which sla1Δ was reintroduced to its chromosomal locus.

It is remarkable that, whereas leu1Δ rescues the slow growth phenotype of sla1Δ mutants in EMM with NH₄⁺ (Figure 2B), leu1Δ does not rescue the rapamycin sensitivity (Figure 3C, 2 and 3). It is also notable that sla1Δ cells appear less sensitive to rapamycin in EMM with proline than with NH₄⁺ (Figure 3C, 2–5), suggesting that NH₄⁺ contributes to the sensitization of sla1Δ cells to rapamycin and that Sla1p promotes leucine uptake and rapamycin resistance via distinct mechanisms.

Because amino acid and tRNA metabolism are altered in sla1Δ cells we expected that slow growth may be accompanied by decreased protein synthesis. We therefore measured 35S-methionine (35S-met) incorporation into protein during log-phase growth in EMM with NH₄⁺ or proline (Figure 3D). Quantitation revealed that 35S incorporation was lower in sla1Δ than in WT in EMM with NH₄⁺ but not with proline (Figure 3D; see normalized values under lanes, left), consistent with sla1Δ growth in these media. We found no difference in 35S-met uptake between sla1Δ and WT measured under the same conditions as 35S-met incorporation (data not shown). Thus 35S-met incorporation in sla1Δ reflects decreased translation rather than limitation of methionine. Slow growth coupled with decreased translation in sla1Δ cells is consistent with involvement of TOR signaling.
Genetic interactions between sla1+ and TOR

S. pombe has two TOR kinases, Tor1p and Tor2p, and whereas tor2+ is essential for vegetative growth, tor1+ is nonessential but is required for normal responses to starvation and other stress (Kawai et al., 2001; Weisman and Choder, 2001). As noted earlier, leu1 mutants are sensitive to rapamycin, dependent on inhibition of tor1- and tor2-dependent amino acid uptake (Weisman and Choder, 2001). This genetic interaction suggests that sla1+ and tor1+ act in parallel to promote growth in EMM. The cumulative data support the existence of genetic interactions between sla1+ and the TOR pathway.

Ectopic expression of los1+ suppresses slow growth and up-regulation of AAM genes in sla1+ cells

Accumulation of aberrant pre-tRNA activates a process termed nuclear surveillance in S. cerevisiae via GCN4 derepression (Qiu et al., 2000). This GCN4-mediated response is reversed by ectopic expression of either RNase P, which processes pre-tRNAs at their 5′ ends, or LOS1, the major tRNA nuclear export factor, and further consistent with this, los1-Δ cells exhibit derepression of GCN4 (Qiu et al., 2000). Moreover, this response can be offset by overexpression of the S. cerevisiae La-homologous protein Lhp1 (Anderson et al., 1998; Calvo et al., 1999). Because sla1+ cells exhibit irregularities in pre-tRNA processing and our data suggest that los1+ activity is limiting in these cells (see later discussion), we asked whether overexpression of los1+ in S. pombe los1+ would offset (supress) their slow growth. Because LOS1 overexpression can be severely toxic (Hellmuth et al., 1998; Sopko et al., 2006), we titrated the activity of the nmt1 promoter driving its expression, with thiamine, including 0.05 μM thiamine, an intermediate level that partially represses nmt1 promoter activity (Javerzat et al., 1996). Figure 5A shows that at 0.05 μM thiamine, los1+ suppresses the slow growth of sla1+ cells. Because 15 μM thiamine is used widely in S. pombe with no reports of toxicity, the loss of suppression in Figure 5A, panel 4 versus panel 3, appears to be due to loss of los1+ expression as a result of more efficacious repression of the nmt1 promoter. No suppression is seen without thiamine (Figure 5A, 2), likely due to toxicity of high-level los1+ overexpression (Hellmuth et al., 1998; Sopko et al., 2006), since under these conditions, los1+ expression is indeed much higher (Figure 5B).

We used Northern analysis to confirm that 0.05 μM thiamine partially repressed expression of los1+ from the ectopic nmt1 promoter (Figure 5B). As expected in no thiamine, los1+ mRNA was expressed at high levels from nmt1-los1+ in the WT and sla1+ cells (Figure 5B, lanes 5–8 vs. 1–4). Using rpl8+ mRNA as a loading control in Figure 5B, middle, we see that lanes 9–16 show that at 0.05 μM thiamine, los1+ mRNA levels were higher in nmt1-los1+ cells (lanes 13–16) than with empty vector (+V, lanes 9–12). Quantitation of the los1+:rpl8+ mRNA ratios in lanes 11/12 and 15/16 confirmed this (Figure 5B, numbers under lanes). The sla1+ cells with nmt1-los1+ expressed los1+ mRNA at 1.9-fold higher levels than in the same cells with empty vector (1.3/0.7 = 1.9-fold; see lanes 15 and 16 vs. 11 and 12, quantitation under lanes). Thus a near-twofold increase in los1+ expression appears to be sufficient to suppress slow growth of sla1+ cells in EMM.

We wanted to determine whether nmt1-los1+–mediated suppression of slow growth was accompanied by suppression of AAM mRNA levels. We examined AAM mRNAs from cells transformed with empty vector or ectopic nmt1-los1+ (Figure 5C), grown in 0.05 μM thiamine and under the same conditions as for Figure 5, A and B. Lanes 1–4 of Figure 5C, top, show that ectopic nmt1-los1+ in WT cells does not affect C132.04 mRNA expression. In striking contrast, the highly elevated C132.04 mRNA in sla1+ cells (Figure 5C, lanes 5 and 6) was completely repressed by ectopic nmt1-los1+ (lanes 7 and 8). Ectopic nmt1-los1+ also and tsc2+ negatively regulate tor2+, and their disruption leads to amino acid uptake deficiency (van Slegtenhorst et al., 2004; Weisman et al., 2005). We therefore deleted tsc1+ in our WT and sla1+ cells (Figure 4B). The tsc1+ mutant exhibited slow growth in NH4+ but not proline, and this phenotype was exacerbated in the sla1+ cells (Figure 4B). This suggests that sla1+ and tsc1+ act in parallel to promote growth in EMM. The cumulative data support the existence of genetic interactions between sla1+ and the TOR pathway.

FIGURE 2: sla1+ cells are sensitive to NH4+ and deficient in leucine uptake. (A–C) Cells were grown in liquid media as indicated, and then 10-fold serial dilutions were spotted on plates containing media indicated above and incubated at 32°C for 2–6 d. (A) Growth of the sla1+ mutant is inhibited by NH4+. Strains: yAS99 (WT) and yAS113 (sla1+). (B) Ectopic leu1+, but not ura4+, suppresses slow growth of sla1+ cells on EMM. Strains: yAS99 (WT) and yAS113 (sla1+) transformed with empty pRep3X (leu1+) or pRep4X (ura4+). The difference in growth of strains in A and C is likely due to partial suppression by ura4+ gene. (D) sla1+ cells are deficient in leucine uptake. Cells were grown in EMM with either NH4+ or proline as nitrogen source and transferred to the same media with 3H-leucine. Strains: yAS99 (WT), yAS113 (sla1+), CY1472 (tor1+), and CY1473 (sla1+ tor1+).
suppressed the up-regulation of C1105 and ppr1′ mRNA in sla1′ (Figure 5C). Therefore overexpression of los1′ suppresses both slow growth and up-regulation of AAM genes in the sla1′ mutant. As data to be presented suggest, ectopic nmt1-los1′ also complements the tRNA export deficiency in these same sla1′ cells in which it suppresses the AAM mRNA up-regulation. nmt1-los1′ also suppresses up-regulation of AAM genes in the sla1′ los1′ double mutant (Figure 5C, lanes 13–16). Figure 5 strengthens the idea that the stress-related growth inhibition and AAM gene up-regulation phenotypes of sla1′ are caused by defects in nuclear pre-tRNA metabolism. Consistent with this, deletion of sla1′ and los1′ have additive effects on growth (Figure 6A), verifying genetic interaction.

**FIGURE 3:** sla1′ cells are sensitive to rapamycin and exhibit decreased protein synthesis. (A, B) Cells were grown in liquid EMM medium with essential supplements, and 10-fold serial dilutions were spotted on the plates containing different media as indicated and incubated at 32°C for 2–6 d. (A) yAS99 (WT) and yAS113 (sla1′) cells transformed with empty pRep4X vector (V) or pRep4X carrying sla1′ as indicated to the left were spotted as 10-fold serial dilutions. (B) Rapamycin sensitivity of an independent sla1′ strain derived from SPG17 is complemented by introduction of chromosomal copy of sla1′. (C) Rapamycin sensitivity of sla1′ cells is independent of leucine auxotrophy. WT and sla1′ strains were transformed with pRep3X (leu1′) and grown in EMM containing either NH₄⁺ or proline as indicated. (D) General translation is reduced in the sla1′ mutant. Cells were grown in EMM with NH₄⁺ or 10 mM proline as the nitrogen source in the presence of [35S]-methionine. [35S] incorporation was quantitated in each lane of the autoradiograph per unit of total protein based on scanning the Coomassie-stained gel and normalized to 1.0 as reflected below the lanes. The WT and sla1′ strains used are as in A and B.

Ectopic expression of los1′ increases low pre-tRNA levels in sla1′ cells and suppresses imbalance of pre-tRNA intermediates

Given the foregoing findings that reveal a relationship between los1′ and sla1′, it might be expected that ectopic los1′ would affect the pattern of pre-tRNAs in sla1′ cells. We assessed this using the same RNA samples in Figure 6B as used for Figure 5C. We examined the intron-containing pre-tRNA^CUU, which is a standard to follow pre-tRNA metabolism in sla1′ cells (Van Horn et al., 1997; Intine et al., 2000; 2002; Huang et al., 2006; Bayfield et al., 2007). An intron probe detects pre-tRNA^CUU intermediates that differ by whether or not their 5′ leaders and/or 3′ trailers have been removed (Van Horn et al., 1997; Huang et al., 2006; Bayfield and Maraia, 2009). The upper band represents nascent pre-tRNA that contains an intact 5′ leader and 3′ trailer. The lowest band has lost both the 5′ leader and the 3′ trailer. The middle band can be a mix of species that lack either an intact 5′ leader or the 3′ trailer, as indicated to the right of Figure 6B, including those that have been nibbled by 3′ exonucleases (Maraia and Lamichhane, 2011). The uppermost band does not accumulate as an intact species in sla1′ cells due to instability (Van Horn et al., 1997; Intine et al., 2000; 2002; Huang et al., 2006; Bayfield et al., 2007; reviewed in Maraia and Lamichhane, 2011). Subtle mobility differences of upper and middle bands can be best appreciated in the Figure 6B, top, by comparing lanes 4/5 and 8/9.

Los1p is a major nuclear exporter of tRNA in yeast; its vertebrate homologue is exportin-t (Xpo-t), and intron-containing pre-tRNAs are substrates for nuclear export by Los1p/Xpo-t (reviewed in Hopper, 2006). Deficiencies in this export pathway are reflected by alteration of the pattern of pre-tRNA intermediates because Los1p/Xpo-t prefers to bind end-processed tRNA species, that is, the intron-containing lower band (L) in Figure 6B. Accumulation of the unspliced L band in los1′ mutants (Hopper et al., 1980; Hurt et al., 1987) reflects that tRNA splicing occurs in the cytoplasm of S. cerevisiae (Yoshihisa et al., 2003; Hopper, 2006). Thus a distinctive pattern of intron-containing pre-tRNAs is observed in cells lacking Los1p because its favored ligand, pre-tRNA with matured 5′ and 3′ ends, specifically accumulates (Arts et al., 1998; Lipowsky et al., 1999; also see Sarkar and Hopper, 1998; Grosshans et al., 2000; Hopper and Shaheen, 2008). We quantified the ratio of the bottom to top or middle bands in Figure 6B (top, ratios given under the lanes). Although this ratio is ~0.3 in wild-type cells, set as the control value of 1.0 and 0.93 in lanes 1 and 2 respectively, it increases 2.64-fold in our los1′ cells (compare lanes 1 and 2 with 9 and 10). Moreover, the L band is depleted in lanes...
11 and 12 relative to 9 and 10, and the ratio is more similar to WT, indicating that in 0.05 μM thiamine, nmt1Δloss1Δ promotes tRNA export. The data provide evidence that our los1-Δ cells are indeed defective for tRNA export as expected and that tRNA splicing occurs in the cytoplasm of S. pombe, consistent with other data (Intine et al., 2002; Bayfield et al., 2007). It is remarkable that this ratio increases 2.6-fold in los1-Δ cells (lanes 5 and 6), suggesting functional limitation of Los1p-mediated tRNA export activity in these cells. Of importance, this ratio normalizes in sla1-Δ cells to near WT levels upon expression of nmt1-loss1Δ in 0.05 μM thiamine (lanes 7 and 8).

The ratio and abundance of the pre-tRNA5′-CUU intermediates differ in sla1-Δ and WT cells (Figure 6B, lanes 1/2 and 5/6). The top band is diminished due to lack of the stabilizing effects of La protein in sla1-Δ cells. The L band is relatively prominent in sla1-Δ cells (Figure 6B, compare lanes 6 and 2). The high ratio of the L/M bands in lanes 5 and 6 relative to lanes 7 and 8 provides evidence that nuclear export of the L species is limited in sla1-Δ cells. Furthermore, ectopic los1-Δ unexpectedly increased the amount of the M species pre-tRNA in sla1-Δ cells to a level that more resembles that in the WT cells (Figure 6B, lanes 1/2 and 7/8). The unexpected increase of the M band by ectopic los1-Δ in lanes 7 and 8 relative to 5 and 6 suggests that Los1p has a stabilizing effect on 5′ leader–containing, 3′ end–processed pre-tRNA5′-CUU in sla1-Δ cells. The cumulative data argue that ectopic los1-Δ helps alleviate response to aberrant nuclear pre-tRNA metabolism in sla1-Δ cells.

We stripped the blot in Figure 6B, top (data not shown), and rehybridized with a probe specific for the 5′ leader of pre-tRNA5′-CUU (Figure 6B, middle). This revealed that the 5′ leader–containing species is at relatively low levels in sla1-Δ cells (lanes 5 and 6) but more prominent in the sla1-Δloss1Δ cells (Figure 6B, middle, lanes 7 and 8; see quantitation for loading by US small nuclear RNA [snRNA] levels under the bottom lanes). These data suggest that overexpression of los1Δ stabilizes pre-tRNA in the absence of Sla1p, potentially compensating, at least in part, for the lack of Sla1p. Based on gel migration and binding properties of Los1p Xpo-t (see Discussion), we suspect that the los1Δ-stabilized pre-tRNA in lanes 7 and 8 contains 3′ CCA, consistent with 3′ exonucleases mediating CCA turnover in S. cerevisiae nuclei (Copela et al., 2008).

**DISCUSSION**

Here we report consequences of disrupting the gene encoding the S. pombe La protein on genome-wide mRNA expression and associated metabolic parameters. The S. pombe response to sla1Δ deletion involves a network of genetic outputs that affects growth and metabolism. Altered pre-tRNA metabolism is a principal effect of sla1Δ deletion, and this appears to be a signal for the response, similar to but distinct from the nuclear surveillance system previously described for S. cerevisiae (Qiu et al., 2000). Thus the conclusion that emerges is that in S. pombe sla1Δ regulates AAM mRNA production through its effects on nuclear tRNA processing and maybe nuclear export.

La proteins associate with, stabilize, and promote the nuclear retention, proper order of 5′ and 3′ processing, and folding of pre-tRNAs, affording opportunity for processing, nucleotide modifications, and proper folding in an orderly manner (Yoo and Wolin, 1997; Intine et al., 2002; Chakshusmathi et al., 2003; Copela et al., 2006; Huang et al., 2006; Bayfield et al., 2007; Bayfield and Mariaa, 2009; Mariaa and Lamichhane, 2011).

Despite involvement of La with specific mRNAs (Cardinali et al., 2003; Intine et al., 2003; Trotta et al., 2003; Costa-Mattioli et al., 2004; Brenet et al., 2009), our results indicate loss of its nuclear function in pre-tRNA metabolism as the cause of the sla1-Δ phenotypes. Evidence for this is that the altered pattern of pre-tRNA intermediates in sla1-Δ cells was accompanied by apparent decrease in los1Δ-mediated tRNA nuclear export activity and that overexpression of los1Δ reversed these effects, as well as AAM gene up-regulation and slow growth of sla1-Δ cells. Limitation of Los1 has also been observed in S. cerevisiae strains that exhibit stress (DNA damage) response (Ghavidel et al., 2007) and perturbations of pre-tRNA biogenesis (Karkusiewicz et al., 2011).

**Aberrant tRNA processing in sla1-Δ cells and a nuclear surveillance–like response**

Defects in tRNA processing or nuclear export in S. cerevisiae lead to a stress response termed nuclear surveillance that induces AAM expression via GCN4 (Qiu et al., 2000). S. pombe AAM genes with promoters similar to Gcn4p-binding sites (Tanay et al., 2005) are activated by sla1Δ deletion. Suppression by los1Δ is consistent with the idea that a sensing component of the S. pombe response is via nuclear pre-tRNA. We note that some effects of sla1Δ deletion may reflect low levels of mature tRNA or increases in uncharged tRNA, as initially considered and later dismissed for the GCN4 response (Vazquez de Aldana et al., 1994; Qiu et al., 2000), and we cannot exclude this possibility.

Stress response analogy may extend further. LOS1 and GCN4 are involved in DNA damage response that leads to a decrease in the G1 cyclin, Cin2p (Ghavidel et al., 2007). sla1Δ cells are hypersensitive to the DNA-damaging agent ethyl methanesulfonate.
La deficiency causes growth defects

...and C869.10, with amino acid starvation, our [sl]Δ cells would appear to differ in response to La deletion since no growth deficiency was observed for [sl]Δ-los1+ in minimal medium (data not shown). In addition, whereas AAM induction in [sl]Δ cells is dependent, at least in part, on Atf1p and/or Pcr1p, Deletions of [sl]Δ and att1+ or pcr1+ show additive effects on some mRNAs for which att1+ and pcr1+ would appear to act independently of each other. Because Pcr1p and Atf1p perform overlapping and distinct functions (Sanso et al., 2008), [sl]Δ may antagonize AAM gene transcription either independently or as a Atf1/Pcr1 heterodimer. Further, since some mRNAs up-regulated in [sl]Δ are not affected by att1+ or pcr1+, these may be controlled by other TFs. In either case this appears to be different from the situation in S. cerevisiae, in which a single TF, Gcn4p, induces all of the target genes (Natarajan et al., 2001).

Another distinction is with regard to Atf1/Pcr1 TF activity, controlled by MAP kinase Spc1/Sty1 under conditions of extreme stress, such as oxidative stress during starvation (Nemoto et al., 2010). There is no apparent involvement of Spc1/Sty1 in our system, based on Sty1p phosphorylation (data not shown). In addition, deleting spc1+ in [sl]Δ did not restore growth of [sl]Δ cells in EMM (data not shown). Therefore a Sty1p-independent function of Atf1/Pcr1 (Lawrence et al., 2007) is likely involved in AAM gene induction, as well as other TFs.

Gcn4p-like function in S. pombe is likely performed by multiple AP-1-related TFs

Our data show that up-regulation of only a subset of the AAM genes tested in [sl]Δ cells is dependent, at least in part, on Atf1p and/or Pcr1p. Deletions of [sl]Δ and att1+ or pcr1+ show additive effects on some mRNAs for which att1+ and pcr1+ would appear to act independently of each other. Because Pcr1p and Atf1p perform overlapping and distinct functions (Sanso et al., 2008), [sl]Δ may antagonize AAM gene transcription either independently or as a Atf1/Pcr1 heterodimer. Further, since some mRNAs upregulated in [sl]Δ are not affected by att1+ or pcr1+, these may be controlled by other TFs. In either case this appears to be different from the situation in S. cerevisiae, in which a single TF, Gcn4p, induces all of the target genes (Natarajan et al., 2001).

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S. pombe mating program is partially derepressed in [sl]Δ cells

Microarray and Northern analysis of [sl]Δ cells in EMM revealed partial derepression of mRNAs that are up-regulated during mating (Supplemental Data), including pcr1+ mRNA, pas1+ (mating-specific cyclin), isp6+ (transcribed during sexual differentiation and induced by nitrogen starvation; Figure 1C), and fbp1+ (data not shown). Atf1p and Pcr1p up-regulate fbp1+ and ste11+, induced in meiosis along with cgs1+ involved in sexual differentiation (Takeda et al., 1995). With regard to links to TOR, Tor2p has been found associated with Ste11p and Mei2p, inhibiting sexual differentiation (Alvarez and Moreno, 2006). This suggests that the stress- and mating-related phenotypes of [sl]Δ cells may be linked.

Overlap between Sl1p and TOR

Growth of wild-type S. pombe is not inhibited by rapamycin, but that of leu1Δ-deficient cells is, due to inhibition of tor1Δ-mediated amino acid uptake (Weisman et al., 2005). Ectopic leu1Δ suppressed slow growth of [sl]Δ, whereas excess leucine in the media did not, consistent with a defect in leucine uptake. Indeed, the [sl]Δ mutant is hypersensitive to rapamycin. When [sl]Δ cells were grown in EMM with NH4+, the translation rate was only ~30% relative to isogenic WT cells, consistent with TOR involvement. Genetic analysis further suggested that Sl1p acts in parallel with Tsc1p and Tor1p to promote growth in EMM.

We examined mRNA levels for the putative permeases 7G5.06 and isp5+ orthologous to S. cerevisiae GAP1+, and C869.10,
orthologous to proline transporter PUT4. Although these were at similar levels in sla1Δ and WT cells, EMM resulted in ~2.5-fold decrease of ptr2Δ mRNA (nitrogen-repressible peptide transporter). However, ectopic ptr2Δ did not restore growth of sla1Δ, whereas ectopic isp5Δ expression did, albeit partially (Supplemental Figure S3A). In summary, decreased leucine uptake and NH4+ sensitivity appear to contribute to slow growth of sla1Δ cells.

We observed that sla1Δ cells formed fewer colonies and colonies of smaller size than sla1Δ+ (Figure 1B), suggesting deficiencies in two different growth parameters—plating efficiency and proliferation. Plating on proline was similar for sla1Δ+ and sla1Δ−, with ~50% cell recovery relative to YES (Supplemental Table S1). By contrast, plating efficiency of sla1Δ− was reproducibly <0.01% on NH4+ but much higher for sla1Δ+ (Supplemental Table S1). Thus sla1Δ− cells suffer from severe plating deficiency in the presence of NH4+.

Sensitivity of leu1Δ mutants to NH4+ is due to impaired amino acid import imposed by pub1Δ (Karagiannis et al., 1999). Deletion of pub1Δ suppressed the slow growth (colony size) of sla1Δ− much more so than the low plating efficiency (Supplemental Figure S3B). Therefore slow growth of sla1Δ− in NH4+ depends on pub1Δ+, and this appears to be genetically separable from low plating efficiency.

Finally, we note the potential importance of C132,04 induction in sla1Δ cells and suppression by ectopic los1Δ (Figure S5). This mRNAs encodes glutamate dehydrogenase (gdh2Δ), a central enzyme at the boundary of carbon and NH4+ metabolism in the TOR pathway (Tate and Cooper, 2003; Tate et al., 2006; Choo et al., 2010).

los1Δ contributes to pre-tRNA metabolism in the absence of Sla1p

That the lower band, L, in the top of Figure 6B accumulates in los1Δ cells and is decreased by ectopic los1Δ reflects that this intermediate is a substrate for Los1p-mediated export. That ectopic los1Δ leads to a reduction in the ratio of the L:U bands is consistent with the idea that Los1p nuclear export activity is quite limiting in sla1Δ cells. Although tRNA export limitation was noted previously (Arts et al., 1998; Qiu et al., 2000; Kuersten et al., 2002; Pierce et al., 2010), our data are distinguishable since overexpression of los1Δ in sla1Δ does not only affects the level of the 5′ and 3′ processed, intron-containing pre-tRNA (L band), but also increases the levels of the more premature, intron-containing pre-tRNA intermediates (Figure 6B). We believe that overcoming the Los1p limitation is quite significant in sla1Δ cells, which lack pre-tRNA 3′ end protection and in which nuclear surveillance pre-tRNA 3′ end decay is active (Maria and Lamichhane, 2011).

We propose that Los1p binding to pre-tRNA provides an important activity offsetting pre-tRNA 3′ end-mediated decay in sla1Δ cells. The 3′ exonucleases that act on pre-tRNAs can also digest the CCA ends of nuclear pre-tRNAs (Copela et al., 2008). A 3′ protective activity of Los1p/Xpo-t should not be unexpected since it is a tRNA CCA-3′ end-binding protein (Cook et al., 2009), as reflected by a requirement of CCA on its cargo (Arts et al., 1998; Lipowsky et al., 1999). A structure of S. pombe Xpo-t/los1p and tRNA shows a binding pocket for a 3′ overhang, CCA-3′ OH (Cook et al., 2009). Most of the binding to the tRNA 3′ region is sequence independent and includes an Asp side chain (D178) contact to the 3′ OH group, somewhat similar to the invariant Asp side chain of La (hLa D33) that binds, sequesters, and protects the 3′ OH terminus of pre-tRNA from 3′ exonucleases (Hu et al., 2007; Toplova et al., 2006).

Pre-tRNAs are susceptible to 3′-mediated decay in sla1Δ cells (Maria and Lamichhane, 2011), which, as reported here, are limited for los1Δ-mediated tRNA export. On the basis of this limitation, the binding properties of Xpo-t/Los1p, and the presence of intron-containing nuclear pre-tRNAs with CCA-3′ OH (Wolfe et al., 1996), we propose that ectopic Xpo-t/Los1p in sla1Δ cells binds those pre-tRNAs that would otherwise be susceptible and stabilizes them from 3′ decay, affording the opportunity for modifications and/or proper folding and export rather than degradation. Increased levels of pre-tRNAs in sla1Δ+los1Δ− cells observed on Northern blots is consistent with this.

**Materials and Methods**

**cDNA microarray analysis**

Total RNA for microarray analysis was obtained from early-log-phase (OD600 = 0.2–0.4) cells grown either in YES or EMM media with NH4Cl or proline as the nitrogen source and prepared as described
Similarly, CY1472 and CY1473 were generated by replacing ttor1::kan MX6, and CY1474 and CY1475 by replacing tsc1::kan MX6 in yAS99 and yAS113, respectively. CY1569 and CY1570 were made by replacing los1::los1 with los1::urod4* (Bahler et al., 1998) in yAS99 and yAS113, respectively. The obtained strains were selected on 5-fluoroorotic acid medium to counterselect for urad4*. All gene disruptions were confirmed by PCR.

Media were prepared according to standard recipes. For some applications, the NH4Cl in EMM was replaced with 10 mM proline. Recipe for EMM: potassium phthalate (3 g/l), Na2SO4 (0.04 g/l), ZnSO4 (0.4 mg/l), Na3HPO4 (2.2 mg/l), pantothenic acid (1 mg/l), FeCl2 (0.2 mg/l), NH4Cl (5 g/l), nicotinic acid (10 mg/l), molybdc acid (0.1 mg/l), MgCl2 (anhydrous) (0.492 g/l), D-biotin (0.01 mg/l), CuSO4 (40 μg/l), CaCl2 (14.7 mg/l), boric acid (0.5 mg/l), citric acid (1 mg/l), KCl (1 g/l), and MnSO4 (0.4 mg/l). When supplemented, EMM also contained leucine, adenine, and uracil, each at 225 mg/l. Rapamycin was used at 100 ng/ml. Thiamine was used at 0.05 μM (intermediate repression) or 15 μM (full repression).

3H-leucine uptake
This was performed as described, in EMM containing 225 mg/l leucine (~1.6 mM) and trace amounts of 3H-leucine (Weisman et al., 2005).

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REFERENCES

Plasmids
pRep4X hLa and pRep hLaA328-344 in Rep4X were described previously (Intine et al., 2000; Fairley et al., 2005). The isp5p open reading frame was PCR amplified using genomic DNA as template with the primers GTGCAGATGAAATACCGGGTTCTCTCC (forward) and GGATCCTTAAACCGAGAAAGATTGACG (reverse), digested with Sall and BamHI, and cloned into Rep4X. los1::los1 open reading frame was PCR amplified with the primers GTGCAGATGGC CGCAGGGATGTC (forward) and GGATCCTACATACATTCTTTATAGCTTGT (reverse), digested with Sall and BamHI, and cloned into Rep4X.

RNA purification and Northern blotting
Total RNA was purified as described (Lyne et al., 2003). For mRNA Northern analysis 5 and 10 μg of total RNA were separated in 1% denaturing agarose gel, transferred to a nylon membrane (GeneScreen Plus; PerkinElmer, Waltham, MA), UV cross-linked, baked, and subjected to hybridization at 42°C overnight with random primed 32P-DNA fragments of genes of interest. Hybridization solution was 5x Denhardt’s, 5x saline–sodium citrate, 50% formamide, 0.2% SDS, 5 mM EDTA, and 100 μg/ml total yeast RNA. Northern blotting of small RNAs was done essentially as described (Intine et al., 2000). Quantitation was done using a PhosphorImager FLA-3000 (Fujifilm, Tokyo, Japan).

35S-methionine incorporation
Ten milliliters of cells was grown exponentially to an OD600 = 0.2 and transferred to medium containing 1 mCi of 35S-methionine (PerkinElmer) and grown for 3.5 h at 32°C. Washed cells were harvested, and 25 and 50 μg of the whole-cell extract were separated on 10% PAGE. Gels were stained with SimplyBlue (Invitrogen, Carlsbad, CA), fixed, and dried, and 35S was quantified with a PhosphorImager FLA3000.


