**SUMO Protease SMT7 Modulates Ribosomal Protein L30 and Regulates Cell-size Checkpoint Function**

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**Short title:**
SUMOylated RPL30 regulates cell size

**One-sentence summary:**
SUMO protease SMT7 regulates cell cycle division number of the mat3 cells through deSUMOylation of SUMOylated RIBOSOMAL PROTEIN L30

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**Abstract**

Proliferating cells actively coordinate growth and cell division to ensure cell-size homeostasis; however, the underlying mechanism through which size is controlled is poorly understood. Defect in a SUMO protease protein, suppressor of mat3 7 (SMT7), has been shown to reduce cell division number and increase cell size of the small-size mutant mating type locus 3-4 (mat3-4), which contains a defective Chlamydomonas retinoblastoma tumor suppressor-related protein. Here we describe development of an in vitro SUMOylation system using Chlamydomonas components and use it to provide evidence that SMT7 is a bona fide SUMO protease. We further demonstrate that the SUMO protease activity is required for supernumerous mitotic divisions of the mat3-4 cells. In addition, we identified RIBOSOMAL PROTEIN L30 (RPL30) as a prime SMT7 target and demonstrated that its SUMOylation is an important modulator of cell division in mat3-4 cells. Loss of SMT7 caused elevated SUMOylated RPL30 levels. Importantly, overexpression of the translational fusion version of RPL30-SUMO4, which mimics elevation of the SUMOylated RPL30 protein in mat3-4, caused a decrease in mitotic division and recapitulated the size-increasing phenotype of the smt7-1 mat3-4 cells. In summary, our study reveals a novel mechanism through which a SUMO protease regulates cell division in the mat3-4 mutant of Chlamydomonas and provides yet another important example of the role that protein SUMOylation can play in regulating key cellular processes, including cell division.
Introduction

Cell size homeostasis in proliferating cells is an evolutionarily conserved trait (Jorgensen and Tyers, 2004; Umen, 2005; Tzur et al., 2009; Goudarzi and Lindstrom, 2016). Cell size control requires coordination of growth and the cell cycle and until now, the underlying mechanism has only been extensively investigated in yeasts. Studies of yeasts have provided crucial evidence that the regulatory topology required for size control is similar to that found in the opisthokont branch of eukaryotes (Cross et al., 2011). In budding yeast, defects in Whiskey 5 (Whi5), the transcriptional inhibitor that controls G1/S transition, cause a small-cell phenotype (Jorgensen et al., 2002). A small-size phenotype is also observed in animals (opisthokonta branch) and the green alga Chlamydomonas (Viridiplantae branch) cells with defective G1/S inhibitors, members of the retinoblastoma (Rb) family (Dannenberg et al., 2000; Sage et al., 2000; Fang et al., 2006) whose sequences are entirely different from Whi5. These findings suggest that the mode of action from similar regulatory circuits rather than homologous proteins have been conserved during evolution.

In addition to the G1/S transition network centered on Rb or Whi5, defects in ribosome biogenesis have been shown to affect cell size. In budding yeast, genome-wide screening for size mutants revealed that defects in Split finger protein 1 (Spf1) transcription factor and Suppressor of cdc25 (Sch9) kinase that govern ribosome biogenesis and translation initiation generate small daughter cells (Jorgensen et al., 2004; Marion et al., 2004; Urban et al., 2007). However, the size threshold of yeasts is not static and is subject to changes in growth rate (Jorgensen et al., 2004; Ferrezuelo et al., 2012; Turner et al., 2012; Chica et al., 2016), a property that makes size control studies in yeasts complicated.

It is extremely challenging to assess cell-size defects in multicellular organisms. Despite this, plant and animal cells within one tissue often display a remarkable uniformity in size (Lloyd, 2013; Ginzberg et al., 2015; Serrano-Mislata et al., 2015; Willis et al., 2016; R. et al., 2017). Recent studies in animal cells reveal that cells adjust both cell cycle length and growth rate to maintain size homeostasis (Cadart et al., 2018; Ginzberg et al., 2018). Growth rate modulation controlled by ribosome-based protein translation has been suggested to regulate size homeostasis (Kafri et al., 2016). Even though deficiencies in the ribosome biogenesis pathway have been found to produce small cells in Drosophila and mouse systems (Montagne et al., 1999; Ruvinsky et al., 2005), evidence of a causal relationship between size homeostasis and ribosome-dependent regulation remains limited.
Protein conjugation by the small-ubiquitin-like modifier (SUMOylation) plays important roles during the cell division cycle (Li and Hochstrasser, 1999; Di Bacco and Gill, 2006; Pelisch et al., 2014). Proteomic studies reveal that many cell cycle-related proteins are conjugated by SUMO (Hendriks et al., 2014; Cubeñas-Potts et al., 2015). Indeed, SUMOylation has been found to be important in regulating several cell cycle proteins. For example, SUMO conjugation of cyclin E contributes to the control of replication firing (Bonne-Andrea et al., 2013). SUMO modification stabilizes cyclin dependent kinase 6 and regulates G1/S transition (Bellail et al., 2014). Forkhead box transcription factor M1 (FoxM1) is extensively SUMOylated and its SUMOylation is important for cell cycle progression and chromosome segregation (Schimmel et al., 2014). SUMO deconjugation enzymes human sentrin-specific protease 1 (SENP1) and SENP2 are important for precise spatial and temporal control of SUMOylation in mitosis (Cubeñas-Potts et al., 2013). Deregulation of these SUMOylation and deSUMOylation enzymes not only causes defects in cell proliferation and genome stability, but recent findings have also shown that different types of cancer cells require a functioning SUMOylation system (Eifler and Vertegaal, 2015).

The unicellular green alga Chlamydomonas reinhardtii (Chlamydomonas) has a well-characterized haploid genetic system (Harris, 1989; Harris and Editor, 2009), sequenced genome (Merchant et al., 2007), relative paucity of gene duplications, a well-equipped molecular toolkit (Leon-Banares et al., 2004; Jinkerson and Jonikas, 2015; Mussgnug, 2015; Shin et al., 2016; Greiner et al., 2017), and recently added mutant libraries (Zhang et al., 2014; Li et al., 2016; Breker et al., 2018) that make it a unique microbial model for cell-size control and cell-cycle studies in the plant superkingdom (Tulin and Cross, 2014; Cross and Umen, 2015; Umen, 2018).

When grown under light-dark cycles (e.g., 12 h light-12 h dark) mimicking the diurnal rhythm found in Nature, Chlamydomonas cells utilize modified cell cycle-multiple fission cell division. Multiple fission is characterized by a long G1 phase, during which cells can grow more than 10 times in size. At the end of G1, mother cells undergo successive rounds of alternating DNA synthesis (S phase) and mitosis (M phase) to produce 2^n daughters of uniform size. At early/mid G1, cells pass the first size checkpoint, “Commitment”, which is equivalent to “Start” in budding yeast (Hartwell et al., 1974; Johnston et al., 1977) and “Restriction Point” in animals (Pardee, 1974; Dolznig et al., 2004), during which cells acquire sufficient mass to
complete the cell cycle. The second size checkpoint occurs during S/M, where mother cells undergo a controlled number of division cycles to generate uniformly-sized daughters. The number of S/M cycles is dictated by the size of mother cells in which larger mother cells divide more times than smaller mother cells, so size homeostasis is achieved (Craigie and Cavaliersmith, 1982; Donnan and John, 1983). Multiple fission allows *Chlamydomonas* cell cultures to be naturally synchronized by an alternating light/dark regime. As a result, cells grow photosynthetically at G1 during the light period, while cell division cycle occurs in the dark. Because cellular functions associated with growth and cell division are temporarily separated, the daughter cell-size can be used as a direct readout to assess the size control mechanism (Umen, 2005; Fang et al., 2006).

The retinoblastoma (Rb) tumor suppressor pathway regulates Commitment size and cell division number in *Chlamydomonas* (Umen and Goodenough, 2001; Fang et al., 2006). Mutations in the *Chlamydomonas Rb* gene, *MATING TYPE LOCUS 3 (MAT3)*, cause these cells to commit at a reduced cell size and to divide supernumerously at the S/M phase in a manner that generates small daughter cells (Umen and Goodenough, 2001). These defects are suppressed by mutations in *E2F* or *DP*, that encode subunits of evolutionarily conserved heterodimeric transcription factor E2F-DP, a regulatory duplex that acts directly downstream of the Rb pathway (Fang et al., 2006). In addition to *E2F* and *DP*, several extragenic partial suppressors of *mat3* (SMTs) have been isolated (Fang and Umen, 2008; Fang et al., 2014). A defect in *SMT7*, which encodes a SUMO protease, in the *mat3* mutant causes size suppression, rendering daughter cells (*smt7-1 mat3-4*) larger than *mat3-4* but smaller than wild-type cells (Supplemental Figure 1).

Interestingly, cells containing the single mutation, *smt7-1*, display no obvious size defect (Fang and Umen, 2008).

Here, we describe establishment of an *in vitro Chlamydomonas* SUMOylation system and provide biochemical evidence that SMT7 possesses SUMO protease activity. In addition, we show that SUMO protease activity of SMT7 is required for MAT3-dependent size checkpoint control. Ribosomal protein L30 (RPL30) was isolated as a SMT7-interacting protein whose SUMO deconjugation is regulated by SMT7. As a result of this interaction, a defect in *SMT7* caused increased levels of RPL30 SUMOylation. Surprisingly, overexpression of RPL30-SUMO4AGG-3XHA protein, which mimics SUMOylated RPL30 protein but not
RPL30-3XHA protein in mat3-4 cells recapitulated smt7-1 mat3-4 cells and led to reduced cell division and size suppression. Together, our study provides unexpected insights into the size-mediated cell division cycle and demonstrates that SUMOylation of a ribosomal protein can have novel regulatory consequences.

Results

Molecular characterization of the SMT7 locus

Even though a defect in a putative SUMO protease SMT7 has been demonstrated to suppress the small cell size of mat3-4 (Fang and Umen, 2008), the structure of SMT7 has not been fully characterized. Despite numerous attempts to amplify SMT7 cDNA, we failed to obtain the full-length cDNA. As an alternative, we combined RT-PCR and 3' RACE-PCR to amplify overlapping SMT7 cDNA fragments (Supplemental Figure 2A) and validate the gene structure of SMT7 (Figure 1A). SMT7 encodes a protein with a distinct N-terminal region followed by a conserved SUMO protease domain (Pfam 02902; Figure 1B). Protein sequence alignment of the SUMO protease domains of SMT7 and SUMO proteases from humans, Arabidopsis, and budding yeast indicated that the canonical catalytic triad (His860-Asp877-Cys928) required for SUMO deconjugation function is evolutionarily conserved (Figure 1C). Phylogenetic analysis revealed that SMT7 is related to SUMO proteases EARLY IN SHORT DAYS4 (ESD4) and its closest homologs (Supplemental Figure 2B). In addition to the SUMO protease domain, one potential nuclear localization sequence (NLS), and two putative SUMO-interacting motifs were identified in the SMT7 protein sequence (Supplemental Figure 3).

SUMO protease activity of SMT7 is required for size control in the mat3 mutant

To detect and purify SMT7 protein, a C-terminal triple hemagglutinin (3XHA) epitope-tagged allele of a genomic SMT7 clone under the control of its native promoter and terminator was constructed (pSMT7-3XHA, Supplemental Figure 4). Immunoblotting with the anti-HA antibody confirmed that the expression of a major protein ran slightly larger than 100 kDa, which is close to the predicted size (~106 kDa) of full-length SMT7-3XHA protein in the smt7-1 mat3-4::SMT7-3XHA strains but not in the untagged wild-type strain (Figure 2A). Expression of SMT7-3XHA protein was able to rescue the function of smt7-1 in the
smt7-1 mat3-4 strain and restore the small-size phenotype of mat3 mutant in three independent transformants (Table 1).

To investigate whether SUMO protease activity is required for size checkpoint function of SMT7, transgenic smt7-1 mat3-4 strains carrying two mutated forms of SMT7 genomic DNAs (pSMT7C928A-3XHA and pSMT7C928S-3XHA) that encode the catalytic dead SMT7 proteins were generated. Three independent strains expressing the SMT7C928A-3XHA or SMT7C928S-3XHA protein were verified by immunoblotting (Figure 2B). The presence of two sizes of SMT7 proteins was also detected in the cell extracts of the smt7-1 mat3-4::SMT7-3XHA, smt7-1 mat3-4::SMT7C928A-3XHA and smt7-1 mat3-4::SMT7C928S-3XHA strains. Unlike the smt7-1 mat3-4::SMT7-3XHA strains, expressing the defective SMT7 protein SMT7C928A-3XHA or SMT7C928S-3XHA failed to restore the small size in the smt7-1 mat3-4 background (Table 1), indicating that SUMO protease activity is required for SMT7 to regulate the size control mechanism in the mat3-4 mutant.

**SMT7 mRNA and protein are diurnally regulated**

To investigate how SMT7 mRNA was regulated, qRT-PCR was initially used to monitor its expression in samples collected at two-hour intervals from previously synchronous 21gr culture (Fang et al., 2014). However, the abundance of SMT7 mRNA was too low to be detected by quantitative PCR (qPCR). As an alternative, RT-droplet digital PCR (RT-ddPCR) was used to determine the expression pattern of SMT7 mRNA. RT-ddPCR has been shown to be more sensitive than qRT-PCR in detecting rare mRNAs (Hindson et al., 2013). SMT7 mRNA levels increased during the G1 phase, reached a peak at the G1/S transition, and then gradually declined as cells completed mitosis (Supplemental Figure 5). The expression pattern of SMT7 in the synchronized cell culture is similar to that previously reported (Zones et al., 2015).

Because our generated SMT7 antibody could not detect endogenous SMT7 protein, a smt7-1::SMT7-3XHA strain was generated by crossing the complemented smt7-1 mat3-4::SMT7-3XHA #3 to a wild-type (mt-, 6145C) strain. Cell size measurement of the segregated progeny confirmed again that expression of the SMT7-3XHA protein in the smt7-1 mat3-4 mutant restored the small cell size (Supplemental Table 1). The expression pattern of SMT7-3XHA protein in the smt7-1::SMT7-3XHA strain was determined every two
hours by HA antibody in synchronized culture by immunoblotting. The culture synchrony was assessed by Commitment and mitotic index assays (Figure 3A), and expression of S/M marker gene CDKB1 (Figure 3B). To investigate dynamic changes of SMT7 protein either as the basis of overall cellular protein concentration or the amount of protein per cell, lysates were loaded on two blots, one with equal amounts of protein in each lane and one with equal numbers of cells in each lane (Figure 3C). SMT7 protein was expressed throughout the cell cycle with its levels increased during G1, reaching the first peak at around the Commitment state, then declining before reaching the second peak at the S/M phase, which is marked by accumulation of the cell cycle marker CYCD3 protein (Bisová et al., 2005; Zones et al., 2015). SMT7 protein level was gradually decreased in post-mitotic cells. The dual accumulation peaks were most prominent when equal numbers of cells were loaded for immunoblot analysis. Together, these results show that SMT7 mRNA and protein are regulated diurnally.

**SMT7 is predominantly localized in the nuclear membrane**

The substrate specificity of SUMO proteases is strongly influenced by their restricted cellular localization (Li and Hochstrasser, 2003). SMT7 protein has one potential nuclear localization sequence (Supplemental Figure 3) and is predicted to be a nuclear protein. To gain insight into where SMT7 is localized to exert its functions, localization of SMT7-3XHA protein was determined in the complemented smt7-1::SMT7-3XHA_I18 strain using cell fractionation followed by immunoblotting. Separation of the subcellular fractions was confirmed using the respective organelle markers. A strong enrichment of a nuclear protein histone3 (H3) was detected in the nuclear fraction but not in the cytosol/membrane fraction (Figure 4A). Thylakoid marker copper response defect 1 (CRD1) protein was only detected in the cytosol/membrane fraction but not in the nuclear extract. Importantly, SMT7-3XHA protein was enriched in the nuclear fraction, indicating SMT7 is predominantly localized to the nuclei.

To gain further detailed information about where SMT7 accumulated in the nucleus, we used indirect immunofluorescence (IF) with anti-HA antibody. Even though the anti-HA antibody was able to successfully detect the SMT7-3XHA protein of smt7-1 mat3-4::SMT7-3XHA and smt7-1::SMT7-3XHA transgenic lines (Figure 2 and Figure 3) by immunoblotting, they failed to generate reliable IF signals in
these complemented strains. To overcome this problem, a glycine-rich linker was inserted between sequences encoding SMT7 and 3XHA tag to generate the \( pSMT7\text{-lin-}3XHA \) construct. Insertion of a flexible glycine-rich linker between a tagged protein and an epitope has been shown to increase epitope sensitivity and accessibility (Sabourin et al., 2007; Reddy Chichili et al., 2013). Expression of SMT7-lin-3XHA restored SMT7 function in the \( \text{smt7-1 mat3-4} \) double mutant (Supplemental Table 2). Thus, \( pSMT7\text{-lin-}3XHA \) was introduced into the wild-type strain and the transgenic strains expressing the SMT7-lin-3XHA protein (Figure 4B) were used for IF studies. Consistent with its expression in the nuclear fraction (Figure 4A), SMT7-lin-3XHA signal was detected as a discontinuous rim-like staining of the nuclear periphery surrounding the DAPI-stained DNA typical of nuclear pore complex (NPC)-associated localization (Figure 4C). The discontinuous rim-like staining of the nuclear periphery in SMT7-lin-3XHA was very similar to the distribution of NPC-associated SUMO protease in budding yeast and mammals (Zhang et al., 2002; Goeres et al., 2011; Chow et al., 2012). Hence, SMT7 is predominately localized in nuclear envelopes and potentially targets to NPCs. Consistent but very weak cytoplasmic SMT7-lin-3XHA staining was also observed. Therefore, SMT7 may be present in the cytoplasm to some extent.

To investigate whether SMT7 is misregulated in the \( \text{mat3-4} \) mutant, we used IF to detect the SMT7-lin-3XHA signal in the complemented \( \text{smt7-1 mat3-4::SMT7\text{-lin-}3XHA} \) strains. Expression of SMT7-lin-3XHA protein was verified in independent complemented \( \text{smt7-1 mat3-4::SMT7\text{-lin-}3XHA} \) strains (Figure 4B). Consistent with the SMT7-lin-3XHA IF signals detected in the \( SMT7\text{-lin-}3XHA \) transgenic strains, SMT7-lin-3XHA staining was also detected in a punctate fashion in nuclear envelopes in the \( \text{mat3-4} \) background (Figure 4D), indicating that MAT3 does not affect localization of SMT7 in the nucleus.

The SUMO protease domain of SMT7 is capable of removing SUMO from SUMOylated Chlamydomonas PCNA protein \textit{in vitro}

To assess the SUMO protease activity of SMT7, an \textit{in vitro} SUMOylation system was constructed. The \textit{Chlamydomonas} SUMOylation components were identified using \textit{Arabidopsis} SUMO activating enzyme 1 (AtSAE1), AtSAE2, E2 conjugating enzyme 1 (AtSCE1), AtSUMO1, and proliferating cell nuclear antigen (AtPCNA) proteins as the BLASTP queries for the \textit{Chlamydomonas} proteome (see Methods) and the
corresponding cDNAs were isolated by RT-PCR. The alignment of protein sequences of individual SUMOylation components are shown in Supplemental Figure 6. Among the SUMO genes we identified, SUMO4 mRNA is the most abundant SUMO gene transcript (Supplemental Figure 7A) and diurnally regulated (Zones et al., 2015). This gene was therefore chosen for the in vitro SUMOylation assay. The alignment of SUMO protein sequences from yeast, Chlamydomonas, Arabidopsis, and human are shown in Supplemental Figure 7B. Because proliferating cell nuclear antigen (PCNA) is known to be SUMO-conjugated in various organisms (Hoege et al., 2002; Leach and Michael, 2005; Gali et al., 2012; Moldovan et al., 2012; Strzalka et al., 2012) and at least one consensus SUMOylation site (K91) of Chlamydomonas PCNA can be predicted with high confidence by multiple prediction programs (Supplemental Figure 8), Chlamydomonas PCNA was used as the substrate for the SUMO conjugation test. Chlamydomonas SAE1 and SAE2 cDNAs were cloned into the pCDFDuet vector. Chlamydomonas SCE1 and SUMO4 were cloned into the pETDuet vector. Chlamydomonas PCNA was cloned into the pRSFDuet-3XHA vector. E. coli cells transformed with pCDFDuet-SAE1-SAE2, pETDuet-SCE1-SUMO4GG, and pRSFDuet-3XHA-PCNA plasmids were selected (Figure 5A, see Methods). Co-expression of SAE1, SAE2, SCE1, SUMO4, and 3XHA-PCNA was verified by immunoblotting (Figure 5B). In addition to detecting a 3XHA-PCNA protein with the predicted size (~37 kDa), a ~ 60 kDa low-mobility SUMO4-conjugated 3XHA-PCNA protein was also detected when all of the SUMOylation components were present (Figure 5B), indicating that PCNA protein is SUMOylated. Importantly, SUMO4 failed to be conjugated to PCNA when one or more of the SUMO conjugation components were missing. Interestingly, the E. coli endogenous proteins were also found to be conjugated with SUMO4 when all the SUMOylation components were present (Figure 5B). Hence, we conclude that Chlamydomonas PCNA is SUMOylated and the in vitro SUMOylation system is established.

To test the SUMO protease activity of SMT7 and determine its ability to remove SUMO from SUMO-conjugated PCNA protein, SMT7 was co-expressed with the SUMOylation components along with 3XHA-PCNA protein. The full-length SMT7 cDNA is extremely GC-rich (~76 %) and was challenging to clone. As an alternative, the SUMO protease domain of SMT7 (SMT7709-972) was amplified and introduced into the pRSFDue-3XHA-PCNA plasmid to make pRSFDue-3XHA-PCNA-SMT7709-972. Expression of all the
corresponding components was verified by immunoblotting (Figure 5C). When SMT7\textsuperscript{709-972} was expressed in the presence of all the SUMOylation components, only unconjugated 3XHA-PCNA was detected, indicating that SMT7\textsuperscript{709-972} is capable of removing SUMO4 from conjugated PCNA. Coincidentally, expression of SMT7\textsuperscript{709-972} caused a decrease in levels of SUMO4 conjugates and an increase in levels of SUMO4 monomers in \textit{E. coli} cell lysates (Figure 5C). Expression of the defective SUMO protease domain, SMT7\textsuperscript{709-972/C928A} or SMT7\textsuperscript{709-972/C928S}, on the other hand, failed to remove SUMO4 from PCNA and SUMO4-conjugated PCNA was detected (Figure 5C). In conclusion, our data provide evidence that the SUMO protease domain of SMT7 possesses SUMO-deconjugating activity.

**SUMO4-conjugated proteins accumulate in the \textit{smt7-1} mutant**

To investigate whether SUMO4-conjugated proteins were regulated by SMT7 \textit{in vivo}, SUMO4 antibody were raised to detect SUMO4-conjugates. The specificity of SUMO4 antibody was confirmed by immunoblotting of recombinant \textit{Chlamydomonas} SUMO1, SUMO2, and SUMO4 proteins (Supplemental Figure 9) and the SUMO4 antibody was therefore used for immunoblotting of \textit{Chlamydomonas} cellular extracts. In addition to SUMO4 monomer migrating close to \(\sim13\) kDa, a larger number of SUMO4-conjugated proteins were detected in the wild-type (Figure 6A) and \textit{mat3-4} (Figure 6B) strains. When SMT7 is missing, the levels of SUMO4 monomer were substantially decreased and the levels of the SUMO4-conjugated proteins were substantially increased, indicating that SMT7 is a major regulator of SUMO4 conjugates in \textit{Chlamydomonas}.

**Identification of ribosomal protein L30 as the critical SMT7-interacting protein in \textit{mat3-4}**

To identify SMT7 targets and their roles in MAT3-mediated size checkpoint control, immunoprecipitation (IP) was conducted to isolate SMT7-interacting proteins. Because SMT7 and its targets may interact transiently, a substrate-trapping-based strategy was used to immunopurify SMT7-3XHA-interacting proteins from cellular extracts prepared from two independent \textit{smt7-1 mat3-4::SMT7\textsuperscript{C928A-3XHA} transgenic lines. Substrate-trapping takes advantage of the catalytic inactive mutant SMT7, SMT7\textsuperscript{C928A-3XHA, which allows substrate binding but greatly slows its dissociation because of the impaired catalytic
activity (Flint et al., 1997; Elmore et al., 2011), to enrich SMT7-interacting proteins. The smt7-1 mat3-4 mutant was used as a negative control for IP. IPs followed by liquid chromatography-mass spectrometry (LC-MS/MS) analysis identified SMT7C928A-3XHA-interacting proteins as the following molecules: RPS2, RPS23, RPL30, a low-CO2-inducible protein, elongation factor 3a-related protein, light harvesting chlorophyll a/b binding protein 2 (Lhca2), a flagellar-associated protein, FtsH-like membrane ATPase/metalloprotease, plastid ribosomal protein S13, and a NAC domain containing protein (Table 2).

Importantly, none of these proteins were detected in several parallel control IP experiments conducted using a smt7-1 mat3-4 strain lacking HA-tagged SMT7C928A. Because RPL30 was isolated six out of ten times in independent IP experiments and ribosome biogenesis has been implicated in cell size control (Jorgensen et al., 2002; Gomez-Herreros et al., 2013), we chose to focus on RPL30.

**SMT7 interacts with RPL30 and regulates the SUMOylation status of RPL30**

To confirm that SMT7 interacts with RPL30 in vivo, polyclonal antibody raised against RPL30 was generated. The purified RPL30 antibody recognized a ~12-kDa band predicted to be the size of RPL30 protein in *Chlamydomonas* cell lysate (Figure 7A). To validate that the antibody recognized endogenous RPL30, the anti-RPL30 antibody were used for IP from lysates prepared from the wild-type strain. Mass spectrometry analysis confirmed that RPL30 was pulled down in the IP pellets (Supplemental Figure 10 and Supplemental Table 3). Hence, the specificity of anti-RPL30 antibody was validated.

A co-immunoprecipitation (co-IP) assay was used to investigate whether RPL30 interacts with SMT7 protein in vivo. HA-tagged SMT7C928A protein (SMT7C928A-3XHA) from a complemented smt7-1 mat3-4::SMT7C928A-3XHA_A22 strain was subjected to IP, and the IP pellets were probed on immunoblots with RPL30-specific antibody. RPL30 protein was found to be associated with SMT7C928A-3XHA protein in the smt7-1 mat3-4::SMT7C928A-3XHA_A22 strain but not in the control extract prepared from the smt7-1 mat3-4 cells nor in the no-antibody cell extract of the smt7-1 mat3-4::SMT7C928A-3XHA_A22 strain (Figure 7A). Taken together, our data suggest that SMT7C928A-3XHA interacts with RPL30 in vivo.

We used the established *in vitro* SUMOylation assay to determine whether *Chlamydomonas* RPL30 can be SUMOylated and whether SMT7 is able to remove the SUMO-conjugated RPL30. Multiple
SUMOylation prediction programs failed to identify a common SUMO acceptor lysine with high confidence in RPL30 (Supplemental Figure 8). Even so, when RPL30 was co-expressed with all of the SUMOylation components, an approximately 20-kDa S-tag RPL30-3XHA protein and two slowly migrated RPL30 signals (~40 and ~50 kDa) were detected by anti-HA antibody (Figure 7B), suggesting two and three SUMO4 were conjugated to RPL30 protein in vitro. As expected, SUMO4-conjugated RPL30 proteins disappeared when SMT7<sup>709-972</sup> was co-expressed (Figure 7B), indicating that the SUMO protease activity of SMT7<sup>709-972</sup> was able to remove SUMO4 from SUMOylated RPL30.

To test whether SUMOylation of RPL30 was regulated by SMT7 in vivo, RPL30 protein was examined in strains lacking SMT7 by immunoblotting. The levels of unconjugated RPL30 protein were slightly but consistently reduced in <i>smt7-1</i> and <i>smt7-1 mat3-4</i> strains compared with the wild-type and <i>mat3-4</i> strains, respectively (Supplemental Figure 11A). A slow-mobility RPL30 signal running at approximately 22 kDa that corresponds to the mono-SUMO conjugated RPL30 was repeatedly detected (Supplemental Figure 11A). However, the polyclonal RPL30 antibody recognized many non-specific proteins and worked poorly for IP analysis (data not shown). To improve specification for RPL30 IP, the <i>mat3-4</i> transformants carrying the HA-tagged version of <i>RPL30</i> (<i>pRPL30-3XHA-Paro, mat3-4::RPL30-3XHA</i>) were generated. Expression of the RPL30-3XHA protein in three independent <i>mat3-4::RPL30-3XHA</i> strains was confirmed by immunoblotting (Figure 7C). RPL30-3XHA and its conjugates were first pulled down by anti-HA magnetic beads followed by IP using the anti-SUMO4 antibody-conjugated beads. Interestingly, a ladder-like migration pattern of RPL30-3XHA (presumably caused by the attachment of multiple SUMO4 molecules) was observed in cell lysates prepared from the <i>mat3-4::RPL30-3XHA</i> but not from the <i>mat3-4</i> control cells (Figure 7D). Intriguingly, the unmodified RPL30-3XHA protein was also pulled down by tandem IP.

To validate this result, a reciprocal purification was carried out. SUMO4 conjugates were first immunopurified by the anti-SUMO4 antibody-conjugated beads and the SUMO4-conjugated RPL30 protein was further purified with anti-HA magnetic beads. Consistently, ladder-like slowly migrating RPL30-3XHA species were detected in the <i>mat3-4::RPL30-3XHA</i> but not in the <i>mat3-4</i> control cells (Figure 7E). Similarly, the unmodified RPL30-3XHA protein was the dominant protein purified by tandem IP. In both cases, four and five SUMO4 conjugates were found to be the dominant species of SUMOylated RPL30,
even though no conspicuous poly-SUMOylated RPL30 was detected in the in vitro system (Figure 7B). The discrepancy in the number of conjugated SUMO proteins between in vitro and in vivo experiments has also been reported previously (Duprez et al., 1999; Johnson and Blobel, 1999; Rodriguez et al., 2001). The absence of SUMO E3 ligase in the in vitro system may contribute to this discrepancy (Takahashi et al., 2003; Leung et al., 2015).

Even though SUMOylation sites were predicted in RPL30 (Supplemental Figure 8), mono- or di-SUMO conjugation cannot explain multiple slowly migrating species of RPL30-3XHA. Protein conjugation by poly-SUMO chains have been reported in budding yeast, mammals, and Trypanosoma brucei and it often leads to modified proteins with a ladder-like migration pattern (Tatham et al., 2001; Bylebyl et al., 2003; Liang et al., 2016; Iribarren et al., 2018). Furthermore, a conserved N-terminal SUMOylation site important for SUMO polymerization is shared by the SUMO proteins that are capable of forming the polymeric chains (Tatham et al., 2001; Bylebyl et al., 2003; Cheng et al., 2006; Iribarren et al., 2018). Interestingly, Chlamydomonas SUMO proteins also contain this N-terminal SUMOylation site (Wang et al., 2008; Shin et al., 2010) (Figure 7F), suggesting CrSUMO4 is able to form a poly-SUMO chain. Therefore, we suspect that RPL30 is conjugated with poly-SUMO4 chains. Whether CrSUMO4 is capable of forming poly-SUMO chains and whether RPL30 is conjugated with poly-SUMO4 chains remain to be determined.

To investigate whether SMT7 regulates SUMOylation of RPL30, the smt7-1 mat3-4 transformants carrying the HA-tagged version of RPL30 (pRPL30-3XHA-Hyg, smt7-1 mat3-4::RPL30-3XHA) were generated. Expression of the RPL30-3XHA protein was confirmed in four independent transformants by immunoblotting (Figure 7G). We chose smt7-1 mat3-4::RPL30-3XHA #3 and smt7-1 mat3-4::RPL30-3XHA #15 for tandem IP experiments because their RPL30-3XHA levels are similar to that of the mat3-4::RPL30-3XHA_C14 strain (Figure 7G).

We prepared total cell lysates of smt7-1 mat3-4::RPL30-3XHA #3, smt7-1 mat3-4::RPL30-3XHA #15, and mat3-4::RPL30-3XHA_C14 for tandem IP starting with anti-HA magnetic beads followed by anti-SUMO4 beads. Cell lysates of smt7-1 mat3-4 were used as a negative control. Equal amounts of input protein of the tested strains were confirmed by Coomassie blue staining (Supplemental Figure 11B). Similar to the mat3-4::RPL30-3XHA (Figure 7D and 7E), multiple SUMO4 proteins were conjugated to RPL30 in
smt7-1 mat3-4::RPL30-3XHA #3 and smt7-1 mat3-4::RPL30-3XHA #15 strains and displayed a ladder-like pattern (Figure 7H). Moreover, the levels of SUMOylated RPL30 conjugates were increased in two independent smt7-1 mat3-4::RPL30-3XHA stains, indicating that RPL30 SUMOylation is regulated by SMT7. Taken together, our data provide evidence that RPL30 is conjugated with SUMO4 in vivo and SMT7 is important for this regulation.

**Overexpression of the RPL30-SUMO4\[^{ΔGG}\] in mat3-4 recapitulates the size-suppressing phenotype of smt7-1 mat3-4**

Because RPL30 was shown to be a target of SMT7 in vitro and in vivo, we hypothesized that SMT7 modulates the SUMOylation status of RPL30 to regulate cell division in a MAT3-dependent fashion. In smt7-1 mat3-4 cells, it is possible that a defect in SMT7 causes failure to replenish unconjugated RPL30, which is rate-limiting for cell division. To test this possibility, RPL30 level was knocked down in mat3-4 cells using an artificial microRNA (amiRNA) approach. If a relatively high amount of unconjugated RPL30 is critical for increased cell division number (and, thus, smaller cell size) in the mat3-4 mutant, it is predicted that mat3-4::amiRPL30 cells would have larger daughters than those of the mat3-4 strain. Down-regulation of RPL30 in mat3-4 strains by three different amiRNA constructs was confirmed in multiple independent transgenic lines by immunoblot analysis (Supplemental Figure 12). However, reducing RPL30 protein in mat3-4 cells failed to phenocopy the size suppression seen in the smt7-1 mat3-4 strain (Table 3), suggesting that the amounts of unconjugated RPL30 are not rate-limiting for cell division.

Alternatively, it is possible that an increase in SUMOylated RPL30 leads to a decrease in the number of cell divisions in the smt7-1 mat3-4 strain and, thus, larger cell size. Consistent with this notion, mono-SUMOylated RPL30 was found to increase in abundance, though slightly but consistently, in smt7-1 and smt7-1 mat3-4 strains (Supplemental Figure 11). To test the second possibility, the expression constructs pRPL30-SUMO4\[^{ΔGG}\]-3XHA and pSUMO4\[^{ΔGG}\]-RPL30-3XHA that allow overexpression of the SUMOylated RPL30 mimicking proteins were generated. The mutations in SUMO4\[^{ΔGG}\] removed two glycine residues of SUMO4 required for cleavage by SUMO protease. Overexpression of SUMO protease-resistant SUMO\[^{ΔGG}\]-chimera proteins has been commonly used to test cellular functions of the SUMOylated proteins (Ross et al.,
2002; Bossis et al., 2005; Cheng et al., 2017). The pRPL30-SUMO4\(^{AGG}\)-3XHA and pSUMO4\(^{AGG}\)-RPL30-3XHA were independently transformed into the mat3-4 cells. For controls, the mat3-4::RPL30-3XHA strains (Figure 7C) and the mat3-4 transformants carrying the unmodified version of SUMO4\(^{AGG}\) (mat3-4::SUMO4\(^{AGG}\)-3XHA) were included. Expression of the RPL30-SUMO4\(^{AGG}\)-3XHA, SUMO4\(^{AGG}\)-RPL30-3XHA, and SUMO4\(^{AGG}\)-3XHA proteins was confirmed by immunoblotting (Figure 8A-C). Unlike mat3-4::RPL30-3XHA and mat3-4::SUMO4\(^{AGG}\)-3XHA strains, whose daughter cell size was similar to that of mat3-4 cells (Table 4), the daughter cells of mat3-4::RPL30-SUMO4\(^{AGG}\)-3XHA strains with a high ratio of RPL30-SUMO4\(^{AGG}\)-3XHA:Rubisco (strain A18, A31, and A34, Figure 8A) were consistently larger than mat3-4 daughters and similar to the size of smt7-1 mat3-4 cells (Table 4 and Figure 8D). However, the daughter cells of mat3-4::SUMO4\(^{AGG}\)-RPL30-3XHA strains had no size defect. We noticed that expression levels of SUMO4\(^{AGG}\)-RPL30-3XHA protein in mat3-4::SUMO4\(^{AGG}\)-RPL30-3XHA strains were lower than those of RPL30-SUMO4\(^{AGG}\)-3XHA in mat3-4::RPL30-SUMO4\(^{AGG}\)-3XHA strains. Therefore, it is possible that SUMO4\(^{AGG}\)-RPL30-3XHA protein accumulation levels were not sufficient to affect size-dependent cell division. Alternatively, we also could not exclude the possibility that SUMO4\(^{AGG}\)-RPL30-3XHA protein did not resemble SUMO4-conjugated RPL30. Regardless, overexpression of the RPL30-SUMO4\(^{AGG}\) protein in mat3-4 cells recapitulated the size-suppressing phenotype of the smt7-1 mat3-4 strain, suggesting that an increase in SUMO-conjugated RPL30 protein contributes to a decrease in the number of cell divisions.

**Discussion**

In previous work, we isolated smt7-1 as a partial suppressor of the small size of mat3 mutant (Fang and Umen, 2008). Here, we characterized the SMT7 molecularly and biochemically and demonstrated that SMT7 encodes a *bona fide* Chlamydomonas SUMO protease. Key properties of SMT7 indicate that the SUMO protease activity of SMT7 is required to maintain cellular SUMO conjugation dynamics and that a defect in SMT7 increased SUMO4-conjugated species (Figure 6). Similar to yeast ULP1, human SENP1 and SENP2, and *Arabidopsis* ESD4 (Hang and Dasso, 2002; Zhang et al., 2002; Li and Hochstrasser, 2003; Panse et al., 2003; Xu et al., 2007), SMT7 was shown to be predominantly localized in discrete regions of nuclear membrane, presumably where the NPCs are. NPC-associated SUMO proteases are found to regulate
SUMOylation of a gene-bound transcription factor (Texari et al., 2013), to maintain nucleoporin homeostasis (Chow et al., 2014), and to regulate repair of double strand DNA breaks (Duheron et al., 2017). Knowing that substrate specificity of SUMO proteases is strongly influenced by their restricted cellular localization, we propose that SMT7 regulates SUMO-conjugated proteins that transport to or from the nucleus.

It is already known that SUMO proteases play important roles in cell division control (Li and Hochstrasser, 1999; Di Bacco et al., 2006; Pelisch et al., 2014; Schimmel et al., 2014; Eifler and Vertegaal, 2015). However, the identities of SUMO protease-modulated target proteins and their links to the cell cycle functions remain incomplete. Despite SMT7 mRNA being a very low abundance gene, ours (Supplemental Figure 5) and Zones’s data (Zones et al., 2015) suggest that SMT7 mRNA is regulated diurnally with its expression elevated before onset of the S/M phase and gradually declines as cells exit mitosis. SMT7 protein, on the other hand, displayed dual expression peaks that are associated with Commitment and S/M phase (Figure 3), implicating the involvement of SMT7 in both size checkpoint functions. The incomplete correlation between expression patterns of SMT7 mRNA and its encoded protein suggests that post-transcriptional regulation of SMT7 may be important for its exerted function.

Further support of SMT7 in size checkpoint function is evidenced by the requirement of its SUMO protease activity in the supernumerous cell divisions of mat3-4 cells. Unlike yeast ulp1- or ulp2-deficient cells that are either inviable or have abnormal cell shape and poor growth rate (Li and Hochstrasser, 1999, 2000), the smt7-1 cells grow at a similar rate to wild-type cells and have normal-size daughters, and the smt7-1 mutation only affects size in the small-size mat3-4 cell background (Fang and Umen, 2008). This suggests that SMT7-dependent cellular activity is not rate-limiting for mitotic cell division when MAT3 is intact. It is possible that protease activities carried out by other SUMO proteases maintain cell division when SMT7 is depleted. This possibility is supported by the presence of multiple SUMO proteases found in the Chlamydomonas genome (Breker et al., 2018). It will be interesting to determine whether other Chlamydomonas SUMO proteases act similarly to SMT7 to regulate cell division.

In this study we found that SMT7-dependent deconjugation of SUMOylated (SUMO4 in particular) targets affects size checkpoint control when MAT3 is defective. We found that RPL30 is apparently the
prime SMT7 target in causing this phenotype and SMT7 is predominantly localized in NPCs. It is therefore reasonable to hypothesize that SMT7 removes SUMO4 from SUMO4-conjugated RPL30 in NPCs. SUMOylation of ribosome components is not unprecedented as many ribosomal proteins are found to be modified by SUMO in animal systems and in budding yeast (Panse et al., 2004; Hendriks and Vertegaal, 2016; Esteras et al., 2017). It has been reported that an impaired SUMO pathway causes defects in 60S subunit biogenesis and its nuclear export in budding yeast (Panse et al., 2006). Coincidentally, some of the pre-60S transacting factors and RPL5 protein are modified by SUMO (Panse et al., 2006). In addition, it has been suggested that nucleoplasm-enriched accumulation of SUMO-conjugated Drosophila RPL22e may possess an extraribosomal function (Kearse et al., 2013). In human cells, SUMO modification stabilizes RPS3 protein (Jang et al., 2011). However, it is not clear how SUMOylation of ribosomal proteins is regulated and whether such modification regulates general translation or provides specialized functions.

Here, we report that RPL30 is SUMOylated and demonstrate that RPL30 is conjugated with multiple SUMO4 molecules in Chlamydomonas. Because the N-terminal end of SUMO4 contains the conserved SUMOylation site, which allows conjugation with the poly-SUMO chains, and SUMOylated RPL30 formed a ladder-like pattern (Figure 8), we speculate that SUMO4 is capable of forming polymeric chains in Chlamydomonas. Poly-SUMO chains are reported to regulate resection of homology-dependent double strand break repair during vegetative growth and meiosis in budding yeast (Chen et al., 2016; Horigome et al., 2016) and to modulate nuclear foci formation and chromatin organization in procyclic Trypanosoma brucei (Iribarren et al., 2018). In addition, SUMO chains regulate the formation of the synaptonemal complex and promyelocytic leukemia nuclear bodies in mammals (Tatham et al., 2008; Watts and Hoffmann, 2011; Leung et al., 2015). The RPL30 SUMOylation process identified in this work may constitute the first step towards assessing the role of the potential polymeric SUMO structures in Chlamydomonas.

During the course of purifying the SUMO4-conjugated RPL30 protein, the unmodified RPL30 was repeatedly co-immunoprecipitated with SUMO4 conjugates. It is possible that the SUMO interacting motifs (SIM, Supplemental Figure 13A) of RPL30 provide the docking sites for poly-SUMO4 chains and/or poly-SUMO4 conjugates. In fact, SIM has been shown to be important for binding to poly-SUMO chains.
The requirement of SIM in RPL30 for binding to the poly-SUMO4 chain remains to be tested.

Overexpression of the SUMO protease-resistant RPL30-SUMO4\(^{ΔGG}\) protein, which mimics SUMOylated RPL30, but not overexpression of the RPL30 or SUMO4 protein in mat3-4 cells recapitulated smt7-1 mat3-4 and led to size suppression of the mat3-4 strain. These data provide compelling evidence to suggest that increased levels of SUMO4-conjugated RPL30 lead to altered cell size regulation in mat3-4 cells and to increased cell size. Even though RPL30 was found to be conjugated by multiple SUMO4 in vivo, an increase in RPL30-SUMO4\(^{ΔGG}\) level was sufficient to affect size in the mat3-4 strain. It will be of future interest to investigate how SUMOylation dynamics of RPL30 are regulated to control size-mediated cell cycle control.

How does an increase in SUMOylated RPL30 level affect cell division? RPL30 is one of the components constituting the large (60S) ribosomal subunit. RPL30 is localized on the surface of the large (60S) ribosomal subunit (Slavov et al., 2015) and is part of the intersubunit bridge that connects the large and small ribosomal subunits (Halic et al., 2005). The evolutionarily conserved residues involved in intersubunit bridges and RNA binding of RPL30 have been identified (Chen et al., 2003) and are conserved in Chlamydomonas RPL30 (Supplemental Figure 13B). It is therefore reasonable to propose that the SUMOylation status of RPL30 protein is involved in modulating RPL30-related ribosome biogenesis or pre-ribosomal particle assembly, translocation, or maturation. The other possibility is that accumulation of SUMOylated RPL30 has an extra-ribosomal function. Many ribosomal proteins in plant and animal systems have been reported to have specialized functions other than translation (Warner and McIntosh, 2009; Nagaraj et al., 2015; Han et al., 2017; Li et al., 2017; Zhang et al., 2017; Dionne et al., 2019). Moreover, SUMO conjugation of mouse RPL11 protein has been shown to regulate p53-mediated stress responses (El Motiam et al., 2018), further supporting the importance of SUMOylation on the extraribosomal functions of ribosomal proteins. It will be of great interest to determine how SUMOylated RPL30 regulates cell division. The underlying cell-size control mechanism governed by the SMT7-RPL30-MAT3 module remains to be investigated.
It is important to note that overexpression of RPL30-SUMO4\(^{\Delta GG}\)-3XHA did not completely restore the size checkpoint defects caused by mat3-4, suggesting that other pathways acting downstream of MAT3/Rb are required for cell division. The mat3-4::RPL30-SUMO4\(^{\Delta GG}\)-3XHA strain may be a valuable tool for investigating size control mechanism. Together, our findings shed light on a novel SMT7-RPL30-MAT3 module for size control.

**Methods**

**Chlamydomonas strains and growth conditions**

*Chlamydomonas reinhardtii* strains 21gr (MT\(^+\)), 6145C (MT\(^-\)), mat3-4, smt7-1, and smt7-1 mat3-4 were as described previously (Umen and Goodenough, 2001; Fang and Umen, 2008). Cells were maintained on a Tris-acetate-phosphate (TAP) agar plate and allowed to grow in liquid TAP medium (Gorman and Levine, 1965) under cool white light (GE F40 CW) with illumination of 250 to 300 \(\mu\)mol photons m\(^{-2}\)s\(^{-1}\) at 24°C aerated with 0.5% (v/v) CO\(_2\) before transformation. Cells were grown photosynthetically in liquid HSM medium (Sueoka, 1960) for synchronization and cell cycle experiments. 21gr was used as a wild-type strain for most of the biochemistry experiments.

To avoid spontaneous suppressors accumulated in the smt7-1 mat3-4 population, smt7-1 (MT\(^-\)) was crossed to unsuppressed mat3-4 mutant (MT\(^+\)) and the fresh smt7-1 mat3-4 strain was generated for the experiments used in this study. To generate the smt7-1::SMT7-3XHA strain for protein studies, smt7-1 mat3-4::SMT7-3XHA (MT\(^+\)) was crossed to 6145C (MT\(^-\)) and segregated smt7-1::SMT7-3XHA strain whose smt7-1 mutation was scored by paromomycin resistance and pSMT7.1-3XHA DNA transgene marked by zeocin resistance was isolated.

**Cloning and epitope tagging of SMT7**

To generate the wild-type SMT7 genomic DNAs C-terminally translationally fused to the triple-hemagglutinin-tagged (3XHA) before the stop codon, a 1.2 Kb AgeI SMT7 genomic DNA fragment with insertion of DNA sequence 5'-

AGTTACCCATACGATGTTTCTGACTATGCGGCTATCCCTATGACGTCCCGGACTATGCAGGATCC
TATCCATATGACGTTCCAGATTACGCT-3', which was flanked by BglII sites and encoded a 3XHA tag (as shown in Supplemental Figure 4), was synthesized, digested, and ligated into AgeI digested pSMT7.1 plasmid (Fang and Umen, 2008) to generate pSMT7.1-3XHA. To generate the catalytically dead SMT7 constructs, the synthesized 1.2 Kb AgeI SMT7 genomic DNA fragment (GENEWIZ) was designed to contain point mutations that either changed amino acid residue 928 to alanine (TGC to GCC, C928A) or serine (TGC to AGC, C928S) along with the sequence encoding the 3XHA tag. The synthesized mutated SMT7 genomic DNA fragment was then digested and ligated to AgeI-digested pSMT7.1 plasmid (Fang and Umen, 2008) to generate pSMT7.1^{C928A}-3XHA or pSMT7.1^{C928S}-3XHA respectively.

**Generation of overexpressing and artificial miRNA strains**

To construct the RPL30 overexpression vector, RPL30 cDNA was amplified by RPL30_fNdel and RPL30_rEcoRI primers (Supplemental Table 4) and cloned into Ndel- and EcoRI-digested pF1.1 (Supplemental Figure 14) to generate pRPL30-3XHA-Paro that confers paromomycin resistance in *Chlamydomonas*. To generate pRPL30-3XHA-Hyg that conferred hygromycin resistance in *Chlamydomonas*, hygromycin cassette amplified from pHyg3 (Berthold et al., 2002) by Hyg3For-NotI and Hyg3Rev-XbaI primers (Supplemental Table 4) was cloned into the NotI- and XbaI- digested pRPL30-3XHA-Paro plasmid to replace paromomycin marker. To make a construct that allows overexpression of the RPL30-SUMO4ΔGG protein, SUMO4 cDNA lacking the double glycine (SUMO4ΔGG) was amplified by SUMO4_fEcoRI and SUMO4ΔGG_rEcoRI primers (Supplemental Table 4) and cloned into the EcoRI-digested pRPL30-3XHA-Paro plasmid to generate pRPL30-SUMO4ΔGG-3XHA. To make a construct that allows overexpression of SUMO4ΔGG-RPL30 protein, SUMO4ΔGG cDNA was amplified by SUMO4_fNdel and SUMO4ΔGG_rNdel primers (Supplemental Table 4) and cloned into the Ndel-digested pRPL30-3XHA-Paro plasmid to generate pSUMO4ΔGG-RPL30-3XHA. To make a construct that allows overexpression of SUMO4ΔGG protein, SUMO4ΔGG cDNA was amplified by SUMO4_fNdel and SUMO4ΔGG_rEcoRI primers (Supplemental Table 4) and cloned into Ndel- and EcoRI- digested pF1.1 to generate pSUMO4ΔGG-3XHA.

To generate RPL30-specific artificial microRNA (amiRNA) constructs, the amiRNA-specific target regions were predicted by the WMD3 program (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi)
The RPL30-specific oligonucleotides (Supplemental Table 5) were allowed to anneal and were cloned into SpeI-digested pChlamiRNA3int plasmid as described previously (Molnar et al., 2009) to generate pChlamiRNA3int-RPL30-2, pChlamiRNA3int-RPL30-3, and pChlamiRNA3int-RPL30-4 respectively.

**RNA isolation, RACE-PCR, RT-PCR, quantitative RT-PCR, and droplet digital RT-PCR**

Total RNA was isolated as described previously (Fang et al., 2014). Five micrograms of RNA was used for cDNA synthesis. cDNAs were reverse transcribed in the presence of a mixture of oligo dT and random primers (9:1) at 50°C for 50 min using ThermoScript Reverse Transcription Kit (Invitrogen) following the manufacturer’s instructions. Each 20 μl PCR reaction contained 1μl of 1/20 fold diluted cDNA, 1X Phusion GC buffer, 0.2 mM dNTPs, 1 μM primers, 3% (v/v) DMSO, 0.5 M betaine, and 0.4 units Phusion High-Fidelity DNA polymerase (New England Biolabs). The PCR program was carried out as follows: 98°C for 1 min, 50 cycles of 98°C for 10 s, 62 or 68°C for 10 s, and 72°C for 20 s per kb of the genes.

To isolate 3’ ends of SMT7 cDNA, rapid amplification of cDNA ends (RACE)-PCR was carried out using the SMARTer RACE cDNA Amplification Kit (Clontech) following the manufacturer’s instructions. Four to six micrograms of RNA was used for cDNA synthesis. The first strand cDNA was synthesized following the manufacturer’s protocol. Approximately 850 bp SMT7 3’ cDNA was amplified by SMT7 gene-specific primer (SMT7_3’RACE) and Nested Universal Primer A (NUP, provided by the SMARTer RACE cDNA Amplification Kit) by Phusion High-Fidelity polymerase (New England Biolabs) and validated by sequencing (Supplemental Figure 2 and Supplemental Table 6).

Because it was extremely difficult to obtain 5’ RACE cDNA, we designed SMT7-specific primers and used RT-PCR to amplify SMT7 cDNA to validate the SMT7 gene model. Approximately 2.7 kb SMT7 cDNA was amplified by SMT7c_f8 and SMT7c_rXhoI (Supplemental Table 6). A 394 bp SMT7 cDNA fragment flanking the start codon was amplified by SMT7c_f9 and SMT7c_r10 (Supplemental Table 6). The 20 μL PCR reaction contained 1 μL cDNA, 1X Phusion GC buffer, 0.2 mM dNTPs, 1 μM of each primer, 3% (v/v) DMSO, 0.5 M betaine, 7.5% ethylene glycol (v/v) (Zhang et al., 2009) and 0.4 units Phusion High-
Fidelity DNA polymerase (New England Biolabs). The PCR program was carried out as follows: 98°C for 1 min, 50 cycles of 98°C for 10 s, 62-65°C for 10 s, and 72°C for 20 s per kb of DNA.

Ten microliters of quantitative RT-PCR (qRT-PCR) reaction contained 2.5 μl of 1/20 diluted cDNA, 0.2 μM of primers, and 5 ul of 2X KAPA SYBR FAST master mix (KAPA Biosystems). The primers used for qRT-PCR are listed in supplemental Table 7. Real-time RT-PCR was carried out by a Bio-Rad CFX96 (Bio-Rad). The following PCR program was used for amplification: 95°C for 2 min, 40 cycles of 95°C for 5s, 63°C for 25s. GBLP was used as an internal control. PCR was performed in triplicate.

For droplet digital RT-PCR (dd RT-PCR), 20 uL of PCR reaction contained 1 ul of 1/20 diluted cDNA, 900 nM of SMT7_1 and SMT7_2, 250 nM of SMT7_P probe, and 10 ul of 2x ddPCR Supermix for Probes (Bio-Rad). The primers used for ddRT-PCR are listed in supplemental Table 7. PCR was carried out using the QX200 Droplet Digital PCR System (Bio-Rad). The following PCR program was used for amplification: 95°C for 10 min, 40 cycles of 94°C for 30s, 60°C for 1 min, followed by 98°C for 10 min to deactivate enzyme. PCR was performed in triplicate.

**Complementation of smt7-1 mat3-4**

For SMT7 complementation, SacI-linearized pSMT7.1-3XHA was transformed into smt7-1 mat3-4 strain by electroporation as described previously (Shimogawara et al., 1998). As a comparison, SacI-linearized pSMT7.1\textsuperscript{C928A}-3XHA or pSMT7.1\textsuperscript{C928S}-3XHA plasmid was transformed into smt7-1 mat3-4 by electroporation. Transformants were selected on a TAP plate supplemented with 5 μg/mL of Zeocin (Invitrogen), which marked pSMT7.1-3XHA, pSMT7.1\textsuperscript{C928A}-3XHA or pSMT7.1\textsuperscript{C928S}-3XHA.

**Cell-size measurement and culture synchronization**

For cell-size measurement, a dark-shifted experiment was carried out as described previously (Fang et al., 2014). Cell-size was measured using Coulter Counter (Beckman Coulter) as described previously (Fang et al., 2006).
For culture synchronization, *Chlamydomonas* cells were cultured in high-salt medium (HSM) in a 12h:12h light:dark cycle with equal fluences of blue (450 nm) and red (630 nm) light (150 μmol photons m$^{-2}$s$^{-1}$ each). Commitment and mitotic assays were carried out as previously described (Fang et al., 2006).

**Identification of Chlamydomonas SUMOylation components**

Protein sequences of the *Arabidopsis* AtSAE1a (At4g24940), AtSAE2 (At2g21470), AtSCE1 (At3g57870), and AtPCNA (At1g07370) were used to as BLASTP queries of the *Chlamydomonas* proteome ([https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Creinhardtii](https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Creinhardtii)) and the highest-scoring hits were identified as *Chlamydomonas* SAE1 (Cre09.g408550; 7.5E-28), SAE2 (Cre06.g296983; 5.6E-142), SCE1 (Cre02.g142000; 7.8E-84), and PCNA (Cre12.g515850, 8.8E-128), respectively.

To identify *Chlamydomonas* SUMO genes, *Arabidopsis* AtSUMO1 (At4g26840) was used as a BLASTP query of the *Chlamydomonas* proteome (E-value of 1e-14 was set as an arbitrary cutoff). Four genes encoding SUMO1 (Cre16.g662550), SUMO2 (Cre16.g675749), SUMO3 (Cre16.g675861), and SUMO4 (Cre16.g675637) were identified. SUMO1, SUMO2, and SUMO4 were previously identified as CrSUMO148, CrSUMO97, and CrSUMO96, respectively (Wang et al., 2008).

During the course of our study, we found that some of the previously annotated SUMOylation components (Wang et al., 2008) are incorrect, which is likely caused by sequencing gaps and assembly errors of the previous genome. The updated annotations of the SUMOylation components and their previous annotations were listed in Supplemental Table 8 as a reference.

**Phylogenetic analysis**

Phylogenetic analysis was conducted using MEGA7 program (Kumar et al., 2016). MUSCLE alignment of protein sequences from the SUMO protease domain of multiple SUMO proteases were used to generate the phylogenetic tree. The unrooted phylogenetic tree (Supplemental Figure 2B) was constructed using the Maximum Likelihood method with 1,000 bootstrap replications. The evolutionary distances were computed by Poisson of amino acid substitution model and the rate variation among sites was determined by
gamma distribution. The heuristic to improve the likelihood of a tree was obtained by the Nearest-Neighbor-Interchange method.

Reconstitution of Chlamydomonas SUMOylation system and *in vitro* SUMOylation assay

The full-length cDNAs for *SAE1, SAE2, SCE1*, mature *SUMO4, PCNA* and *RPL30* were amplified by RT-PCR using the specific primer pairs listed in Supplemental Table 9. The *SAE2* cDNA (2272 bp) was digested with *BamHI* and *HindIII* and cloned into the expression vector pCDFDuet (Promega) to generate pCDFDuet-*SAE2* plasmid. The *SAE1* cDNA (1193 bp) was then digested by *BglII* and *XhoI* and cloned into pCDFDuet-*SAE2* to generate pCDFDuet-*SAE1*-*SAE2* (Figure 5A).

The SUMO4*GG* cDNA (292 bp) encoding the mature SUMO4 protein was digested by *BamHI* and *HindIII* and cloned into the pETDuet vector (Promega) to generate pETDuet-*SUMO4*GG. The *SCE1* cDNA (497 bp) fragment digested by *BglII* and *XhoI* was cloned into the pETDuet-*SUMO4*GG plasmid to generate pETDuet-*SCE1*-*SUMO4*GG (Figure 5A). The DNA sequence encoding the 3XHA tag was amplified by 3XHA*_f*BglII and 3XHA*_r*EcoRV primers (Supplemental Table 9) from pSMT7.1-*3XHA* plasmid and cloned into *BglII*- and *EcoRV*-digested pRSFDuet (Promega) to generate pRSFDuet-*3XHA*-PCNA and pRSFDuet-*3XHA*-RPL30 respectively.

The SUMO protease domain of *SMT7* cDNA was PCR amplified using primers SMT7*709-972*_f*BamHI and SMT7*709-972*_r*HindIII (Supplemental Table 9) and cloned into *BamHI*- and *HindIII*-digested pRSFDuet-*3XHA*-PCNA and pRSFDuet-*3XHA*-RPL30 to generate pRSFDuet-*3XHA*-PCNA-SMT7*709-972* (Figure 5A) and pRCFDuet-*3XHA*-RPL30-*SMT7*709-972* respectively. The catalytic-dead versions of the SUMO protease domain of *SMT7* cDNA were created using primers SMT7_C*928A*_f* and SMT7_C*928A*_r*, or SMT7_C*928S*_f* and SMT7_C*928S*_r* (Supplemental Table 9) and PCR amplified using primers SMT7*709-972*_f*BamHI and SMT7*709-972*_r*HindIII to generate SMT7*709-972/C928A* and SMT7*709-972/C928S* cDNA respectively. All constructs were sequenced to verify the DNA sequences.

For *E. coli*-based *in vitro* SUMOylation, pCDFDuet-*SAE1*-*SAE2*, pETDuet-*SCE1-*SUMO4*GG, and pRSFDuet-*3XHA*-PCNA or pRSFDuet-*3XHA*-RPL30 plasmids were introduced into *E. coli* Rosetta (DE3)
cells. Ten nanograms of each plasmid were transformed together into *E. coli Rosetta (DE3)* by electroporation (Bio-Rad 1 mm cuvette, 18000 V/cm, 200Ω, 25 μF). Transformed *E. coli* cells were selected on an LB agar plate containing 100 μg/ml carbenicillin, 50 μg/ml kanamycin, and 50 μg/ml streptomycin. Transformants were cultured in 5 ml of LB broth containing 100 μg/ml carbenicillin, 50 μg/ml kanamycin, and 50 μg/ml streptomycin at 37°C for 16 hours. Approximately 50 to 100 μl of overnight cultured cells were inoculated in 5 ml LB broth containing antibiotics and incubated at 37°C until the OD<sub>600</sub> reached 0.5–0.7.

For protein induction, 0.2 mM IPTG was added to the culture and the culture was incubated at 24°C for an additional 2.5 hours with vigorous shaking. Cells (with the OD<sub>600</sub> reaching up to approximately 1.0) were harvested from 1 ml culture by centrifugation. Cell pellets were resuspended in 200 μl 1X SDS sampling buffer (10% glycerol, 60 mM Tris/HCl pH 6.8, 2 % SDS, 0.01% bromophenol blue, and 0.1 M DTT) and incubated at 95°C for 5 mins. Induction of each protein component was verified by immunoblotting.

**Antibody generation and immunoblotting**

To generate polyclonal antibodies specific for SMT7, SUMO4, CYCD3, and RPL30 proteins, SMT7 peptide EFPKNIPTQRNGC (GeneScript), RPL30 peptide ITDPGDSDIKTVE (GeneScript), SUMO4 peptide FDGNRAKPDSTPEALGME (LTK BioLaboratories Taiwan), and CYCD3 peptide EDDVLEDDEDRFYRP (LTK BioLaboratories Taiwan) were synthesized respectively to generate rabbit polyclonal anti-sera. Polyclonal antibodies raised against SMT7, SUMO4, CYCD3, and RPL30 were affinity purified respectively.

Approximately 3 × 10<sup>7</sup> cells were harvested by centrifugation at 3220 x g for 5 min at RT. Cells were homogenized in 200 μL of 1X Urea-based protein extraction buffer (250 mM Tris-HCl, pH 6.8, 3.5 % SDS, 10% Glycerol, 1M Urea) supplemented with 1X protease inhibitor cocktail (Sigma-Aldrich), 1 mM PMSF, 1 mM benzamidine, and 10 μM MG132 at 4°C for 15 min. Cell debris was removed by centrifugation at 16,200 x g for 30 min at 4°C. Supernatant was then transferred to a new tube and protein concentration was determined using a Bio-Rad DC Protein Assay Kit.
Protein samples were resolved in a standard 10% or 12% SDS-PAGE or Bolt 4-12% Bis-Tris Plus gel (Invitrogen) and transferred to Immobilon-P PVDF membrane (Merck Millipore). Blots were blocked in 1 x TBST (20 mM Tris base, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h at room temperature (RT) and incubated with primary antibodies diluted in 1 x TBST with 5% non-fat milk at 4°C overnight. Anti-RPL30 antiserum was incubated at 35°C for 1 h. Antibody dilutions were as follows: anti-HA (1:1000, 3F10, Roche 11867423001), anti-SUMO4 (1:3000), anti-RPL30 (1:3000), anti-CYCD3 (1:3000), anti-RbcL (1:5000, Agrisera AS03037), anti-AtpB (1:5000, Agrisera AS05085), anti-CRD1 (1:3000, Agrisera AS06122), anti-Histone 3 (1:5000, Agrisera AS10710), anti-S (1:5000, Novagen 71549-3), anti-polyHistidine (1:3000, Sigma-Aldrich H1029), and anti-SMT7 (1:3000).

Blots were washed three times for 15 min each and then incubated with horseradish peroxidase (HRP)-conjugated goat-anti-mouse-IgG (1:20000, Jackson ImmunoResearch Laboratories 115-035-003), goat-anti-rat-IgG (1:20000, Jackson ImmunoResearch Laboratories 112-035-003) or goat-anti-rabbit-IgG (1:20000, PerkinElmer NEF812) for 1 h at RT. After washing in 1 x TBST for 15 min three times, the blots were processed for chemiluminescence detection using Advansta WesternBright ECL or Amersham ECL select (for detection of HA-tagged protein) Western Blotting detection reagent. The images were acquired by a ChemiDoc XRS+ imager (Bio-Rad).

**Immunofluorescence**

A linker sequence 5′-GGCGGCAGCGGCGGCGAGCGCGCcAGCGGCGG (encoding a peptide sequence: GGSGGSGGSGG) was introduced into the coding region between SMT7 and 3XHA by DNA synthesis (GENEWIZ); therefore, the pSMT7.1-lin-3XHA plasmid was generated. The pSMT7.1-lin-3XHA plasmid was transformed into 21gr and smt7-1 mat3-4 strains to generate SMT7-lin-3XHA and smt7-1 mat3-4::SMT7-lin-3XHA strains, respectively. One milliliter of cells (~10^6 cells mL^-1) growing in the TAP medium was allowed to attach to polylysine-coated coverslips for 15 min at RT. The attached cells were fixed by immersion into two changes of pre-cooled -20°C 200 μl of 100% methanol for 10 min and rehydrated in 500 μl of 1X phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) at RT for 15 min. Coverslips were then blocked in 150 μl blocking solution (5% BSA and
5% Normal Goat Serum (Jackson ImmunoResearch Laboratories) in 1X PBS) at RT for 90 min. After blocking, coverslips were incubated with anti-HA (Roche 3F10) antibody diluted at a ratio of 1:50 in blocking solution at 4°C overnight. Coverslips were washed three times with 1X PBST (1X PBS supplemented with 0.1 % Tween 20), 10 min each in a coplin jar at RT. Goat anti-rat Alexa Fluor 488-conjugated secondary antibody (ThermoFisher Scientific) was then diluted in blocking solution at 1:500 and incubated with coverslips for 120 min at RT in darkness. Following washing in 1X PBST for 10 min, coverslips were stained with DAPI (2 μg/mL in 1X PBS) for 10 min at RT. Following four washes with 1X PBST with 10 min per wash at RT, coverslips were mounted with Vectashield antifade medium (Vector laboratories H-1000). Zeiss 710 confocal microscopy with filter sets (488 nm laser for Alexa Fluor 488; 405 nm UV laser for DAPI) were used to capture the images.

**Immunoprecipitation and mass spectrometry**

The smt7-1 mat3-4 and smt7-1 mat3-4::SMT7C928A-3XHA strains growing in HSM medium under continuous light were used for IP. Approximately 3 × 10⁸ cells were harvested by centrifugation at 3220 x g for 5 min at RT. Cells were washed two times with 10 mL of 1X PBS at RT. Cell pellets were then resuspended in 1 mL of 1X PBS containing 2 mM of Disuccinimidyl Glutarate (DSG, ThermoFisher Scientific) or ethylene glycol bis(succinimidy succinate) (EGS, ThermoFisher Scientific) for cross-linking. Cells were incubated on ice for 30 min, and then quenched by adding Tris-HCl (pH 7.5) to 200 mM and incubated on ice for another 5 min. Cross-linked cells were washed twice with 10 mL 1X PBS at 4°C. Cell pellets were resuspended in 1 mL of RIPA buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM EGTA, 10% Glycerol) containing 1X protease inhibitor cocktail (Sigma-Aldrich), 1 mM PMSF, 1 mM benzamidine, and 10 μM MG132 and vortexed for 45 min at 4°C in the presence of 500 μL zirconium beads (1 mm in diameter; Biospec). Cell lysates were transferred to a new 1.5 mL tube and centrifuged at 16,200 x g for 30 min at 4°C to remove cell debris. Supernatant was then transferred to a new tube and protein concentration was determined using a Bio-Rad DC Protein Assay Kit.

Cell lysates were subjected to IP with anti-HA conjugated magnetic beads (Pierce). Thirty-five microliters (10 mg/ ml) of anti-HA magnetic beads (Pierce) were washed once with 1 mL 1X TBST and
incubated with 3500 to 4000 μg protein lysates at 4°C for 4 h with 15 rpm end-over-end mixing. After incubation, magnetic beads were washed five times with 1 mL of RIPA buffer containing 1X protease inhibitor cocktail (Sigma-Aldrich), 1 mM PMSF, 1 mM benzamidine, and 10 μM MG132 for 10 min at 4°C. Immunoprecipitates were sent to the Proteomics Core Laboratory (Academia Sinica) for LC-MS/MS analysis following the standard protocol (http://ipmb.sinica.edu.tw/proteomics/Services.html (Shevchenko et al., 1996)). Peptides and proteins were identified using Proteome Discoverer software (v.2.1, Thermo Fisher Scientific) with the SEQUEST search engine against Creinhardtii 281 v.5.5. protein database in Phytozome v.12.

For tandem immunopurification, 10 mg total cellular proteins were used for IP. IP was carried out as described above except using the modified 1X RIPA buffer supplemented with 0.1% SDS, 0.5% sodium deoxycholate, 20 mM N-ethylmaleimide (NEM, SUMO protease inhibitor), 1X protease inhibitor cocktail (Sigma-Aldrich), 1 mM PMSF, 1 mM benzamidine, and 10 μM MG132. The immunoprecipitates from the first purification were eluted from magnetic beads using 2.5% SDS solution. The eluate was diluted by adding 10 ml of modified RIPA buffer and concentrated using an Amicon Ultra-4 centrifugal filter unit (Merck) before secondary IP. Immunoprecipitates were then eluted by 2.5% SDS solution and subjected to immunoblotting analysis. Horse radish peroxidase-conjugated anti-HA antibody (1:1000, Cell signaling, # 2999) was used for immunoblotting.

**Co-immunoprecipitation of SMT7^{C928A}-3XHA and RPL30**

The cell lysates of *smt7-1 mat3-4::SMT7^{C928A}-3XHA* transformant expressing SMT7^{C928A}-3XHA protein were subjected to IP with anti-RPL30 antibody conjugated beads. The cell lysates of *smt7-1 mat3-4* were used as a negative control. Approximately 40 μg anti-RPL30 antibody was conjugated to 30 μL of protein A Magnetic Sepharose beads (GE Healthcare). Briefly, RPL30 antibody was incubated with beads in 1 x TBS binding buffer (20 mM Tris, 150 mM NaCl, pH 7.5) at RT for 60 min. After removing 1 x TBS binding buffer, RPL30 antibody was cross-linked to beads by incubating in 500 μL of 200 mM triethanolamine (pH 8.9) supplemented with 50 mM dimethyl pimelimidate dihydrochloride (DMP) (Sigma-Aldrich) at RT for 60 min. After removing the crosslinking solution, 500 μL of 100 mM ethanolamine (pH
8.9) was added and incubated at RT for another 15 min to stop the crosslinking reaction. The un-bound antibody was removed by 500 μL of elution buffer (0.1 M Glycine-HCl with 2 M Urea, pH2.9). The RPL30 antibody-conjugated beads were washed twice with 500 μL 1 x TBS binding buffer and subjected to the IP procedure described previously with slight modifications. In brief, 1200 μg *Chlamydomonas* cell lysates were pre-cleaned by incubating with 30 μL of protein A Magnetic Sepharose beads (GE Healthcare) at 4°C for 2 h. The pre-cleaned cell lysates were then incubated with RPL30 antibody-conjugated beads supplemented with 0.1% SDS and 0.5% Sodium deoxycholate (SDC) at 4°C for 4 h.

**Nuclei isolation from *Chlamydomonas***

Nuclei were isolated as described previously (Ning et al., 2013) with slight modifications. Briefly, 2 × 10⁸ cells were harvested from cultures growing in HSM medium under continuous illumination (GE F40 CW and GE F32T8) of 250 to 300 μmol photons m⁻²s⁻¹ at 24°C. The cell wall was removed by incubating cells in 20 mL of autolysin at RT overnight. NP-40 was used to test the effectiveness of autolysin treatment. Autolysin-treated cells were then harvested, resuspended, and incubated in 5 mL of 1X NIBA buffer (1X nuclei isolation buffer in CelLytic Plant Nuclei Isolation/Extraction kit, Sigma-Aldrich, containing 1X protease inhibitor cocktail (Sigma-Aldrich), 1 mM PMSF, 1mM benzamidine, 10 μM MG132, and 20 mM N-Ethylmaleimide (NEM)) on ice for 10 min. Homogenized cells were harvested by centrifugation at 1260 g for 10 min and resuspended in 10 mL 1X NIBA with 2% NP-40 on ice for 60 min. Nuclei were harvested by centrifugation at 1000g for 30 min at 4°C. The harvested nuclear pellets were washed twice with 10 mL of 1X NIBA solution and resuspended in 1 mL RIPA buffer containing 1X protease inhibitor cocktail (Sigma-Aldrich), 1 mM PMSF, 1 mM benzamidine, 10 μM MG132, 20 mM NEM.

**Accession Numbers**

CrSMT7, Cre16.g692600; CrRPL30, Cre10.g420750; CrSUMO1, Cr16.g662550; CrSUMO2, Cre16.g675749, CrSUMO3, Cre16.g675861; CrSUMO4, Cre16.g675637; CrSAE1, Cre09.g408550; CrSAE2, Cre06.g296983; CrSCE1, Cre02.g142000.
Supplemental Data

Supplemental Figure 1. Nomarski images of daughter cells of the indicated strains.

Supplemental Figure 2. SMT7 encodes a SUMO protease.

Supplemental Figure 3. Schematic of SMT7 protein showing the SUMO-interacting motifs (purple) predicted by GPS-SUMO (Zhao et al., 2014); the predicted nuclear localization sequences (NLS, blue) predicted by NLS mapper (Kosugi et al., 2009), and SUMO protease domain (pfam 02902, yellow).

Supplemental Figure 4. The synthesized SMT7 genomic fragment.

Supplemental Figure 5. Expression pattern of SMT7 mRNA in a synchronized wild-type (21gr) culture (Fang et al., 2014) by droplet digital PCR.

Supplemental Figure 6. Clustal Omega alignment of protein sequences of *Chlamydomonas* SUMOylation components.

Supplemental Figure 7. Transcript abundance and protein alignment of *Chlamydomonas* SUMOs.

Supplemental Figure 8. SUMOylation sites prediction.

Supplemental Figure 9. Immunoblot detection of the recombinant SUMO1, SUMO2, and SUMO4 proteins using SUMO4 (α-SUMO4), His-tag (α-His), and SUMO96 (α-SUMO96) antibodies.

Supplemental Figure 10. Detection and identification of the immunoprecipitated RPL30 protein.

Supplemental Figure 11. SUMOylation of RPL30.

Supplemental Figure 12. Immunoblotting was used to confirm down-regulation of RPL30 in multiple transgenic mat3-4 strains carrying the *amiRPL30-2*, *amiRPL30-3*, or *amiRPL30-4* construct.

Supplemental Figure 13. Potential important features of RPL30 protein.

Supplemental Figure 14. Schematic representation of pF1.1 vector.

Supplemental Table 1. Modal daughter cell sizes (the size that appears most frequently in the cell size distribution data) of the indicated strains derived from crosses of *smt7-1 mat3-4 pSMT7.1-3XHA* #3 and 6145C (mt+).

Supplemental Table 2. Modal daughter cell sizes (the size that appears most frequently in the cell size distribution data) of the indicated strains.
Supplemental Table 3. Proteins identified by immunoprecipitation with the RPL30 antibody followed by Mass Spectrometry analysis.

Supplemental Table 4. Primers used for generation of the RPL30, RPL30-SUMO4ΔGG, and SUMO4ΔGG-RpL30 overexpressing constructs.

Supplemental Table 5. The DNA sequences used for generating RPL30 artificial miRNA constructs.

Supplemental Table 6. RACE and RT-PCR primers used to amplify the SMT7 cDNA fragments.

Supplemental Table 7. Primers used for RT-PCR of the CDKB1 and RPL30, and for droplet RT-PCR of SMT7.

Supplemental Table 8. Nomenclature conversion table for annotation of SUMOylation components.

Supplemental Table 9. Primers used to amplify Chlamydomonas SUMOylation components for in vitro SUMOylation assay.

Supplemental Dataset 1. Alignments used to generate the phylogeny presented in Supplemental Figure 2B.

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AUTHOR CONTRIBUTION
SCF conceived the idea and coordinated the study. SCF and YLL designed the experiments. YLL, CLC, CHC, and MHC conducted the experiments. YLL and SCF analyzed the data and wrote the manuscript.
**Table 1.** Modal cell sizes (i.e., the size that appears most frequently in the cell size distribution data) of the indicated strains. Standard deviation (SD) values were derived from at least three independent experiments.

| Genotype                          | Daughter cell size (µm³ ± SD) |
|-----------------------------------|------------------------------|
| Wild-type (21gr)                  | 65.6 ± 6.9                   |
| *mat3-4*                          | 24.1 ± 1.7                   |
| *smt7-1 mat3-4*                   | 37.0 ± 0.5                   |
| *smt7-1 mat3-4::SMT7-3XHA #1*     | 25.8 ± 2.5                   |
| *smt7-1 mat3-4::SMT7-3XHA #2*     | 22.8 ± 1.5                   |
| *smt7-1 mat3-4::SMT7-3XHA #3*     | 21.4 ± 0.7                   |
| *smt7-1 mat3-4::SMT7*C928A*-3XHA_A11* | 42.8 ± 3.6                   |
| *smt7-1 mat3-4::SMT7*C928A*-3XHA_A19* | 34.6 ± 3.6                   |
| *smt7-1 mat3-4::SMT7*C928A*-3XHA_A22* | 33.5 ± 3.6                   |
| *smt7-1 mat3-4::SMT7*C928S*-3XHA_A22* | 38.8 ± 3.1                   |
| *smt7-1 mat3-4::SMT7*C928S*-3XHA_S20* | 33.8 ± 2.1                   |
| *smt7-1 mat3-4::SMT7*C928S*-3XHA_S22* | 35.3 ± 3.5                   |
Table 2. SMT7-interacting proteins isolated by immunoprecipitation.

| Gene ID         | Description                                                | Number of times being identified from 10 independent IP experiments |
|-----------------|-------------------------------------------------------------|---------------------------------------------------------------|
| Cre10.g420750   | Ribosomal protein L30                                      | 6                                                             |
| Cre12.g493950   | Plastid ribosomal protein S13                              | 3                                                             |
| Cre04.g222700   | Elongation factor 3a-related                               | 3                                                             |
| Cre10.g452800   | Low-CO2-inducible protein                                  | 3                                                             |
| Cre12.g485800   | FtsH-like membrane ATPase/metalloprotease                  | 3                                                             |
| Cre01.g039250   | Ribosomal protein S2                                       | 2                                                             |
| Cre12.g504200   | Ribosomal protein S23                                      | 2                                                             |
| Cre09.g393450   | Flagellar associated protein                               | 2                                                             |
| Cre12.g508750   | Light harvesting chlorophyll a/b binding protein 2 (Lhca2) | 2                                                             |
| Cre15.g635600   | Nascent polypeptide associated complex alpha-subunit related | 2                                                             |
Table 3. Modal cell sizes of *mat3-4, smt7-1 mat3-4*, and three independent *mat3-4::amiRPL30* strains. Standard deviation (SD) values were derived from three to five independent experiments.

| Genotype                          | Daughter cell size (μm³ ± SD) |
|----------------------------------|-------------------------------|
| mat3-4                           | 24.6 ± 2.4                    |
| smt7-1 mat3-4                    | 38.5 ± 4.5                    |
| mat3-4::amiRPL30-2 #20           | 24.7 ± 2.0                    |
| mat3-4::amiRPL30-2 #22           | 25.1 ± 0.5                    |
| mat3-4::amiRPL30-2 #33           | 27.4 ± 4.5                    |
| mat3-4::amiRPL30-3 #9            | 27.7 ± 1.8                    |
| mat3-4::amiRPL30-3 #19           | 24.2 ± 1.6                    |
| mat3-4::amiRPL30-4 #2            | 27.8 ± 3.2                    |
| mat3-4::amiRPL30-4 #13           | 24.1 ± 2.6                    |
| mat3-4::amiRPL30-4 #16           | 23.1 ± 2.3                    |
Table 4. Modal cell sizes of mat3-4, smt7-1 mat3-4, independent mat3-4::RPL30-SUMO4ΔGG-3XHA, mat3-4::SUMO4ΔGG-RPL30-3XHA, mat3-4::RPL30-3XHA, and mat3-4::SUMO4ΔGG-3XHA strains. Standard deviation (SD) values were derived from three to six independent experiments.

| Genotype                                           | Daughter cell size (μm³ ± SD) |
|----------------------------------------------------|------------------------------|
| mat3-4                                             | 23.8 ± 2.1                   |
| smt7-1 mat3-4                                      | 34.3 ± 1.6                   |
| mat3-4::RPL30-SUMO4ΔGG-3XHA_A18                    | 31.2 ± 1.6*                 |
| mat3-4::RPL30-SUMO4ΔGG-3XHA_A31                    | 29.4 ± 2.3*                 |
| mat3-4::RPL30-SUMO4ΔGG-3XHA_A34                    | 30.9 ± 0.9*                 |
| mat3-4::RPL30-SUMO4ΔGG-3XHA_B1                     | 27.7 ± 3.6                  |
| mat3-4::RPL30-SUMO4ΔGG-3XHA_B32                    | 27.2 ± 2.8                  |
| mat3-4::SUMO4ΔGG-RPL30-3XHA_A5                     | 23.2 ± 2.5                  |
| mat3-4::SUMO4ΔGG-RPL30-3XHA_A8                     | 21.9 ± 0.7                  |
| mat3-4::SUMO4ΔGG-RPL30-3XHA_A15                    | 22.4 ± 2.4                  |
| mat3-4::RPL30-3XHA_C14                             | 21.4 ± 3.0                  |
| mat3-4::RPL30-3XHA_C17                             | 26.1 ± 4.0                  |
| mat3-4::RPL30-3XHA_C36                             | 25.6 ± 1.5                  |
| mat3-4::SUMO4ΔGG-3XHA #6                           | 22.1 ± 3.5                  |
| mat3-4::SUMO4ΔGG-3XHA #12                           | 24.0 ± 1.8                  |
| mat3-4::SUMO4ΔGG-3XHA #17                           | 22.7 ± 3.3                  |
| mat3-4::SUMO4ΔGG-3XHA #18                           | 24.3 ± 4.9                  |
| mat3-4::SUMO4ΔGG-3XHA #19                           | 23.7 ± 2.5                  |

*Indicates a significant increase in cell size of mat3-4::RPL30-SUMO4ΔGG strains compared with mat3-4 by a two-tailed nonparametric t-test (P < 0.05).
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Figure 1. Molecular characterization of SMT7. (A) Schematic representation of the SMT7 gene model. Black lines indicate introns. Black conserved catalytic triad of SUMO proteases. Blue line indicates the SUMO protease domain. **HsSENP3** (Q9H4L4.2), HsSENP5 (Q9H610.3), HsSENP6 (Q9GZR1.2), HsSENP7 (Q9BQF6.4), *Saccharomyces cerevisiae* (ScULP2, AT1G26470) and *Arabidopsis thaliana* (AtESD4, AT4G15880), and AtESD4L (AT3G06910). **,** conserved residue (in red). "**", residue showing strong similarity among sequences. ",", residue showing weak similarity among sequences. Red triangles represent the conserved catalytic triad of SUMO proteases. Blue line indicates the SUMO protease domain.
Figure 2. Immunoblot analysis of total protein lysates of the smt7-1 complemented strains (A) Immunoblot detection of SMT7-3XHA protein with the anti-HA antibody from total protein extracts of three independent transgenic smt7-1 mat3-4 strains carrying the pSMT7.1-3XHA construct, mat3-4, smt7-1, and wild type (21gr) strains. SMT7-3XHA protein was shown as a single band predicted at the molecular mass ~106 kDa. Immunoblot with Ribulose bisphosphate carboxylase large chain (RbcL) antibody was used as a loading control. (B) Immunoblot detection of SMT7\textsuperscript{C928S}-3XHA or SMT7\textsuperscript{C928S}-3XHA protein with the anti-HA antibody from total protein extracts of the independent transgenic smt7-1 mat3-4 strains carrying the pSMT7.1\textsuperscript{C928A}-3XHA or pSMT7.1\textsuperscript{C928S}-3XHA construct respectively. Immunoblot with ATP synthase subunit beta (AtpB) antibody was used as a loading control. An asterisk marks a putative degraded SMT7-3XHA protein. Total protein extracts were separated by a 10% SDS-PAGE (A) or a 4%-12% gradient gel (B).
Figure 3. Expression of SMT7 protein is diurnally regulated. (A) Graph showing the percentage of cells that passage through commitment (dashed lines) and mitotic index (solid lines) of the synchronized smt7-1::SMT7-3XHA culture. The complemented smt7-1::SMT7-3XHA cell culture entered the S/M phase at approximately 12 h. The bar above the graph indicates the cell cycle phases. Synchronized cultures were maintained in 12-h-light/12-h-dark cycles. (B) Expression of S/M phase marker CDKB1 determined by qRT-PCR analysis. Standard errors are shown by error bars. (C) Immunoblot detection of SMT7-3XHA from total protein extracts of the synchronized complemented smt7-1::SMT7-3XHA strain with the anti-HA antibody. Protein samples were collected at the indicated time points. The amounts of protein per lane for the immunoblotting and Coomassie blue staining were normalized by equal protein amount (upper three panels) or equal cell number (lower three panels). Protein expression of cyclin D3 (CrCYCD3) is used as a S/M phase marker.
Figure 4. Subcellular localization of SMT7 protein. (A) Immunoblots of total cellular protein (whole cell) and the nuclear and cytoplasmic/membrane fractions prepared from smt7-1, and complemented smt7-1 strain expressing a triple HA-tagged SMT7 protein (smt7-1::SMT7-3XHA_I18). Histone 3 (H3) was used as a nuclear marker and CRD1 protein was used as a chloroplast membrane marker (Allen et al., 2008). Equal amounts of protein were loaded in each lane. The experiment was repeated three times, yielding an identical result, with representative data shown. (B) Immunoblots of total cellular protein from transgenic strains expressing triple HA-tagged SMT7 protein with linker (smt7-1::SMT7-lin-3XHA) in the wild-type and smt7-1 mat3-4 background. Immunoblots with RbcL antibodies were used as a loading control. (C) Confocal immunofluorescence microscopy of wild-type and SMT7-lin-3XHA-expressing cells stained with anti-HA antibody and the DNA dye DAPI. High magnification image of the cell in red boxed region was shown. (D) Confocal immunofluorescence microscopy of smt7-1 mat3-4 and SMT7-lin-3XHA-expressing smt7-1 mat3-4 cells stained with anti-HA antibody and the DNA dye DAPI. High magnification image of the cell in red boxed region was shown. GFP/DAPI, merged signals from the GFP and DAPI channels. DIC, differential interference contrast images of cells.
Figure 5. A reconstituted in vitro SUMO conjugation and de-conjugation system for *Chlamydomonas*. (A) Expression vectors carrying the cDNAs encoding the individual SUMOylation components as indicated were generated and introduced into *E. coli* cells. (B) *Chlamydomonas* PCNA was conjugated with SUMO4. The SAE2 and SUMO4GG are N-terminally fused to 6XHis. SAE1, SCE1, and PCNA are C-terminally fused to the S tag. PCNA is N-terminally fused to the 3XHA tag. (C) SUMOylated *Chlamydomonas* PCNA was deconjugated by SMT7709-972. Expression of each SUMOylation component was confirmed by immunoblotting using the corresponding antibodies. Expression of SMT7 was confirmed using SMT7-specific antibody. Asterisks indicate the SUMOylated PCNA. Coomassie brilliant blue (CBB) staining showing equal loading of total protein amount. Immunoblotting showing two independent *E. coli* strains, depicted by “1” and “2”, carrying the indicated constructs. Total protein extracts were separated on a 12% SDS-PAGE gel.
Figure 6. Increased SUMO4 conjugates in \textit{smt7-1} and \textit{smt7-1 mat3-4} mutants. (A) Immunoblots of total cellular protein prepared from wild-type 21 gr, \textit{smt7-1} and three independent complemented \textit{smt7-1::SMT7-3XHA} strains with anti-SUMO4 antibody. (B) Immunoblots of total cellular protein prepared from \textit{mat3-4}, \textit{smt7-1 mat3-4}, three independent \textit{smt7-1 mat3-4::SMT7-3XHA}, two independent \textit{smt7-1 mat3-4::SMT7\textsuperscript{C928A}-3XHA}, and two independent \textit{smt7-1 mat3-4::SMT7\textsuperscript{C928S}-3XHA} strains with anti-SUMO4 antibody. Expression of SMT7-3XHA protein was confirmed by immunoblots with HA antibody. CBB staining and immunoblots with AtpB antibody were used to show equal loading of total protein amount. Total protein extracts were separated on a 4–12% gradient gel.
Figure 7. A defect in SMT7 affects the deSUMOylation of SUMO conjugated RPL30. (A) Immunoblot detection of HA-tagged SMT7C928A protein from anti-RPL30 immunoprecipitation of smt7-1 mat3-4 and the complemented smt7-1 mat3-4::SMT7C928A-3XHA_A22 strains. No antibody (No Ab) was used as a negative control for IP. ** marks the non-specific protein. (B) In vitro was used as a negative control. (E) Immunoblot detection of SUMO4-conjugated RPL30 proteins from the mat3-4::RPL30-3XHA_C14 cell lysates immunoprecipitated by anti-HA antibody followed by anti-SUMO4 antibody. The mat3-4 cell lysate was used as a negative control. (G) Immunoblot detection of RPL30-3XHA protein from total cell lysates prepared from mat3-4::RPL30-3XHA_C14 strain and the complemented smt7-1 mat3-4::SMT7C928A-3XHA_A22 strains with the anti-HA antibody. The mat3-4 cell lysate was used as a negative control. (H) Immunoblot detection of SUMO4-conjugated RPL30 proteins from cell lysates of smt7-1 mat3-4, mat3-4::RPL30-3XHA_C14, and two independent smt7-1 mat3-4::RPL30-3XHA strains immunoprecipitated with anti-HA antibody followed by anti-SUMO4 antibody. Red asterisks followed by a number mark the number of SUMO4 proteins conjugated with RPL30 protein.
Figure 8. Characterization of mat3-4::RPL30-SUMO4ΔGG-3XHA, mat3-4::SUMO4ΔGG-RPL30-3XHA, and mat3-4::SUMO4ΔGG-3XHA transgenic strains. (A) Immunoblot detection of RPL30-SUMO4ΔGG-3XHA protein from total cell lysates prepared from mat3-4 and five independent mat3-4::RPL30-SUMO4ΔGG-3XHA strains using the anti-HA antibody. The relative ratios of RPL30-SUMO4ΔGG-3XHA/RbcL protein are shown. The ratio of RPL30-SUMO4ΔGG-3XHA_B1/RbcL was arbitrarily set to one. (B) Immunoblot detection of SUMO4ΔGG-RPL30-3XHA protein from total cell lysates prepared from mat3-4 and six independent mat3-4::SUMO4ΔGG-RPL30-3XHA strains using the anti-HA antibody. (C) Immunoblots of total cell lysates prepared from mat3-4 and five independent mat3-4::SUMO4ΔGG-3XHA strains using the anti-HA antibody. (D) Cell size distribution of daughter cells of the indicated strains described in the panel. A18, A31, and A34 represent independent mat3-4::RPL30-SUMO4ΔGG-3XHA strains.
SUMO Protease SMT7 Modulates Ribosomal Protein L30 and Regulates Cell-size Checkpoint Function

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