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BREAKTHROUGH REPORT

Phosphorus Availability Regulates TORC1 Signaling via LST8 in Chlamydomonas[OPEN]

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Target of rapamycin complex 1 (TORC1) is a central regulator of cell growth. It balances anabolic and catabolic processes in response to nutrients, growth factors, and energy availability. Nitrogen- and carbon-containing metabolites have been shown to activate TORC1 in yeast, animals, and plants. Here, we show that phosphorus (P) regulates TORC1 signaling in the model green alga Chlamydomonas (*Chlamydomonas reinhardtii*) via LST8, a conserved TORC1 subunit that interacts with the kinase domain of TOR. P starvation results in a sharp decrease in LST8 abundance and downregulation of TORC1 activity. A hypomorphic *lst8* mutation resulted in decreased LST8 abundance, and it both reduced TORC1 signaling and altered the cellular response to P starvation. Additionally, we found that LST8 levels and TORC1 activity were not properly regulated in a mutant defective in the transcription factor PSR1, which is the major mediator of P deprivation responses in Chlamydomonas. Unlike wild-type cells, the *psr1* mutant failed to downregulate LST8 abundance and TORC1 activity when under P limitation. These results identify PSR1 as an upstream regulator of TORC1 and demonstrate that TORC1 is a key component in P signaling in Chlamydomonas.

INTRODUCTION

Target of rapamycin (TOR) is a kinase that is an evolutionarily conserved protein and is an essential regulator of cell growth. It perceives the cell's nutritional status and transmits appropriate signals to the cell growth machinery. In the presence of required nutrients, TOR promotes cell growth by activating anabolic processes such as protein synthesis and ribosome biogenesis and by repressing catabolic processes such as autophagy (Loewith and Hall, 2011; González and Hall, 2017; Saxton and Sabatini, 2017). In metazoans and fungi, TOR exists in two structurally and functionally distinct multiprotein complexes termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2). These complexes were originally described in *Saccharomyces cerevisiae* (Loewith et al., 2002) but are conserved in many other eukaryotes (Soulard et al., 2009; Eltschinger and Loewith, 2016; González and Hall, 2017; Saxton and Sabatini, 2017).

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The core components of TORC1 include the TOR kinase, Raptor/KOG1, and LST8, whereas TORC2 is composed of TOR, LST8, Sin1/AVO1, and Rictor/AVO3 (Loewith et al., 2002). Besides the TOR kinase, LST8 is the only protein present in both TORC1 and TORC2. LST8 binds to the kinase domain of TOR, and this binding is needed for full catalytic activity of both TORC1 and TORC2 (Kim et al., 2003; Wullschleger et al., 2005; Aylett et al., 2016). Nutrients are important regulators of TORC1 activity in all eukaryotes. In yeast, preferred nitrogen (N) sources and amino acids, particularly Leu and Glu, promote TORC1 function by activating Ras-related GTP-binding (RAG) GTPases Gtr1 and Gtr2 in association with the EGO complex (Binda et al., 2009; Loewith and Hall, 2011; Hatakeyama and De Virgilio, 2016; González and Hall, 2017). Similar, but more intricate, mechanisms operate in animal cells to regulate mTORC1. Amino acids, growth factors, and energy status send signals to mTORC1 via different pathways: Leu and Glu induce mTORC1 via RAG GTPases (Jewell et al., 2013; Bar-Peled and Sabatini, 2014) and glutaminolysis (Durán et al., 2012); growth factors activate mTORC1 via the small GTPase RHEB (González and Hall, 2017; Saxton and Sabatini, 2017); and Glc availability regulates mTORC1 through AMPK (Yuan et al., 2013).

TORC1 is structurally and functionally conserved in plants. Early studies in the model plant Arabidopsis (*Arabidopsis thaliana*)

IN A NUTSHELL

Background: Because nutrient stress seriously affects healthy growth in photosynthetic organisms, understanding how nutrients are sensed is extremely important. The kinase TOR (target of rapamycin) is a master regulator of growth that is present in all eukaryotes. TOR translates environmental and nutritional cues into permissive or restrictive regulation of cell metabolism and growth. In plants and algae, TOR is present in the TORC1 complex along with LST8 and Raptor proteins. Nitrogen and carbon metabolites activate TORC1 signaling in yeast, animals and plants. However, the role of other macronutrients such as phosphorus (P) in TORC1 signaling has received little attention. Although the development of a reliable kinase assay for TOR is fundamental to investigate this pathway in all eukaryotes, this basic tool has been missing in green algae.

Question: Is TORC1 involved in P sensing? To answer this, we developed a molecular tool to measure the activity of the TOR kinase in the model unicellular green alga *Chlamydomonas reinhardtii*.

Findings: We found that TORC1 is involved in the regulation of P signaling in *Chlamydomonas* and that the TORC1 subunit LST8 plays an important role in this signaling pathway. LST8 binds to the kinase domain of TOR, and this binding is needed for full catalytic activity of TORC1. Using genetic and biochemical approaches, we found that when LST8 levels are compromised, *Chlamydomonas* cells displayed an altered response to P starvation, hyperactivating cell recycling and over accumulating lipids. We also found that the transcription factor PSR1, the major mediator of P deprivation responses in *Chlamydomonas*, works upstream of TORC1 to regulate the response to P starvation. To validate these results, we developed a TOR kinase assay in *Chlamydomonas* to monitor the phosphorylation state of RPS6, a downstream target of TORC1.

Next steps: Phosphorus is an essential nutrient for sustaining life, but plants and algae must acclimate to periods of P insufficiency to survive in the natural environment. Next, we hope to identify novel components in the TORC1 pathway that regulate P starvation. This will help us to elucidate the signaling networks that control nutrient stress sensing in the green lineage.

demonstrated that TOR is essential for cell growth since disruption of its single *TOR* gene is lethal (Menand et al., 2002). Plant genomes encode homologs of yeast and animal TORC1 core components, but not TORC2-specific proteins (Dobrenel et al., 2016a). Arabidopsis has two Raptor-encoding genes, *Raptor3g* and *Raptor5g*, and mutant lines disrupted in both genes display impaired growth but are viable, indicating that these genes are not essential (Anderson et al., 2005; Deprost et al., 2005). Two LST8-encoding genes are present in Arabidopsis, but only one of them, *LST8-1*, is significantly expressed (Moreau et al., 2012). Mutations in this gene are not lethal but result in retarded growth, delayed flowering, and hypersensitivity to short-to-long-day transitions (Moreau et al., 2012). A recent study demonstrated that growth of the Arabidopsis *lst8-1* mutant could be restored by mutations of the YAK1 kinase, revealing that this kinase acts in the plant TOR pathway, likely by mediating stress signals (Forzani et al., 2019).

The development of a reliable assay for TOR activity has been fundamental to investigating this pathway in plants. TOR directly phosphorylates the AGC-kinase S6K, which in turn phosphorylates RPS6 in animal cells, and recent studies have demonstrated that the TOR-S6K-RPS6 axis of the mTORC1 pathway is highly conserved in plants. Arabidopsis has two S6K proteins, and TOR phosphorylates both of them at conserved Thr-449 and Thr-455 residues (Mahfouz et al., 2006; Schepetilnikov et al., 2011, 2013; Xiong et al., 2013). Active S6K in turn phosphorylates RPS6 in a TOR-dependent manner (Dobrenel et al., 2016b). Therefore, phosphorylation of S6K and RPS6 has been successfully used to monitor TOR activity in Arabidopsis. Plant TORC1 is activated by sugar, light, energy, sulfur, and hormones (reviewed by Dobrenel et al., 2016a; Schepetilnikov and Ryabova, 2018; Wu et al., 2019). TORC1 also seems to integrate biotic stress signals in plants given that viral and bacterial infections induce this pathway (Schepetilnikov et al., 2011, 2013; Schepetilnikov and Ryabova, 2018). In response to these stimuli, TORC1 not only regulates specific processes, such as mRNA translation and autophagy, but also affects transcriptional and metabolic programs involved in cell division and the synthesis of starch and lipids, the two major forms of carbon and energy storage in plants.

TOR, Raptor, and LST8 homologs have been identified in the model green alga Chlamydomonas (*Chlamydomonas reinhardtii*; Crespo et al., 2005; Díaz-Troya et al., 2008a) and are widely conserved in algal genomes, including freshwater and marine species from distant clades (Shemi et al., 2015). As in plants, TORC2 core proteins Rictor/AVO3 and Sin1/AVO1 are missing in algae (Díaz-Troya et al., 2008b; Shemi et al., 2015). The presence of a large TORC1 complex has been shown in Chlamydomonas. Biochemical evidence indicated that Chlamydomonas LST8 binds to the kinase domain of TOR and that both proteins reside in a high-molecular-mass complex (Díaz-Troya et al., 2008a). Moreover, yeast complementation assays revealed that Chlamydomonas LST8 is functionally and structurally conserved (Díaz-Troya et al., 2008a). Growth of Chlamydomonas is sensitive to rapamycin due to the ability of the immunophilin FKBP12 to bind to this drug (Crespo et al., 2005). The sensitivity of Chlamydomonas to rapamycin has been exploited to investigate TORC1 signaling in this organism. Treatment of Chlamydomonas with rapamycin blocked protein synthesis (Díaz-Troya et al., 2011) and induced autophagy (Pérez-Pérez et al., 2010). Chemical inhibition of TORC1 in Chlamydomonas also resulted in the accumulation of lipid bodies containing TAG and the upregulation of genes involved in TAG synthesis (Imamura et al., 2015).

A recent study in Chlamydomonas used quantitative phosphoproteomics following TORC1 inhibition to identify a number of

proteins with altered phosphorylation that are involved in translation, carotenoid biosynthesis, or autophagy (Roustan and Weckwerth, 2018; Werth et al., 2019). Metabolomic and transcriptomic studies of Chlamydomonas cells treated with rapamycin showed an important role of TOR in the regulation of primary metabolism, particularly in the de novo synthesis of amino acids (Jüppner et al., 2018; Mubeen et al., 2018). The similarities found between TORC1 inhibition and N limitation suggest that N might regulate TORC1 signaling in Chlamydomonas (Pérez-Pérez et al., 2010, 2017; Imamura et al., 2015; Mubeen et al., 2018). However, there is currently no experimental evidence showing that TORC1 responds to nutrients in Chlamydomonas, largely due to the lack of a TOR kinase activity assay. In this study we aimed to investigate the relationship between phosphorus (P) availability and TOR in Chlamydomonas. Our results demonstrated that P regulates TORC1 signaling via LST8, and we have identified the transcription factor PSR1 as a key component of this pathway.

RESULTS

The *lst8-1* Mutant Is Hypersensitive to TOR Inhibition

To investigate the function of LST8 in Chlamydomonas, we took advantage of a newly available library of Chlamydomonas indexed insertional mutants (Li et al., 2016). Although no insertions were found in the *LST8* coding region, we identified a mutant with a predicted insertion of the *AphVIII* paromomycin resistance cassette in the 3′ UTR of *LST8* (Cre17.g713900; Supplemental Figure 1). We named this allele *lst8-1* and confirmed its insertion location using PCR amplification of flanking sequences (Supplemental Figure 1). We first determined the abundance of the LST8 protein in *lst8-1* using an antibody raised against Chlamydomonas LST8 (Díaz-Troya et al., 2008a) and found it was reduced to ~30 to 40% compared with isogenic wild-type cells (Figure 1A). *lst8-1* cells had no detectable growth defect in standard conditions as defined in Methods (Figure 1B;

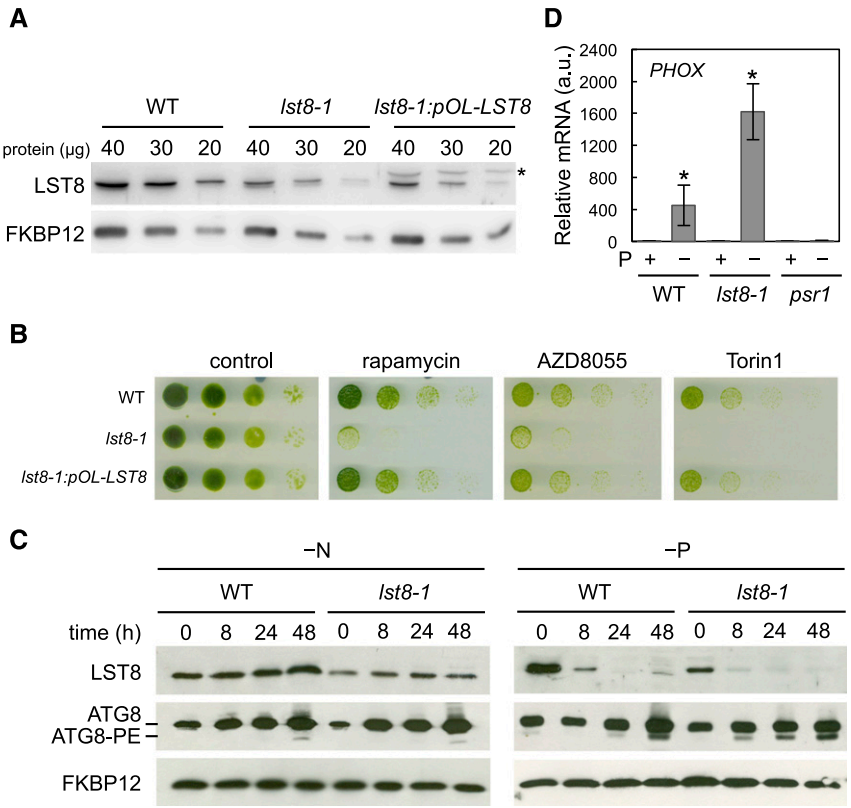

Figure 1. The *lst8-1* Mutant Has Reduced Levels of LST8.

(A) Immunoblot analysis of different amounts of total protein extracts from cells of wild-type (WT), *lst8-1*, and the complemented line *lst8-1:pOL-LST8*. Endogenous LST8 and ectopic OLLAS-tagged LST8 (asterisk) were detected using the LST8 antibody. FKBP12 was used as the loading control.

(B) 10-fold serial dilutions of Chlamydomonas wild-type, *lst8-1*, and *lst8-1:pOL-LST8* cells were spotted onto TAP agar plates containing 500 nM rapamycin, 500 nM Torin1, or 500 nM AZD8055. TAP plates were used as a control.

(C) Immunoblot analysis of LST8 and ATG8 in wild-type and *lst8-1* cells under N or P limitation for 0, 8, 24, and 48 h. Lipidated ATG8 (ATG8-PE) is indicated at the left. FKBP12 was used as the loading control.

(D) RT-qPCR analysis of *PHOX* mRNA abundance in wild-type, *lst8-1*, and *psr1* Chlamydomonas cells shifted to P starvation for 24 h (a.u., arbitrary units). Three biological replicates with three technical replicates were analyzed for each condition. Error bars represent SD of the mean values. Asterisks represent significant differences according to Student's *t* test, $P < 0.01$ (Supplemental File).

Supplemental Figure 2), but their growth was hypersensitive to the TOR inhibitors rapamycin, AZD8055, and Torin1 (Figure 1B). To rescue the *lst8-1* mutation, LST8 tagged at the N terminus with the OLLAS epitope tag was expressed under the control of the HSP70A/RbcS2 promoter. The expression of OLLAS-tagged LST8 (OL-LST8) allowed simultaneous detection of the endogenous and tagged forms of this protein with the LST8 antibody (Figure 1A). The *lst8-1:pOL-LST8*-expressing strain had the same sensitivity to TOR inhibitors as did wild-type cells (Figure 1B), indicating that the hypersensitivity of *lst8-1* cells to these inhibitors was due to the decreased abundance of LST8 in this mutant.

LST8 Abundance Decreases When Starved for P

The TOR kinase regulates cell growth by monitoring the availability of nutrients, and inhibition of the TOR signaling pathway elicits cellular responses typical of nutrient starvation such as cell growth arrest and autophagy. We hypothesized that the decreased level of LST8 in the *lst8-1* mutant might affect the response of these cells to nutrient limitation. Given that TOR responds to N limitation in yeasts and plants, we first monitored growth of wild-type and *lst8-1* cells under N limitation. However, the *lst8-1* mutant displayed similar growth kinetics, as did wild-type cells in low-N medium where N levels are limiting for growth rate and culture density (Supplemental Figure 2A). Moreover, N limitation triggered autophagy with the same kinetics in both wild-type and *lst8-1* cells (Figure 1C).

P, like N, is an essential macronutrient and is among the elements considered most limiting to plant growth (Raghothama, 1999). Therefore, the response of the *lst8-1* mutant to P starvation was also assessed. Unlike the case for N limitation, growth of *lst8-1* cells was hypersensitive to P starvation (Supplemental Figure 2B). Expression of OLLAS-LST8 in the *lst8-1* mutant restored the P deprivation growth response to that of wild-type cells (Supplemental Figure 2B). We also analyzed LST8 protein abundance in wild-type and *lst8-1* cells under N or P starvation. Interestingly, in wild-type cells, the level of LST8 protein decreased within 8 h following P starvation, whereas no effect on LST8 abundance was observed in response to N limitation (Figure 1C). The reduced LST8 abundance observed in wild-type cells upon P starvation preceded the activation of autophagy, which took place after 24 to 48 h of P deprivation (Figure 1C; Couso et al., 2018). LST8 protein abundance also decreased in *lst8-1* cells following 8 h of P starvation to nearly undetectable levels (Figure 1C). However, in contrast to wild-type cells, lipidation of ATG8 was detected within 8 h of P starvation in *lst8-1* cells (Figure 1C), indicating a faster activation of autophagy in this mutant by P starvation. In contrast, induction of autophagy under N limitation occurred with similar timing in wild-type and *lst8-1* cells (Figure 1C). In addition to rapid increases in autophagy under P-limited conditions, *lst8-1* cells also displayed higher mRNA levels for the alkaline phosphatase PHOX (Figure 1D), which is strongly upregulated by P starvation (Moseley et al., 2006). In summary, our results suggest that modulation of LST8 protein levels play an important role in the P-deprivation response in Chlamydomonas.

TOR Regulates Phosphorylation of RPS6 on Ser-245

To further characterize the phenotype of *lst8-1* cells, we developed a method to monitor TOR kinase activity. Because previous efforts to detect TORC1 activity in Chlamydomonas with commercial anti-phosphoS6K antibodies have failed (Couso et al. 2016; Werth et al., 2019), we instead generated antibodies against Chlamydomonas RPS6, a well-defined target of S6K whose phosphorylation is controlled by TORC1 signaling in different organisms, including plants (Dobrenel et al., 2016b). An amino acid alignment of the C terminus of Chlamydomonas, Arabidopsis, yeast (*Saccharomyces cerevisiae*), and human RPS6 proteins revealed that Ser-245 from Chlamydomonas is highly conserved in other RPS6 proteins (Figure 2A). Because this Ser residue is phosphorylated by S6K in a TORC1-dependent manner in both the yeast and human RPS6 proteins (Dobrenel et al., 2016b), we generated antibodies that specifically recognize phosphorylated Ser-245 from Chlamydomonas RPS6. Experiments designed to validate these antibodies indicated that phosphorylation on Ser-245 is indeed regulated by TORC1 in Chlamydomonas. Results showed that phosphorylation on Ser-245 strongly decreased in Chlamydomonas cells treated with the TOR inhibitors rapamycin, Torin1, and AZD8055 (Figure 2B). The Chlamydomonas *rap2* mutant, which lacks FKBP12 and is not sensitive to rapamycin, served as a control for off-target effects of this drug (Crespo et al., 2005). Results showed that rapamycin had no effect on the phosphorylation of Ser-245 in *rap2* mutant cells, whereas Torin1 and AZD8055, which directly target the kinase domain of TOR, decreased phosphorylation (Figure 2B). Taken together, these results indicate that phosphorylation of RPS6 on Ser-245 can be used to monitor TOR function in Chlamydomonas.

TORC1 Signaling Is Attenuated in the Chlamydomonas *lst8-1* Mutant

The hypersensitivity of *lst8-1* cells to TOR inhibitors (Figure 1B) suggested decreased TORC1 activity in this mutant. To explore this hypothesis, we analyzed the phosphorylation state of RPS6 on Ser-245 in wild-type, *lst8-1*, and complemented *lst8-1:pOL-LST8* strains. Decreased RPS6 phosphorylation was detected on Ser-245 in *lst8-1* cells grown in P-replete medium, which could be recovered in the rescued line (Figure 2C). The abundance of TOR kinase protein was examined in these strains with an antibody raised against Chlamydomonas TOR (Díaz-Troya et al., 2008a), and no significant effect was observed in the *lst8-1* mutant (Figure 2C). From these results, we concluded that the low abundance of LST8 protein in *lst8-1* cells downregulates TORC1 activity in this mutant without affecting the overall stability and abundance of TOR kinase.

Loss of LST8 protein abundance under P starvation suggested that TORC1 signaling responds to this nutrient in Chlamydomonas. We examined this possibility further by analyzing the phosphorylation of RPS6 in Chlamydomonas cells shifted to P-free medium. Previous work from our laboratory has shown that P deprivation triggers autophagy in Chlamydomonas, which leads to the degradation of RPS6 in the vacuole (Couso et al., 2018). Activation of autophagy by P starvation is not a fast process in

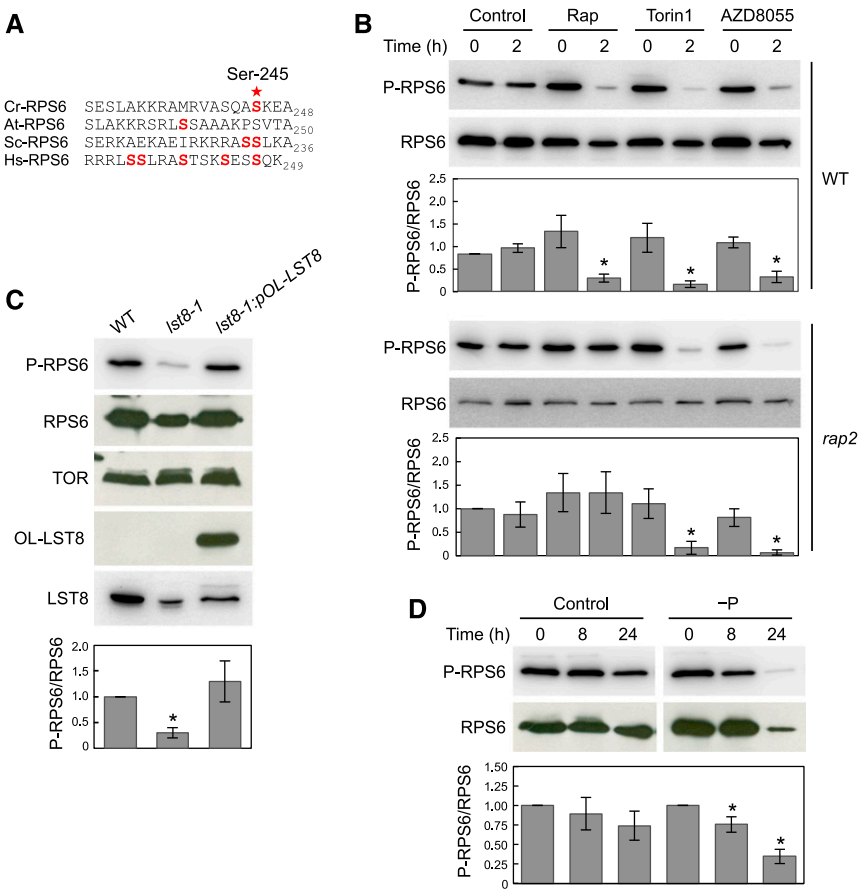

Figure 2. TORC1 Kinase Activity Is Downregulated in the *lst8-1* Mutant.

(A) Amino acid alignment of the C termini of RPS6 proteins from Chlamydomonas (Cr), *Arabidopsis thaliana* (At), *Saccharomyces cerevisiae* (Sc), and *Homo sapiens* (Hs). Ser residues whose phosphorylation is regulated by TOR are shown in red. Chlamydomonas Ser-245 is highlighted and marked with an asterisk.

(B) TOR regulates phosphorylation of RPS6 on Ser-245. Immunoblot analysis of RPS6 and phosphorylated RPS6 (P-RPS6) in wild-type (WT; top) and *rap2* cells (bottom) under control conditions or following TOR inhibition with 500 nM rapamycin (Rap), 500 nM Torin1, or 500 nM AZD8055. TORC1 activity was determined as the ratio of phosphorylated to total RPS6 protein. At least two biological replicates were analyzed for quantification. Error bars represent SD of the mean values. Asterisks represent significant differences according to Student's *t* test, $P < 0.05$ (Supplemental File).

(C) Immunoblot analysis in total protein extracts from wild-type, *lst8-1*, and *lst8-1:pOL-LST8* strains using antibodies against P-RPS6, RPS6, TOR, OLLAS, and LST8. Cells were grown in TAP medium to exponential phase. TORC1 activity was determined as described in (B).

(D) Immunoblot analysis of P-RPS6 and total RPS6 proteins in wild-type cells subjected to P starvation for 0, 8, and 24 h. Cells shifted to P-replete medium were used as the control. TORC1 activity was determined as described in part **(B)**.

Chlamydomonas (Couso et al., 2018), likely due to the large reservoir of polyphosphates it stores under P-replete conditions (Ruiz et al., 2001). Accordingly, lipidation of ATG8 is not detected until 24/48 h of P starvation (Figure 1C; Couso et al., 2018). In contrast, we found that RPS6 phosphorylation on Ser-245 decreased after 8 h of P deprivation, while the level of RPS6 protein remained stable within this time (Figure 2D). These results indicated that P starvation inhibits TORC1 signaling in Chlamydomonas in concert with reduced LST8 levels.

PSR1 Is a Key Component of the TORC1 Signaling Pathway

In Chlamydomonas, P starvation responses are regulated by PSR1, a conserved Myb family transcription factor whose orthologs regulate P deprivation responses in land plants (Shimogawara et al., 1999; Wykoff et al., 1999; Moseley et al., 2006; Bajhaiya et al., 2016). This protein regulates inorganic phosphate (Pi) uptake through transcriptional upregulation of genes for Pi transporters and phosphatases. Consequently, *psr1* mutant cells are defective in specific responses to P starvation (Shimogawara et al., 1999; Wykoff et al., 1999). Here, we found that the *psr1* mutant is partially resistant to the TOR inhibitors rapamycin, AZD8055, and Torin1 (Figure 3A), suggesting a link between PSR1 and TORC1 signaling in Chlamydomonas. To investigate this potential link, the abundance of LST8 in the *psr1* mutant was determined, and we found abnormal regulation of LST8 in *psr1*. In P-replete medium, the level of LST8 protein was lower in *psr1* than in wild-type cells, similar to the levels we found in

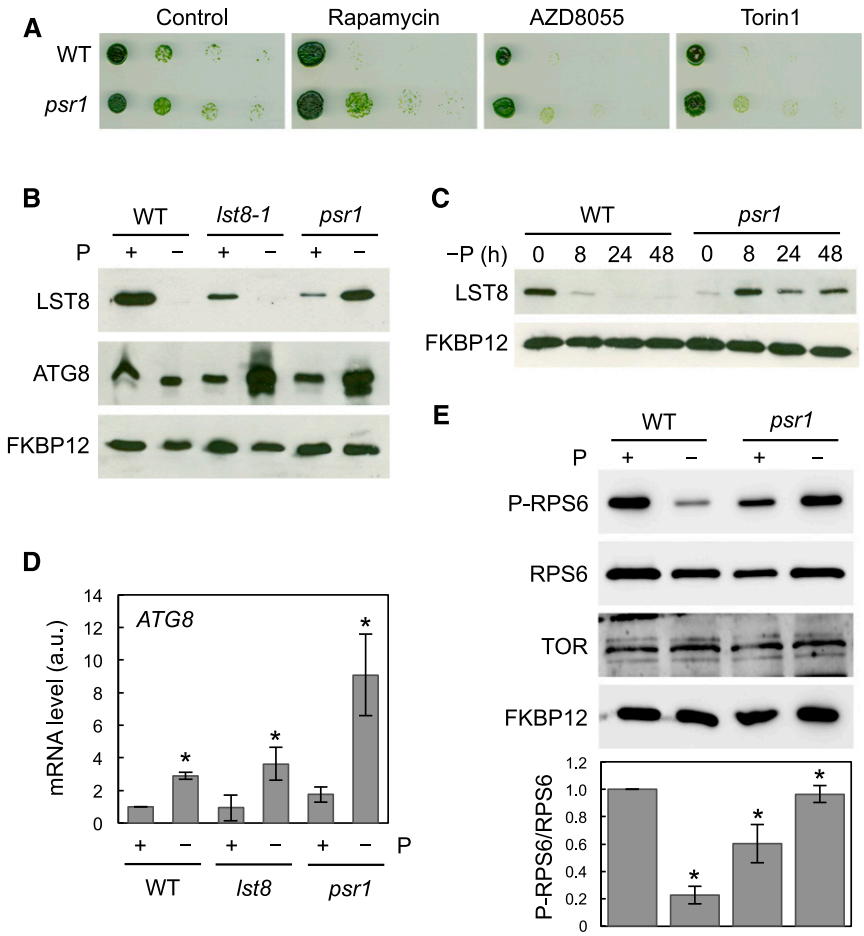

Figure 3. PSR1 Is a Key Component of the TORC1 Signaling Network.

(A) 10-fold serial dilutions of wild-type (WT; CC-125) and *psr1* (CC-4267) cells were spotted onto TAP agar plates containing 500 nM rapamycin, 500 nM Torin1, or 500 nM AZD8055.

(B) Immunoblot analysis of LST8, ATG8, and FKBP12 proteins in wild-type, *lst8-1*, and *psr1* cells. When indicated, cells were subjected to P limitation for 24 h. FKBP12 was used as the loading control.

(C) Immunoblot analysis of LST8 and FKBP12 proteins in wild-type and *psr1* strains. Cells were shifted from P-replete to P-free medium for 0, 8, 24, and 48 h. FKBP12 was used as the loading control.

(D) Analysis of *ATG8* transcript abundance by RT-qPCR in wild-type, *lst8-1*, and *psr1* cells shifted from P-replete to P-free medium for 24 h (a.u., arbitrary units). Three biological replicates with three technical replicates were analyzed for each condition. Error bars represent standard deviations of the mean values, and asterisks represent SD according to Student's *t* test, $P < 0.05$ (Supplemental File).

(E) Immunoblot analysis of P-RPS6, RPS6, FKBP12, and TOR proteins in wild-type and *psr1* cells grown in TAP medium to exponential phase and subjected to P starvation for 24 h. TORC1 activity was determined as the ratio of phosphorylated and total RPS6 protein. Three replicates were analyzed for quantification ($P < 0.05$).

the *lst8-1* mutant (Figure 3B). Moreover, unlike the case in wild type, LST8 was not downregulated in the *psr1* mutant under P starvation. Instead, the abundance of LST8 increased and remained high after 48 h of P starvation (Figures 3B and 3C). Activation of autophagy in *psr1* mutant cells in response to P starvation was also examined. The basal level of autophagy in this mutant was comparable to the levels in wild-type and *lst8-1* strains in P-replete medium. However, ATG8 abundance strongly increased in *psr1* and *lst8-1* mutants, but not in wild-type cells following 24 h of P deprivation (Figure 3B), indicating a rapid activation of autophagy in these mutants. In close agreement, *ATG8* mRNA levels accumulated to higher levels in *psr1* mutant cells either subjected to P starvation (Figure 3D) or treated with rapamycin (Supplemental Figure 3).

To further investigate the relationship between PSR1 and TORC1, we analyzed TORC1 activity in wild-type and *psr1* strains under P-replete versus P-starvation conditions by monitoring RPS6 phosphorylation on Ser-245. In P-replete medium, decreased phosphorylation of RPS6 was observed in *psr1* mutant cells (Figure 3E), similar to what was observed in the *lst8-1* mutant (Figure 2C). Remarkably, P deprivation had opposite effects on TORC1 activity in wild-type and *psr1* cells. While P starvation in

wild-type cells led to reduced RPS6 phosphorylation, a significant increase was detected in the *psr1* mutant under the same nutrient stress (Figure 3E). We also analyzed whether enhanced TORC1 activity in P-starved *psr1* cells might be due to an increase in the abundance of TOR, but the level of this protein remained stable (Figure 3E). These results indicated that TORC1 activity is misregulated in *psr1* mutant cells under P deprivation, suggesting that PSR1 might participate in the regulation of TORC1 in response to P availability (Figure 3B).

TORC1 regulates nutrient-responsive transcription factors in yeast, mammals, and plants (Crespo and Hall, 2002; Dobrenel et al., 2016a; Saxton and Sabatini, 2017; Wu et al., 2019). Thus, we next explored whether PSR1 might be regulated by TORC1 in Chlamydomonas. Given that *PSR1* transcript abundance increases around eightfold under P starvation (Moseley et al., 2006; Bajhaiya et al., 2016), we analyzed PSR1 mRNA levels in rapamycin-treated cells. qPCR analysis revealed a five- to sixfold increase in the *PSR1* transcript level in TOR-inhibited cells (Supplemental Figure 4). However, we failed to detect upregulation of *PHOX*, a PSR1-regulated gene whose expression is strongly induced under P starvation (Moseley et al., 2006). These results suggest that inhibition of TORC1 signaling by rapamycin is not sufficient to elicit a P starvation response in Chlamydomonas.

lst8-1 Cells Accumulate High Levels of Triacylglycerol

TORC1 is an important regulator of lipid metabolism in Chlamydomonas, and the inhibition of this signaling pathway results in the accumulation of triacylglycerol (TAG) (Imamura et al., 2015). Given the low TORC1 activity of *lst8-1* mutant, we analyzed the level of TAG in this strain using Nile red, a dye that fluoresces upon binding to neutral lipids such as TAG. Our results indicated that *lst8-1* cells accumulate more TAG than wild-type cells in P-replete medium (Figures 4A and 4B), which agrees with the lower TORC1 activity detected in *lst8-1* cells (Figure 2C). The rescued *lst8-1:pOL-LST8* strain displayed a similar lipid body content compared with wild-type cells (Figures 4A and 4B). TAG content was also measured by gas chromatography and was significantly increased in the *lst8-1* mutant (Figure 4C). In Chlamydomonas, P starvation leads to the accumulation of TAGs (Bajhaiya et al., 2016; Couso et al., 2018), and this induction was strongly enhanced in the *lst8-1* strain compared with wild-type or rescued strains (Figures 4A to 4C).

DISCUSSION

The TORC1 multiprotein complex is a central regulator that promotes cell growth in response to nutrients in all eukaryotes. In yeast, preferred N sources are converted to Glu and stimulate TORC1 activity. In animal cells, mTORC1 responds to glucose and amino acids, particularly Leu and Glu, directly or via glutaminolysis and α-ketoglutarate production (Durán et al., 2012; Jewell et al., 2013; Saxton and Sabatini, 2017). In plants, TORC1 activity is regulated by intracellular sugar availability, energy, N, and sulfur (Dobrenel et al., 2016a; Dong et al., 2017; Schepetilnikov and Ryabova, 2018; Wu et al., 2019). This study shows a link connecting P starvation to TORC1 signaling in Chlamydomonas through modulation of the TORC1 subunit LST8.

First, we developed and validated an assay for RPS6 phosphorylation, which for the first time enables us to measure TORC1 activity in Chlamydomonas. Phosphorylation of RPS6 at the C terminus is regulated by the highly conserved TORC1-S6K pathway and has been used to monitor TORC1 activity in different organisms, including Arabidopsis (Dobrenel et al., 2016b). Here, we demonstrate that RPS6 phosphorylation on Ser-245 is controlled by TORC1 in Chlamydomonas, and this modification can be used to determine TORC1 activity in this organism (Figure 2). A mass spectrometry approach has also shown that phosphorylation of RPS6 on Ser-245 is regulated by N limitation in Chlamydomonas (Roustan et al., 2017). Using our TORC1 activity assay, we showed that P starvation inhibited TORC1 activity (Figure 2D) and that this reduced activity was associated with a large reduction in LST8 protein abundance, which occurred under P stress and was not observed under N limitation (Figure 1C). LST8 is a core component of both TORC1 and TORC2, and it is needed for TOR activation (Loewith et al., 2002; Kim et al., 2003; Wullschleger et al., 2005). We therefore propose that inhibition of TORC1 by P starvation is linked to the downregulation of LST8 protein abundance.

How is LST8 degraded in Chlamydomonas cells under P starvation? While P deprivation triggers autophagy in Chlamydomonas (Couso et al., 2018), the decrease of LST8 preceded the activation of this catabolic process (Figure 1C), suggesting that this protein is not degraded via autophagy. Another possibility is that LST8 is degraded by the proteasome. In mammals, mLST8 is polyubiquitinated, although this modification seems to balance the presence of mLST8 in TORC1, and TORC2 and has no effect on the stability of the protein (Wang et al., 2017).

Genetic evidence also supports a link between P and TORC1 signaling in Chlamydomonas via modulation of LST8. The *lst8-1* mutant has reduced levels of LST8 protein, most likely due to a reduction in LST8 mRNA or its translation caused by insertion of a marker gene in its 3′ UTR (Figures 1A; Supplemental Figure 1), and it also has reduced TORC1 activity (Figure 2C). P is an essential nutrient for anabolic processes such as DNA replication, ribosome biogenesis, and translation (Raghothama, 1999), and therefore it is conceivable that TORC1 senses P availability to regulate these processes. To our knowledge, phosphate starvation sensing by TORC1 has previously only been shown in the human fungal pathogen *Candida albicans*. A recent study revealed that *C. albicans* TORC1 monitors P availability in addition to N and C (Liu et al., 2017). Signaling of P availability via TORC1 in *C. albicans* requires the Pi transporter Pho84 (Liu et al., 2017), but the role of LST8 was not examined.

TOR has also been connected to inositol polyphosphate (InsP) metabolism in Chlamydomonas. The *vip1-1* mutant is defective in an inositol hexakisphosphate kinase, resulting both in decreased $InsP_7$ and $InsP_8$ levels and in hypersensitivity to TOR inhibitors. This suggests that TOR signaling is impaired in this mutant (Couso et al., 2016). Whether InsPs regulate TOR is currently unknown. TORC1 may also sense other nutrients such as N, but this has not been yet demonstrated in Chlamydomonas. The well-characterized molecular mechanisms connecting TOR to nutrients in yeast and mammals will likely differ in algae and plants, since upstream regulators of this pathway such as Gtr1/2, RHEB, or TSC1/2 do not seem to be conserved in the green lineage

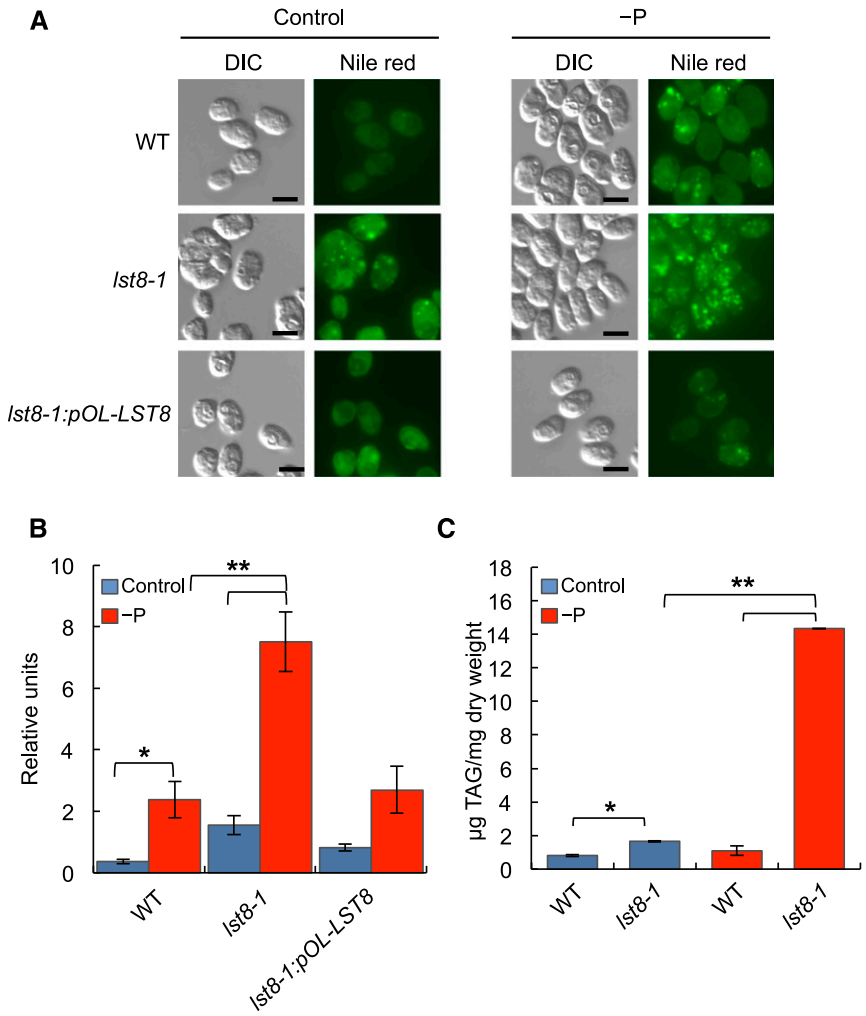

Figure 4. *lst8-1* Mutant Cells Accumulate High Levels of TAG.

(A) Chlamydomonas wild-type (WT), *lst8-1*, and *lst8-1:pOL-LST8* cells were grown in TAP medium or subjected to P starvation (−P) for 24 h. Lipid bodies were stained with Nile red and imaged by differential interference contrast (DIC) and fluorescence microscopy. Bar, 8 μm.
(B) Nile red fluorescence was analyzed and quantified using Image J software in the indicated strains (see Methods). R.U., relative units.
(C) Quantification of TAG from wild-type and *lst8-1* cells under P replete or P limitation during 24 h. For **(B)** and **(C)**, error bars represent SD of the mean values, and asterisks represent significant differences at $^{*}P < 0.05$ or $^{**}P < 0.001$ according to the Student's *t* test (Supplemental File). The analyses in **(B)** were done on ~100 cells per strain/condition. For **(C)**, four biological replicates were analyzed for each strain/condition.

(Dobrenel et al., 2016a). In plants, it was recently proposed that TORC1 acts as a central integrator to perceive and transmit C, N, S, and P nutrient signals to promote cell growth (Wu et al., 2019), although this model awaits experimental verification.

P metabolism is controlled by the transcription factor PSR1 in Chlamydomonas. PSR1 governs the uptake and assimilation of Pi through the transcriptional activation of genes encoding Pi transporters and phosphatases (Shimogawara et al., 1999; Wykoff et al., 1999; Bajhaiya et al., 2016) and has also been connected to the modification of nucleic acid metabolism to optimize P reallocation within cells under P deprivation (Yehudai-Resheff et al., 2007). In this study, we found a novel link between TORC1 signaling and PSR1 that further supports a specific connection between P starvation responses and TORC1 signaling in Chlamydomonas. Our results indicated that TORC1 signaling is misregulated in the *psr1* mutant (Figure 3E). *psr1* mutant cells are defective in a number of specific responses to P-limited conditions, including upregulation of Pi scavenging and uptake genes under this nutrient stress (Shimogawara et al., 1999; Wykoff et al., 1999). We found that *psr1* cells have reduced LST8 protein under P-replete conditions but fail to down-regulate LST8 and TORC1 activity upon P starvation. In wild-type cells, LST8 abundance decreased following P starvation. This is in striking contrast to the increase in LST8 protein detected in *psr1* cells under the same nutrient deficit.

The misregulation of LST8 protein levels and TORC1 activity in P-starved *psr1* mutant cells provides further evidence that TORC1 activity is a downstream effector of PSR1 during P starvation,

though the signaling relationship between these two pathways may be more complex. Notably, *PSR1* mRNA levels, which normally increase under P deprivation, were also increased by TORC1 inhibition, though this increase in PSR1 mRNA did not lead to increases in a *PSR1* downstream target gene mRNA (Supplemental Figure 4). Given the key role that PSR1 plays in the cellular response to P starvation and that this nutrient regulates TORC1 signaling in Chlamydomonas, we favor a model (Figure 5) in which the high TORC1 activity in *psr1* mutant cells under P deficiency might be due to the loss of P homeostasis. The *psr1* mutant was originally isolated by its inability to properly acclimate to P starvation (Shimogawara et al., 1999). The link between PSR1 and TORC1 reported in this study suggests that the phenotype of the *psr1* mutant is a result of defective P sensing and/or P starvation signaling in Chlamydomonas.

TOR is a key regulator of TAG synthesis in green and red algae (Imamura et al., 2015), and PSR1 was recently identified as an important regulator of lipid metabolism in Chlamydomonas (Ngan et al., 2015; Bajhaiya et al., 2016). A link between TORC1 signaling, P homeostasis, and PSR1 is further supported by our finding that *lst8-1* mutant cells synthesized and accumulated higher levels of TAG compared with wild-type cells under both P-replete and P-limited conditions (Figure 4C). Therefore, we propose that the large amount of TAG synthesized in *lst8-1* mutant cells under P starvation is due to defective signaling of this nutrient by TORC1 and the loss of P homeostasis. The precise mechanism by which TORC1 perceives P availability in Chlamydomonas is currently unknown, although our results pinpoint LST8 as a key protein connecting P sensing to TORC1 activity.

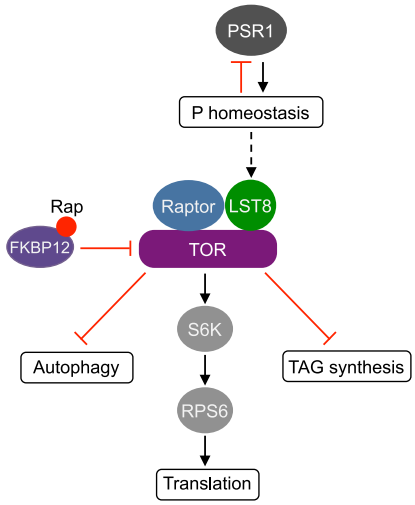

Figure 5. Proposed Model for P-Regulated TORC1 Signaling in Chlamydomonas.

P availability is sensed by TORC1 via subunit LST8. In the presence of P, TORC1 is active and promotes translation via the S6K/RPS6 pathway while negatively regulating autophagy and TAG synthesis. P deprivation activates PSR1, which is required to maintain P homeostasis through the transcriptional activation of genes encoding Pi transporters and phosphatases. Decreased intracellular P levels negatively affect LST8 protein stability by an unidentified mechanism (dashed arrow), resulting in downregulation of TORC1 activity.

METHODS

Strains, Media, and Growth Conditions

Chlamydomonas (*Chlamydomonas reinhardtii*) wild-type CC-4533 (mt−), *lst8-1* LMJ.RY0402.210199 (mt−; Li et al., 2016), *psr1* CC-4267 (mt−), and its parental strain CC-125 (mt+; Shimogawara et al., 1999) were obtained from the Chlamydomonas Resource Center (http://www.chlamycollection.org). *lst8-1* complemented strain expressing OL-LST8 was generated in this study as described below. Chlamydomonas cells were grown under continuous illumination (50 $\mu E\ m^{-2}\ s^{-1}$ from Systion light-emitting diodes with a correlated color temperature rating of 4000K) at 25°C in Tris-acetate phosphate (TAP) pH 7.0 medium as described (Harris, 1989), Tris-acetate pH 7.0 medium for P starvation experiments (Quisel et al., 1996) or low N TAP medium containing 1 mM ammonium chloride. Growth curves were constructed using mean values of optical density 750 nm measurements performed in triplicate. When required, cells in exponential growth phase (10^6 cells mL^{-1}) were treated with rapamycin (Cayman Chemical; 53123-88-9), AZD 8055 (Cayman Chemical; 1009298-09-2), or Torin1 (Cayman Chemical; 1222998-36-8.

lst8-1 Genetic Analysis and Genotyping

Gamete preparation, mating, and segregation analyses were done as described previously (Harris, 1989). Random meiotic progeny isolated from thousands of zygote colonies were individually grown in 96-well microtiter plates and pinned onto agar media plates supplemented with paromomycin or rapamycin. Half of the analyzed colonies were resistant to these drugs, indicating their genetic linkage. Segregation (2:2) was then checked. Genotyping was done using 0.5 μL of total genomic DNA prepared using a fast method described at http://www.chlamycollection.org/methods/quick-and-easy-genomic-dna-prep-for-pcr-analysis/. The LST8 wild-type allele was scored using LST8 primers (5′ and 3′; Supplemental Figure 1; Supplemental Table 1), and the *lst8-1* allele was scored using a combination of LST8, IMP5′, and IMP3′ primers (Supplemental Figure 1; Supplemental Table 1). PCR was performed using Taq polymerase in a final reaction volume of 20 μL in the presence of 1× Taq buffer, 1 μM primers, 80 μM deoxynucleotide triphosphate, 2% DMSO, and 0.5 μL of genomic DNA. PCR conditions were as follows: 96°C for 2 min and then 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

Complementation of the *lst8-1* Mutant Using OL-LST8

Chlamydomonas genomic DNA was isolated according to Crespo et al. (2005) and was used as a template for PCR amplification of three fragments containing the complete *LST8* gene. The OLLAS tag was inserted into the LST8 C terminus using the following combination of primers: LST8F1 with LST8_OLL_R and LST8_OLL_F with LST8R1 (Supplemental Table 1). The PCR products were gel purified and cloned into pBluescript-SK (Agilent) using an In-Fusion HD kit (Clontech) following the manufacturer's instructions. pHYG, which confers resistance to hygromycin, was used together with the previous construct to cotransform *lst8-1* cells. Positive clones expressing OL-LST8 were selected by immunoblot blot analysis of hygromycin-resistant clones with an anti-OLLAS antibody. A single band with the expected molecular mass (34 kD) was detected by immunoblot in total extracts from Chlamydomonas cells expressing OL-LST8.

Protein Preparation and Immunoblot Analysis

Chlamydomonas cells from liquid cultures were collected by centrifugation (4000*g* for 5 min), washed in 50 mM Tris-HCl (pH 7.5) buffer, and resuspended in a minimal volume of the same solution. Cells were lysed by

two cycles of slow freezing to −80°C followed by thawing at room temperature. The soluble cell extract was separated from the insoluble fraction by centrifugation (15,000*g* for 20 min) in a microcentrifuge at 4°C. For immunoblot analyses, total protein extracts (20 to 30 μg) were subjected to 7.5% (TOR determination), 12% (LST8, P-RPS6, and RPS6 determination), or 15% (FKBP12 and ATG8 determination) SDS-PAGE and then transferred to polyvinylidene fluoride membranes previously activated in methanol (Millipore, IPVH00010). Primary antibodies raised against CrTOR (Díaz-Troya et al., 2008a), CrLST8 (Díaz-Troya et al., 2008a), CrATG8 (Pérez-Pérez et al., 2010), CrFKBP12 (Crespo et al., 2005), CrRPS6, P-CrRPS6 (this work), and anti-OLLAS (Thermo Scientific, MA5-16125/RE2204908), were diluted 1:1000, 1:2000, 1:3000, 1:5000, 1:2000, 1:2000, and 1:1000, respectively. Secondary anti-rat (Thermo Fisher Scientific, A18866/QD217492) and anti-rabbit (Sigma, A6154/SLBV9141) antibodies were diluted 1:5000 and 1:10,000, respectively, in phosphate-buffered saline, pH 7.4, containing 0.1% (v/v) Tween-20 (Applichem, A4974) and 5% (w/v) milk powder. The Luminata Crescendo Millipore immunoblotting detection system (Millipore, WBLUR0500) was used to detect the proteins. Proteins were quantified using the Coomassie dye binding method (Bio-Rad, 500-0006).

Nile Red Staining and Fluorescence Microscopy

Chlamydomonas wild type, *lst8-1*, and the complemented strain *lst8-1:pOL-LST8* were treated as follows under P-replete and P-depleted conditions for 24 h. Cells were fixed on ice for 20 min with 2% paraformaldehyde (Sigma-Aldrich, 158127) and then washed with phosphate-buffered saline buffer twice. Lipid body staining was performed as described (Couso et al., 2018). Microscopy was performed with a DM6000B microscope (Leica) using a ×100 oil immersion objective with differential interference contrast optics or wide field fluorescence equipped with an L5 filter cube (Leica; excitation bandpass 480/40 nm; dichroic 505 nm; emission bandpass 527/530 nm) and an ORCAER camera (Hamamatsu). After visualization of lipid bodies using Nile red staining, we made a first approximation of lipid body numbers per cell using a semiquantitative method. Image J (https://imagej.nih.gov/ij) particle count analysis was done on ~100 cells per strain and condition. Errors bars indicate SD of the values.

Lipid Analysis

TAGs were analyzed as previously described (Couso et al., 2018). In brief, 4 mL of $CHCl_3$:methanol (2:1) were added to 20 mg of freeze-dried cells and then mixed by vortexing. Samples were heated at 42°C for 30 min followed by addition of 2.5 mL of 0.1 N HCl:1 M NaCl and additional mixing by vortexing. Samples were centrifuged for 2 min at 500*g* at room temperature and then the aqueous (upper) phase was discarded. The organic phase was washed twice with ultrapure water and then dried under nitrogen gas. Samples were resuspended in 1 mL of hexane. The analysis of TAGs was performed by injecting 1 μL of the lipid extraction into the gas chromatograph (Agilent 6890 GC), using hydrogen as the carrier gas. The injector and detector temperatures were both 370°C, the oven temperature was 335°C, and a head pressure gradient from 70 kPa to 120 kPa was applied. The GC column used was a Quadrex Aluminum-Clad 400-65HT (30 m length, 0.25 mm inside diameter, 0.1 μm film thickness; Woodbridge), and a linear gas rate of 50 cm s^{-1}, a split ratio 1:80, and a flame ionization detector were used. The TAG species were identified according to Fernández-Moya et al. (2000) and quantified by applying the correction factors reported by Carelli and Cert (1993). TAG content was normalized to cellular dry weight. Four biological replicates were analyzed for each condition. Errors bars indicate SD of the values.

RNA Isolation and RT-qPCR Analysis

Chlamydomonas cells were collected by centrifugation (4000*g*, 5 min) and washed once in 50 mM Tris-HCl (pH 7.5) buffer, and then cell pellets were frozen in liquid N and immediately stored at −80°C until use. Total RNA was isolated from frozen cell pellets as described previously (Crespo et al., 2005). First-strand cDNA was generated using the iScript cDNA Synthesis kit (BioRad; 170-8891) in a 20-μL reaction mixture containing 1 μg of total RNA. qPCR was performed on an iCycler apparatus (Bio-Rad). The PCR mixtures were performed in a final volume of 18 μL using the SsoAdvanced Universal SYBR Green Supermix (BioRad; 172-5271). The data were normalized to CBLP expression, a constitutively expressed gene encoding a protein homologous to the β-subunit of a G protein that is used as an internal control. The primer pairs used in this study are listed in Supplemental Table 1. All reactions were performed in triplicate with three biological replicates.

Accession Numbers

Sequence data for this article can be found in the Phytozome (https://phytozome-next.jgi.doe.gov), Yeast Genome (https://www.yeastgenome.org), TAIR (https://www.arabidopsis.org/index.jsp), and NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) databases under the following accession numbers: *Cr-TOR* (Cre09.g400553); *Cr-LST8* (Cre17.g713900); *Cr-PSR1* (Cre12.g495100); *Cr-ATG8* (Cre16.g689650); *Cr-FKBP12* (Cre09.g400553); *Cr-RPS6* (Cre09.g400650); *Cr-PHOX* (Cre04.g216700); *Cr-CBLP* (Cre06.g278222); *At-RPS6* (At4g31700); *Sc-RPS6* (S000000385); *Hs-RPS6* (gene ID 6194). Strains used were wild-type CC-4533 (mt−), *lst8-1* LMJ.RY0402.210199 (mt−; Li et al., 2016), *psr1* CC-4267 (mt−), and its parental strain CC-125 (mt+).

Supplemental Data

Supplemental Figure 1. The *lst8-1* mutant carries an insertion at the *LST8* 3′ UTR

Supplemental Figure 2. Growth of wild-type and *lst8-1* strains under N or P limitation

Supplemental Figure 3. RT-qPCR analysis of *ATG8* in wild-type and *psr1* strains

Supplemental Figure 4. RT-qPCR analysis of *PSR1* and *PHOX* following rapamycin treatment

Supplemental Table 1. Nucleotide sequences of primers used in this study

Supplemental File. Student's *t* test analyses

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AUTHOR CONTRIBUTIONS

J.L.C., M.E.P.-P., and I.C. designed the research. I.C. and M.E.P.-P. performed research. E.M.-F. performed the lipid analysis. I.C., M.E.P.-P.,

M.M.F., E.M.-F., L.M.H., J.G.U., and J.L.C. analyzed data. J.L.C., I.C., and M.E.P.-P. wrote the manuscript with input from the other authors.

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