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*.**

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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). Absence of *S. cerevisiae* La (Lhp1p) disrupts pre-tRNA 5′ processing by RNase P (Yoo and Wolin, 1997). *S. pombe* La (*sla1)* mutants that are defective in nuclear retention and export their pre-tRNA ligands to the cytoplasm cause premature splicing (Intine *et al.*, 2002; Bayfield *et al.*, 2007), whereas other *sla1* mutants distinguish 3′ end protection from RNA chaperone-like activity for structurally impaired pre-tRNAs (Huang *et al.*, 2006; Bayfield and Maraia, 2009). Thus La directs multiple aspects of pre-tRNA metabolism, and its absence from *S. pombe* causes imbalances in the

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Abbreviations used: AAM, amino acid metabolism; EMM, Edinburgh minimal medium; pre-tRNA, precursor tRNA; TOR, target of rapamycin.

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 Maraia, 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 Maraia 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 *sla1*-Δ 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, *sla1*-Δ cells also up-regulate nitrogen and mating genes. Leucine auxotrophic *sla1*-Δ 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; Wullschleger *et al.*, 2006). We tested the idea that aberrant nuclear pre-tRNA metabolism in *sla1*-Δ cells may contribute to the stress responses. Ectopic expression of *los1*$^+$, which encodes a major tRNA exporter, suppresses the decrease in pre-tRNAs in *sla1*-Δ 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), *ppr1*$^+$ (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-aminotriazol (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 does 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). hLaΔSBM, 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 Maraia, 2009), this suggested that the slow growth of *sla1*-Δ is due to defective pre-tRNA metabolism.

Strain	Genotype	Source or reference
yAS99	*h⁻ ade6-704 leu1-32 ura4⁻ sla1⁺*	Intine *et al.* (2000)
yAS113	*h⁻ ade6-704 leu1-32 ura4⁻ sla1⁻*	Intine *et al.* (2000)
SPJ83	*h90 leui-32 his2 ura4DS/E ade2-210 Kint2::URA4⁺*	Jia *et al.* (2004)
SPJ193	*h90 leui-32 his2 ura4DS/E ade2-210 Kint2::URA4⁺ pcr1Δ::kanMX6*	Jia *et al.* (2004)
SPJ266	*h90 leui-32 his2 ura4DS/E ade2-210 Kint2::URA4⁺atf1Δ::kanMX6*	Jia *et al.* (2004)
CY1391	*h90 leui-32 his2 ura4DS/E ade2-210 Kint2::URA4⁺sla1::NAT*	This study
CY1392	*h90 leui-32 his2 ura4DS/E ade2-210 Kint2::URA4⁺ pcr1Δ::kanMX6 sla1::NAT*	This study
CY1393	*h90 leui-32 his2 ura4DS/E ade2-210 Kint2::URA4⁺atf1Δ::kanMX6 sla1::NAT*	This study
CY1404	*h90 leui-32 his2 ura4DS/E ade2-210 Kint2::URA4⁺atf1Δ::kanMX6 pcr1::NAT*	This study
CY1407	*h⁻ ade6-704 leu1-32 ura4⁻ sla1⁺ pub1::kan MX6*	This study
CY1408	*h⁻ ade6-704 leu1-32 ura4⁻ sla1⁻ pub1::kan MX6*	This study
CY1472	*h⁻ ade6-704 leu1-32 ura4⁻ sla1⁺ tor1::kan MX6*	This study
CY1473	*h⁻ ade6-704 leu1-32 ura4⁻ sla1⁻ tor1::kan MX6*	This study
CY1474	*h⁻ ade6-704 leu1-32 ura4⁻ sla1⁺ tsc1::kan MX6*	This study
CY1475	*h⁻ ade6-704 leu1-32 ura4⁻ sla1⁻ tsc1::kan MX6*	This study
CY1569	*h⁻ ade6-704 leu1-32 ura4⁻ sla1⁺ los1⁻*	This study
CY1570	*h⁻ ade6-704 leu1-32 ura4⁻ sla1⁻ los1⁻*	This study
SPG17	*h90 leu1-32 his2 ura4Δ ade2-M210*	Grewal and Klar (1997)
CY1604	*h90 leu1-32 his2 ura4Δ ade2-M210 sla1::NAT*	This study
CY1627	*h90 leu1-32 his2 ura4Δ ade2-M210 sla1::NAT sla1⁺ URA4⁺*	This study

TABLE 1: Yeast strains.

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 1× and 2× amounts). Using *rpl8⁺* mRNA as a loading control with sequential probings of the same blots, we found that *atf1⁺* or *pcr1⁺* deletion in *sla1⁺*-replete 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). *isp6⁺* (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*-Δ but less so to *pcr1⁺* deletion (Figure 1C, lanes 9–14). We conclude that up-regulation of a subset of AAM genes in *sla1⁻* 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⁺* had little effect on *atf1⁻* or pcr*1⁻* 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).

sla1⁻ cells are defective in leucine uptake and hypersensitive to NH_4Cl and rapamycin

We analyzed different ingredients of YES and EMM for effects on *sla1⁻* cell growth (data not shown) and found that the NH_4Cl in EMM was inhibitory. Replacing NH_4^+ 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 NH_4^+ to YES also resulted in very significant growth inhibition of *sla1⁻* (Figure 2A). Thus growth of *sla1⁻* cells is highly sensitive to NH_4Cl.

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

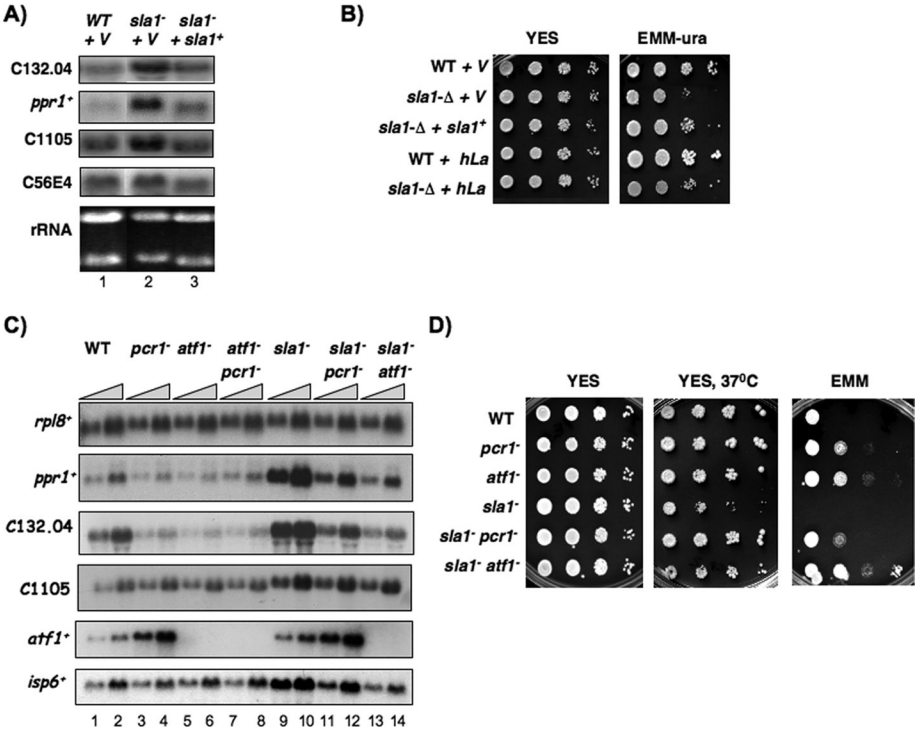

FIGURE 1: Stress-response phenotypes of *sla1*-Δ cells, including AAM gene expression, involve genetic interactions with *atf1*$^+$ and *pcr1*$^+$. (A) Ectopic *sla1*$^+$ suppresses up-regulation of AAM genes in the *sla1*$^-$ mutant. Northern blot of 10 µg of total RNA isolated from cells grown exponentially in EMM with essential supplements probed sequentially for the gene sequences indicated to the left. Bottom, ethidium bromide staining prior to transfer. Strains yAS99 (WT; *leu1-32 ura4-*Δ; Table 1) and yAS113 (*slal-*Δ *leu1-32 ura4-*Δ) were transformed with the *ura4*$^+$-containing pRep4X plasmid vector (V) or pRep4X carrying *sla1*$^+$ as indicated above the lanes. (B) yAS99 (WT) and yAS113 (*slal-*Δ) cells grown in liquid EMM lacking uracil (EMM-ura) were spotted on agar plates as 10-fold serial dilutions and incubated at 32°C for 2–6 d. The strains were transformed with either the empty pRep4X (*ura4*$^+$) plasmid vector (V) or pRep4X carrying *sla1*$^+$ or *hLa* as indicated to the left. (C) A Northern blot loaded with 5 and 10 µg of total RNA from each of the WT or mutants indicated above the lanes was probed, stripped, and reprobed sequentially for the gene sequences indicated to the left. Strains: SPJ83 (WT), SPJ193 (*pcr1*$^-$), SPJ266 (*atf1*$^-$), CY1391 (*sla1*$^-$), CY1392 (*sla1*$^-$ *pcr1*$^-$), CY1393 (*sla1*$^-$ *atf1*$^-$), and CY1404 (*atf1*$^-$ *pcr1*$^-$). Cells were grown in YES media; *rpl8*$^+$ is a loading control. (D) Deletion of *atf1*$^+$ or *pcr1*$^+$ suppresses slow growth in EMM and heat sensitivity of the *sla1*$^-$ mutant. Strains are as in C. The relative growth differences of the *sla1*$^-$ strains in B and D are attributed to differences in the strain backgrounds, in particular, the auxotrophic markers. Supplemental Figure S2 shows that *S. pombe* strains obtained from different labs exhibit different growth rates in EMM.

created a *tor1*-Δ 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 NH_4^+ or proline (Sychrova *et al.*, 1989; Karagiannis *et al.*, 1999; Matsumoto *et al.*, 2002; Weisman *et al.*, 2005). Figure 2D (top) shows that *sla1*$^-$ cells are quite defective in leucine uptake in EMM (containing NH_4^+), even relative to the *tor1*$^-$ mutant. The rate of leucine uptake by *sla1*$^-$ cells appeared to be less compromised in proline than in NH_4^+ (Figure 2D; compare top and bottom). Note that whereas decreased leucine uptake characterizes *sla1*$^-$ 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 tumors, although some develop resistance (Choo and Blenis, 2009; Gibbons *et al.*, 2009; Zhou *et al.*, 2010). Whereas wild-type *S. pombe* is naturally resistant to rapamycin, regulation of leucine uptake is sensitive to rapamycin (Weisman *et al.*, 2005). We found that *sla1*$^-$ mutants are sensitive to rapamycin relative to their isogenic WT strains. Complementation of rapamycin sensitivity of *sla1*$^-$ by ectopic *sla1*$^+$ was partial but significant (Figure 3A, compare 2 and 3) and appeared to depend on the activity of the *nmt1* promoter driving Sla1p expression, which is partially repressible by thiamine (Figure 3A). The *nmt1* promoter remains significantly active in the presence of thiamine (Forsburg, 1993). It was previously found that pRep4X-*sla1*$^+$ in *sla1*$^-$ cells produces fourfold higher Sla1p levels than produced from the chromosomal *sla1*$^+$ locus (R. V. Intine and R. J. Maraia, unpublished results). When *sla1*$^-$ was grown in EMM lacking thiamine, minimal complementation of rapamycin sensitivity by ectopic *sla1*$^+$ was observed relative to WT (Figure 3A, 3). Thiamin significantly improved complementation of rapamycin sensitivity by ectopic *sla1*$^+$ (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 NH_4^+ versus proline (e.g., see Figure 1, B vs. D, and Supplemental Figure S2). To examine rapamycin sensitivity in another genetic background, we deleted *sla1*$^+$ 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 *sla1*-deleted SPG17, designated CY1604, is sensitive to rapamycin relative to its parent SPG17 and to CY1627, in which *sla1*$^+$ was reintroduced to its chromosomal locus.

It is remarkable that, whereas *leu1*$^+$ rescues the slow growth phenotype of *sla1*$^-$ mutants in EMM with NH_4^+ (Figure 2B), *leu1*$^+$ does not rescue the rapamycin sensitivity (Figure 3C, 2 and 3). It is also notable that *sla1*$^-$ cells appear less sensitive to rapamycin in EMM with proline than with NH_4^+ (Figure 3C, 2–5), suggesting that NH_4^+ contributes to the sensitization of *sla1*$^-$ cells to rapamycin and that Sla1p promotes leucine uptake and rapamycin resistance via distinct mechanisms.

Because amino acid and tRNA metabolism are altered in *sla1*$^-$ cells we expected that slow growth may be accompanied by decreased protein synthesis. We therefore measured ^{35}S-methionine (^{35}S-met) incorporation into protein during log-phase growth in EMM with NH_4^+ or proline (Figure 3D). Quantitation revealed that ^{35}S incorporation was lower in *sla1*$^-$ than in WT in EMM with NH_4^+ but not with proline (Figure 3D; see normalized values under lanes, left), consistent with *sla1*$^-$ growth in these media. We found no difference in ^{35}S-met uptake between *sla1*$^-$ and WT measured under the same conditions as ^{35}S-met incorporation (data not shown). Thus ^{35}S-met incorporation in *sla1*$^-$ reflects decreased translation rather than limitation of methionine. Slow growth coupled with decreased translation in *sla1*$^-$ cells is consistent with involvement of TOR signaling.

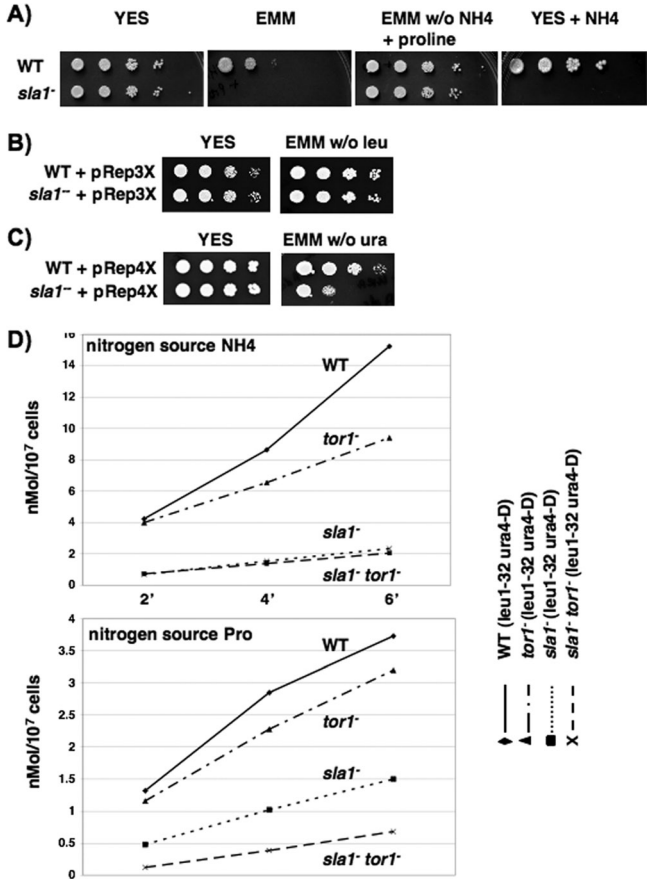

FIGURE 2: *sla1*⁻ cells are sensitive to NH_4^+ and deficient in leucine uptake. (A–C) Cells were grown in liquid media as indicated, and then 10-fold serial dilutions were spotted on plates containing media indicated above and incubated at 32°C for 2–6 d. (A) Growth of the *sla1*⁻ mutant is inhibited by NH_4^+. 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 (C) 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 NH_4^+ 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*⁻).

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*⁻ dependent amino acid uptake (Weisman *et al.*, 2005). Our data that show loss of *sla1*⁺ causes leucine uptake deficiency, as well as NH_4^+ and rapamycin sensitivity, strongly suggest genetic interactions between *sla1*⁺ and *tor1*⁺. Whereas the *tor1*⁻ mutant does not exhibit growth deficiency in NH_4^+, this deletion has an additive effect on growth in combination with *sla1*⁻ mutation (Figure 4A). This suggests that *tor1*⁺ and *sla1*⁺ have overlapping yet distinct functions during vegetative growth.

S. pombe tsc1⁺ and *tsc2*⁺ are homologues of tuberous sclerosis genes, which have been linked to the TOR pathway, tumorigenesis, and nutrient availability (Serfontein *et al.*, 2010). *tsc1*⁺ 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 NH_4^+ 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.

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 *S. pombe los1*⁺ would offset (suppress) 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

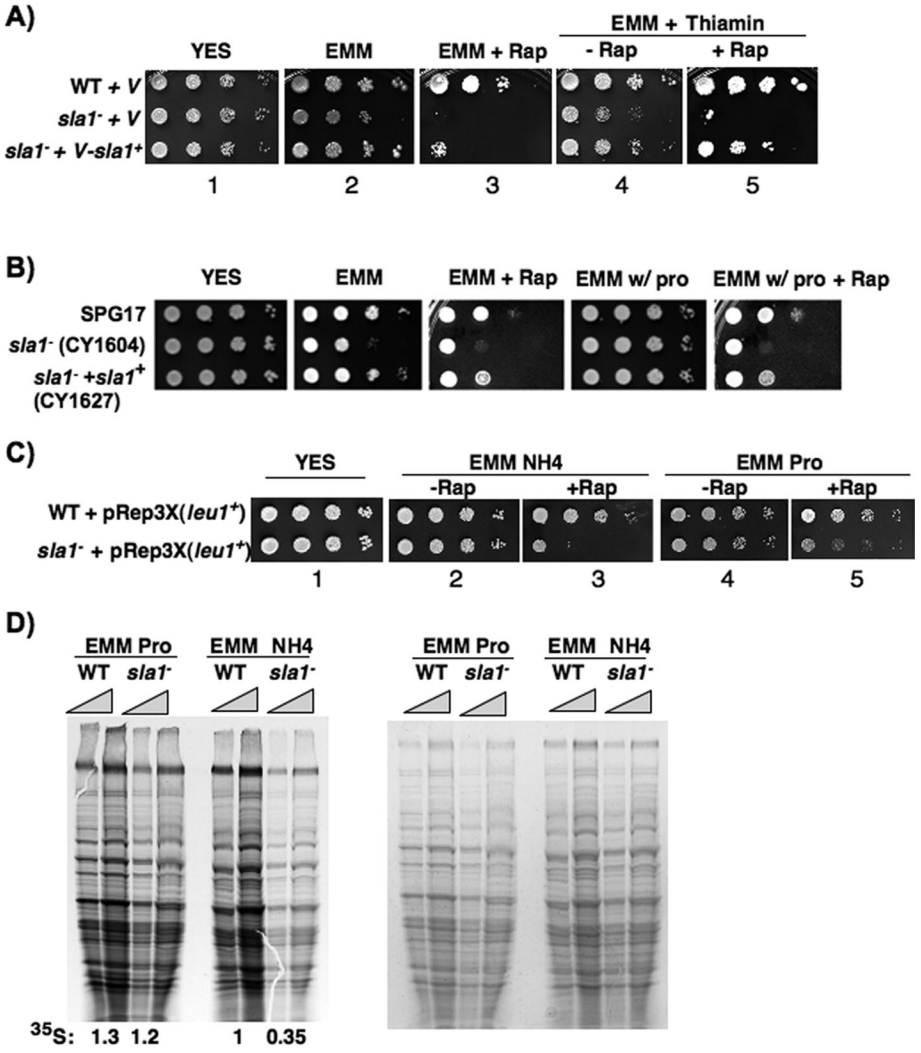

FIGURE 3: *sla1*⁻ cells are sensitive to rapamycin and exhibit decreased protein synthesis. (A, B) Cells were grown in liquid EMM medium with essential supplements, and 10-fold serial dilutions were spotted on the plates containing different media as indicated and incubated at 32°C for 2–6 d. (A) yAS99 (WT) and yAS113 (*slal*⁻) 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 *slal*⁻ 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 NH_4^+ or proline as indicated. (D) General translation is reduced in the *sla1*⁻ mutant. Cells were grown in EMM with NH_4^+or 10 mM proline as the nitrogen source in the presence of ^{35}S-methionine. ^{35}S 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.

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 a 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.

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^{Lys}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^{Lys}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

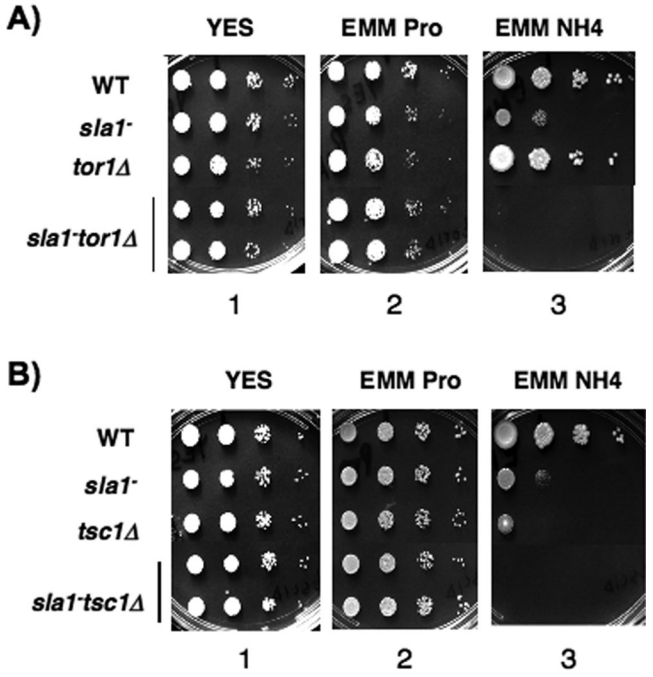

FIGURE 4: Genetic interactions between *sla1*$^+$ and the TOR pathway. Cells were grown in liquid EMM with essential supplements, and 10-fold serial dilutions were spotted onto the indicated media and incubated at 32°C for 2–6 d. (A) Deletions of *sla1*$^+$ and *tor1*$^+$ have additive effects on slow growth in EMM. Strains: yAS99 (WT), yAS113 (*sla1*$^-$), CY1472 (*tor1*$^-$), and CY1473 (*sla1*$^-$ *tor1*$^-$). (B) Deletions of *sla1*$^+$ and *tsc1*$^-$ have additive effects on slow growth in EMM. Strains: yAS99 (WT), yAS113 (*sla1*$^-$), CY1472 (*tor1*$^-$), CY1473 (*sla1*$^-$ *tor1*$^-$), CY1474 (*tsc1*$^-$), and CY1475 (*sla1*$^-$ *tsc1*$^-$).

11 and 12 relative to 9 and 10, and the ratio is more similar to WT, indicating that in 0.05 μM thiamine, *nmt1-los1*$^+$ 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 *sla1*-Δ (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-los1*$^+$ in 0.05 μM thiamine (lanes 7 and 8).

The ratio and abundance of the pre-tRNA^{Lys}CUU intermediates differ in *sla1*$^-$ 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*$^-$ cells. The L band is relatively prominent in *sla1*$^-$ 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 limiting in *sla1*$^-$ cells. Furthermore, ectopic *los1*$^+$ unexpectedly increased the amount of the M species pre-tRNA in *sla1*$^-$ 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*$^+$ 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-tRNA^{Lys}CUU in *sla1*$^-$ cells. The cumulative data argue that ectopic *los1*$^+$ helps alleviate response to aberrant nuclear pre-tRNA metabolism in *sla1*$^-$ cells.

We stripped the blot in Figure 6B, top (data not shown), and rehybridized with a probe specific for the 5′ leader of pre-tRNA^{Lys}CUU (Figure 6B, middle). This revealed that the 5′ leader–containing species is at relatively low levels in *sla1*$^-$ cells (lanes 5 and 6) but more prominent in the *sla1*$^-$+*los1*$^+$ cells (Figure 6B, middle, lanes 7 and 8; see quantitation normalized for loading by U5 small nuclear RNA [snRNA] levels under the bottom lanes). These data suggest that overexpression of *los1*$^+$ 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 *los1*$^+$-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 *sla1*$^+$ deletion involves a network of genetic outputs that affects growth and metabolism. Altered pre-tRNA metabolism is a principal effect of *sla1*$^+$ 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 sla1*$^+$ 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 Maraia, 2009; Maraia 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 *los1*$^+$-mediated tRNA nuclear export activity and that overexpression of *los1*$^+$ 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*$^-$ 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 *sla1*$^+$ deletion. Suppression by *los1*$^+$ 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 *sla1*$^+$ 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,*Cln2p* (Ghavidel *et al.*, 2007). *sla1*$^-$ cells are hypersensitive to the DNA-damaging agent ethyl methanesulfonate

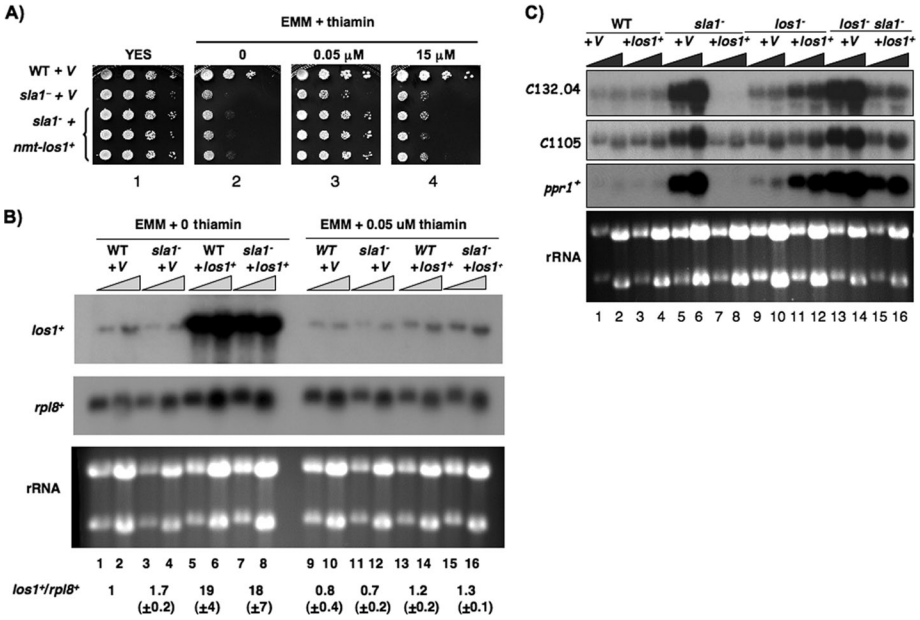

FIGURE 5: Ectopic *los1*$^+$ suppresses slow growth and up-regulation of AAM genes in the absence of *sla1*$^+$. (A) Effect of thiamine-mediated titration of *nmt1-los1*$^+$ expression on growth of *sla1*$^-$ cells. Growth assay was done as for Figure 4; strains are yAS99 (WT) and yAS113 (*sla1*$^-$) transformed with empty pRep4X (V) or *los1*$^+$ cloned into pRep4X (thiamine-repressible *nmt1* promoter, see text). (B) Northern blot analysis of *los1*$^+$ mRNA from cells grown in the presence or absence of thiamine. *rpl8*$^+$ mRNA on the same blot was used as loading control for quantitation; normalized *los1*$^+$ mRNA levels relative to *rpl8*$^+$ are indicated below the lanes. Strains are yAS99 (WT) and yAS113 (*sla1*$^-$), transformed with empty pRep4X (V), or *los1*$^+$ in pRep4X as in A, grown in EMM containing 0 (lanes 1–8) or 0.05 μM thiamine (lanes 9–16) as indicated. (C) Ectopic *los1*$^+$ suppresses up-regulation of AAM genes in *sla1*$^-$ cells. Northern blot of C132.04, C1105, and *ppr1*$^+$ mRNAs using strains as in B plus CY1569 (*los1*$^-$) and CY1570 (*sla1*$^-$ *los1*$^-$) transformed with empty pRep4X (V) or *los1*$^+$ in pRep4X and grown in EMM with 0.05 μM thiamine. Bottom, rRNA.

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 *sla1*$^-$ cells is dependent, at least in part, on Atf1p and/or Pcr1p. Deletions of *sla1*$^+$ and *atf1*$^+$ or *pcr1*$^+$ show additive effects on some mRNAs for which *atf1*$^+$ and *pcr1*$^+$ would appear to act independently of each other. Because Pcr1p and Atf1p perform overlapping and distinct functions (Sanso *et al.*, 2008), Sla1p may antagonize AAM gene transcription either independently or as a Atf1/Pcr1p heterodimer. Further, since some mRNAs up-regulated in *sla1*$^-$ are not affected by *atf1*$^+$ *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*$^+$/*sty1*$^+$ did not restore growth of *sla1*$^-$ 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.

(data not shown) and have approximately twofold less *cyc17*$^+$/*cig2*$^+$ mRNA relative to WT cells in EMM but not YES (Supplemental Figure S1). Indeed, unexpected sensitivity to alterations of Los1p levels was evident in some of our experiments. For example, whereas pre-tRNA distribution was distorted in *los1*-Δ cells relative to wild type, ectopic *nmt1*$^+$-*los1*$^+$ in 0.05 μM thiamine did not fully complement this (Figure 6B), presumably because Los1p levels produced by *nmt1*$^+$-*los1*$^+$ do not match that in wild-type *los1*$^+$ cells. Consistent with this assumption, *los1*$^+$ mRNA levels were significantly lower in the *los1*-Δ $^+$*nmt1*$^+$-*los1*$^+$ cells than in the wild-type *los1*$^+$ cells (data not shown). These findings support the idea that regulation of Los1p homeostasis is critical to normal *S. pombe* metabolism.

Although our data reflect likeness to *S. cerevisiae* nuclear surveillance, there are distinctions. The *GCN4* response is triggered by excess unprocessed pre-tRNA, whereas *sla1*$^-$ cells have a deficiency, suggesting that imbalance of pre-tRNA levels or processing is a commonality. Second, no genome-wide mRNA profiling of *S. cerevisiae lhp1*-Δ or analysis of sensitivity to NH_4^+ has been reported. Third, the two yeasts would appear to differ in response to La deletion since no growth deficiency was observed for *S. cerevisiae lhp1*-Δ. In addition, whereas AAM induction in *S. cerevisiae* occurs with amino acid starvation, our *sla1*-Δ cells exhibit AAM induction in EMM, a standard growth medium that normally does not induce starvation-related responses. Finally, we tested the *S. cerevisiae lhp1*-Δ mutant, and it did not show growth deficiency under amino acid starvation or in minimal medium (data not shown).

S. pombe mating program is partially derepressed in *sla1*$^-$ cells

Microarray and Northern analysis of *sla1*$^-$ 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 *sla1*$^-$ cells may be linked.

Overlap between Sla1p and TOR

Growth of wild-type *S. pombe* is not inhibited by rapamycin, but that of *leu1*$^-$deficient cells is, due to inhibition of *tor1*$^+$-mediated amino acid uptake (Weisman *et al.*, 2005). Ectopic *leu1*$^+$ suppressed slow growth of *sla1*-Δ , whereas excess leucine in the media did not, consistent with a defect in leucine uptake. Indeed, the *sla1*$^-$ mutant is hypersensitive to rapamycin. When *sla1*$^-$ cells were grown in EMM with NH_4^+, the translation rate was only ~30% relative to isogenic WT cells, consistent with TOR involvement. Genetic analysis further suggested that Sla1p 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,

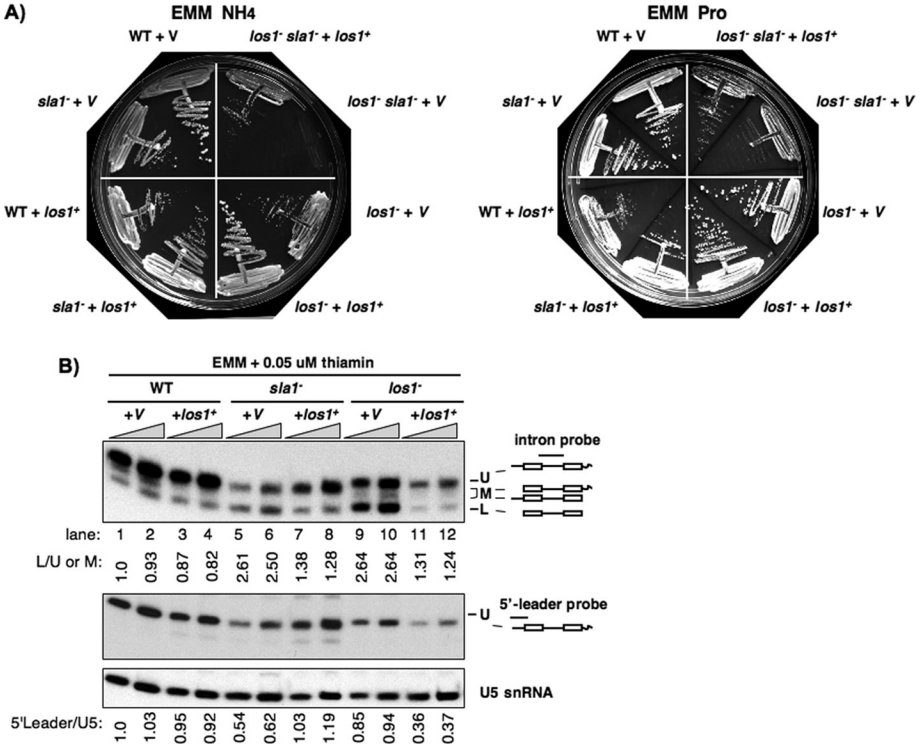

FIGURE 6: Ectopic *los1*$^+$ stabilizes tRNA precursors in the absence of *sla1*$^+$. (A) Growth in EMM with NH_4^+ (left) or proline (right) of various strains as indicated. (B) Northern analysis of small RNAs from foregoing strains transformed with empty pRep4X (V) or *los1*$^+$ in pRep4X, grown in EMM with 0.05 μM thiamine, and probed for the RNA indicated to the right as follows. Top, intron-containing pre-tRNA^{Lys}CUU; quantitation of the bands at the top is shown as a ratio below the lanes normalized as indicated to lane 1; middle, 5′ leader–containing pre-tRNA^{Lys}CUU. U, M, and L indicate positions of the pre-tRNA^{Lys}CUU intermediates, as schematized to the right; location of the probes used is also indicated. Bottom, the same blot probed for U5 snRNA. All three probings were of the same blot. Quantitation of the band in the middle was corrected for loading by U5 snRNA and normalized to lane 1 as indicated below the lanes at the bottom.

orthologous to proline transporter *PUT4*. Although these were at similar levels in *sla1*$^-$ and WT cells, EMM resulted in ~2.5-fold decrease of *ptr2*$^+$ mRNA (nitrogen-repressible peptide transporter). However, ectopic *ptr2*$^+$ did not restore growth of *sla1*$^-$, whereas ectopic *isp5*$^+$ expression did, albeit partially (Supplemental Figure S3A). In summary, decreased leucine uptake and NH_4^+ sensitivity appear to contribute to slow growth of *sla1*$^-$ cells.

We observed that *sla1*$^-$ cells formed fewer colonies and colonies of smaller size than *sla1*$^+$ (Figure 1B), suggesting deficiencies in two different growth parameters—plating efficiency and proliferation. Plating on proline was similar for *sla1*$^+$ and *sla1*$^-$, with ~50% cell recovery relative to YES (Supplemental Table S1). By contrast, plating efficiency of *sla1*$^-$ was reproducibly <0.01% on NH_4^+ but much higher for *sla1*$^+$ (Supplemental Table S1). Thus *sla1*$^-$ cells suffer from severe plating deficiency in the presence of NH_4^+.

Sensitivity of *leu1* mutants to NH_4^+ is due to impaired amino acid import imposed by *pub1*$^+$ (Karagiannis *et al.*, 1999). Deletion of *pub1*$^+$ suppressed the slow growth (colony size) of *sla1*$^-$ much more so than the low plating efficiency (Supplemental Figure S3B). Therefore slow growth of *sla1*$^-$ in NH_4^+ depends on *pub1*$^+$, and this appears to be genetically separable from low plating efficiency.

Finally, we note the potential importance of C132.04 induction in *sla1*$^-$ cells and suppression by ectopic *los1*$^+$ (Figure 5C). This mRNA encodes glutamate dehydrogenase (*gdh2*$^+$), a central enzyme at the boundary of carbon and NH_4^+ metabolism in the TOR pathway (Tate and Cooper, 2003; Tate *et al.*, 2006; Choo *et al.*, 2010).

los1$^+$ contributes to pre-tRNA metabolism in the absence of Sla1p

That the lower band, L, in the top of Figure 6B accumulates in *los1*-Δ cells and is decreased by ectopic *los1*$^+$ reflects that this intermediate is a substrate for Los1p-mediated export. That ectopic *los1*$^+$ 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 *sla1*-Δ 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 *los1*$^+$ in *sla1*-Δ 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 *sla1*-Δ cells, which lack pre-tRNA 3′ end protection and in which nuclear surveillance pre-tRNA 3′ end decay is active (Maraia and Lamichhane, 2011).

We propose that Los1p binding to pre-tRNA provides an important activity offsetting pre-tRNA 3′ end–mediated decay in *sla1*-Δ 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-OH 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 (Huang *et al.*, 2006; Teplova *et al.*, 2006).

Pre-tRNAs are susceptible to 3′-mediated decay in *sla1*-Δ cells (Maraia and Lamichhane, 2011), which, as reported here, are limited for *los1*$^+$-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$^-$3′ OH (Wolfe *et al.*, 1996), we propose that ectopic Xpo-t/Los1p in *sla1*$^-$ 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 *sla1*$^-$+*los1*$^+$ 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 (OD_{600} = 0.2–0.4) cells grown either in YES or EMM media with NH_4Cl or proline as the nitrogen source and prepared as described

(Lyne *et al.*, 2003). RNA labeling, microarray hybridization, data processing, and normalization were carried out as previously described (Lyne *et al.*, 2003).

Yeast strains and growth media

Yeast strains are listed in Table 1. CY1391, CY1392, and CY1393 were constructed by replacing *sla1*$^+$ with *sla1::NAT* (Sato *et al.*, 2005) in SPJ83, SPJ193, and SPJ266 respectively. CY1404 was made by replacing *pcr1*$^+$ with *pcr1::NAT* in SPJ266. CY1407 and CY1408 were generated from yAS99 and yAS113, respectively, by replacing *pub1*$^+$ with *pub1::kan MX6*. Similarly, CY1472 and CY1473 were generated by replacing *tor1*$^+$ with *tor1::kan MX6*, and CY1474 and CY1475 by replacing *tsc1*$^+$ with *tsc1::kan MX6* in yAS99 and yAS113, respectively. CY1569 and CY1570 were made by replacing *los1*$^+$ with *los1::ura4*$^+$ (Bahler *et al.*, 1998) in yAS99 and yAS113, respectively. The obtained strains were selected on 5-fluoroorotic acid medium to counterselect for *ura4*$^+$. All gene disruptions were confirmed by PCR.

Media were prepared according to standard recipes. For some applications, the NH_4Cl in EMM was replaced with 10 mM proline. Recipe for EMM: potassium phthalate (3 g/l), Na_2SO_4 (0.04 g/l), $ZnSO_4$ (0.4 mg/l), Na_2HPO_4 (2.2 g/l), pantothenic acid (1 mg/l), $FeCl_2$ (0.2 mg/l), NH_4Cl (5 g/l), nicotinic acid (10 mg/l), molybdic acid (40 µg/l), dextrose (20 g/l), inositol (10 mg/l), potassium iodide (0.1 mg/l), $MgCl_2$ (anhydrous) (0.492 g/l), D-biotin (0.01 mg/l), $CuSO_4$ (40 µg/l), $CaCl_2$ (14.7 mg/l), boric acid (0.5 mg/l), citric acid (1 mg/l), KCl (1 g/l), and $MnSO_4$ (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).

Plasmids

pRep4X hLa and *pRep hLaΔ328-344* in Rep4X were described previously (Intine *et al.*, 2000; Fairley *et al.*, 2005). The *isp5*$^+$ open reading frame was PCR amplified using genomic DNA as template with the primers GTCGACATGAATAATTAC-GGGGTCTCTTCC (forward) and GGATCCTTAAACGCAGAAAGATAGGACG (reverse), digested with *Sal*I and *Bam*HI, and cloned into Rep4X. *los1*$^+$ open reading frame was PCR amplified with the primers GTCGACATGTCGGC-CCAGGATGTC (forward) and GGATCCTCATACATTACCACTTTT-TAATGCTTG (reverse), digested with *Sal*I and *Bam*HI, 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 5× Denhardt's, 5× 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).

^{35}S-methionine incorporation

Ten milliliters of cells was grown exponentially to an OD_{600} = 0.2 and transferred to medium containing 1 mCi of ^{35}S-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 ^{35}S was quantified with a PhosphorImager FLA3000.

^{3}H-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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