Revised annotation and extended characterizations of components of the *Chlamydomonas reinhardtii* SUMOylation system

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**Abstract**

Small ubiquitin-like modifier (SUMO) conjugation, or SUMOylation, is a reversible post-translational modification that is important for regulation of many cellular processes including cell division cycle in the eukaryotic kingdom. However, only a portion of the components of the Chlamydomonas SUMOylation system are known and their functions and regulation investigated. The present studies are aimed at extending discovery and characterization of new components and improving the annotation and nomenclature of all known proteins and genes involved in the system. Even though only one copy of the heterodimerized SUMO-activating enzyme, *SAE1* and *SAE2*, was identified, the number of SUMO-conjugating enzymes (*SCEs*) and SUMO proteases/isopeptidase was expanded in Chlamydomonas. Using the reconstituted SUMOylation system, we showed that *SCE1*, *SCE2*, and *SCE3* have SUMO-conjugating activity. In addition to SUMOylation, components required for other post-translational modifications such as NEDDylation, URMylation, and UFMylation, were confirmed to be present in Chlamydomonas. Our data also showed that besides isopeptidase activity, the SUMO protease domain of SUPPRESSOR OF MAT3 7/SENTRIN-SPECIFIC PROTEASE 1 (SMT7/SENP1) has endopeptidase activity that is capable of processing SUMO precursors. Moreover, the key cell cycle regulators of Chlamydomonas E2F1, DP1, CDKG1, CYCD2, and CYCD3 were SUMOylated in vitro, suggesting SUMOylation may be part of regulatory pathway modulating cell cycle regulators.

**KEYWORDS**
cell cycle, *Chlamydomonas reinhardtii*, deSUMOylation, SUMOylation

**1 | INTRODUCTION**

Post-translational modifications increase the diversity of protein functions and provide an additional layer of regulation in eukaryotic cells. SUMOylation represents a reversible post-translational modification that allows small ubiquitin-related modifier (SUMO) to be conjugated to intracellular proteins (Vierstra, 2012). SUMO encodes an ~100-amino acid, β-grasp protein structure that is related to ubiquitin and other ubiquitin-like proteins. SUMO conjugation has been shown to regulate many cellular processes including...
transcription regulation, DNA repair, control of the cell cycle, chromatin remodeling, stress regulation, and cell fate reprogramming (Castro, Tavares, Bejarano, & Azevedo, 2012; Cossec et al., 2018; Eifler & Vertegaal, 2015; Hickey, Wilson, & Hochstrasser, 2012; Jackson & Durocher, 2013; Wotton, Pemberton, & Merrill-Schools, 2017).

Unlike ubiquitination that often leads to protein degradation, SUMO conjugation regulates diverse cellular processes including protein stability, protein-protein interaction, protein subcellular localization, and enzyme activity (Geiss-Friedlander & Melchior, 2007; Müller, Hoege, Pyrowolakis, & Jentsch, 2001). Similar to ubiquitination, SUMOylation is also catalyzed through an ATP-dependent E1-E2-E3 conjugation cascade (Figure 1). Before conjugation, SUMO precursors need to be processed by the endopeptidase activity of SUMO proteases to reveal the C-terminal diglycine motif and generate mature SUMO proteins. This diglycine motif is required for efficient adenylation by a heterodimeric SUMO-specific E1-activating enzyme (SAE1/SAE2) in an ATP-dependent manner, during which a thioester bond is formed between the C-terminal carboxyl group of SUMO and the active cysteine residue of SAE2 (Desterro, Rodriguez, Kemp, & Hay, 1999; Gong, Li, Millas, & Yeh, 1999; Johnson, Schwienhorst, Dohmen, & Blobel, 1997). The activated SUMO is then transferred to the SUMO-specific conjugating enzyme (SCE) otherwise referred to as ubiquitin-conjugating enzyme 9 (UBC9) in budding yeast and mammals by forming a thiol-transfer reaction (Transfer). SUMO transfer from SCE to target protein (Target) can occur through a SUMO E3 ligase (E3)-dependent or E3-independent manner, during which an isopeptide bond between the C-terminal glycine residue of SUMO and the Lys side chain of the target protein (Desterro, Thomson, & Hay, 1997; Johnson & Blobel, 1997; Lee et al., 1998). Even though the SUMO E2 enzyme in some cases can transfer SUMO directly to the target proteins, SUMO E3 protein ligases are often required to facilitate this process (Johnson & Gupta, 2001; Kahyo, Nishida, & Yasuda, 2001; Miura et al., 2009; Siramachandran et al., 2019; Takahashi, Kahyo, Toh, Yasuda, & Kikuchi, 2001; Takahashi, Toh, & Kikuchi, 2003; Yunus & Lima, 2009). Indeed, the presence of multiple unique SUMO E3 ligase species within the cell increases the breadth and specificity of target protein selection.

SUMOylation is a reversible process (Figure 1). Conjugated SUMOs can be removed from SUMOylated proteins by SUMO proteases otherwise referred to as Ubl-specific proteases (ULPs) or sentrin-specific proteases (SEPNPs). SUMO proteases are cysteine proteases that catalyze cleavage of the isopeptide bond formed between the SUMO carboxyl terminal glycine and the ε-amino group of lysine (K) of the target protein (Li & Hochstrasser, 1999, 2000). As noted above, the endopeptidase activity of SUMO proteases also are required to process SUMO precursors to produce mature SUMOs (Colby, Matthai, Boeckelmann, & Stuible, 2006; Di Bacco et al., 2006; Gong & Yeh, 2006).

Protein modification by SUMOylation is important for cell cycle regulation. Defect in the SUMO-conjugating enzyme UBC9 or SUMO protease/isopeptidase ULP1 in budding yeast leads to G2/M arrest during the cell cycle (Li & Hochstrasser, 1999; Seufert, Futcher, & Jentsch, 1995). Yeast SUMO protease Ulp2 is essential for spindle dynamics and cell cycle progression (Schwartz, Felberbaum, & Hochstrasser, 2007). Defect in SUMO protease SENP5 leads to decreased cell proliferation (Di Bacco & Gill, 2006). Additionally, mice with defect in UBC9 fail to progress through embryonic development.

**FIGURE 1** SUMOylation pathway. SUMOs are covalently conjugated to proteins via an ATP-dependent E1-E2-E3 enzymatic cascade. SUMO precursor protein is processed by the endopeptidase activity of SUMO proteases (SUMOp) to reveal its C-terminal diglycine (labeled in red) and generate mature SUMO protein (Maturation). Mature SUMO is activated by E1 heterodimeric SAE1-SAE2 in an ATP-dependent reaction (Activation), which allows formation of a thioester bond (blue line) between its C-terminal glycine and an active cysteine residue (C) in SAE2. The activated SUMO is subsequently transferred to a cysteine (C) in the active site of E2 enzyme (SCE) through a thioltransfer reaction (Transfer). SUMO transfer from SCE to target protein (Target) can occur through a SUMO E3 ligase (E3)-dependent or E3-independent manner, during which an isopeptide bond (green line) between the C-terminal glycine of SUMO and a lysine (K) residue in the target is formed (Conjugation). SUMO conjugation is reversible. SUMO conjugates can be removed from target protein by isopeptidase activity of SUMO proteases (DeSUMOylation).
due to DNA hypocondensation and genome instability (Nacerddine et al., 2005). Similarly, deletion of SUMO1 and SUMO2 causes embryonic lethality in Arabidopsis (Saracco, Miller, Kurepa, & Vierstra, 2007; van den Burg, Kini, Schuurink, & Takken, 2010). Moreover, recent genetic and large-scale proteomic studies identify numerous cell cycle regulators as SUMOylation targets, further supporting the importance of SUMOylation in cell cycle regulation (Armstrong, Mohideen, & Lima, 2012; Blomster et al., 2009; Dawlaty et al., 2008; Hendriks & Vertegaal, 2016; Lamoliatte, McManus, Maarifi, Chelbi-Alix, & Thibault, 2017; Mukhopadhyay, Arnaoutov, & Dasso, 2010; Nie, Xie, Loo, & Courey, 2009; Schimmel et al., 2014). SUMOylation has been shown to regulate the passage from the G1 to the S phase. For example, SUMOylation of human Rb promotes G1/S transition by increasing interaction between Rb and CDK2, and level of phosphorylated Rb, which results in the release of E2F transcription factor and consequent gene expression promoting DNA replication (Meng, Qian, Yue, Li, & Xue, 2016). In Arabidopsis, SUMOylated DPa dissociates the E2Fa/DPa complex and inhibits the expression of genes involved in DNA synthesis (Liu et al., 2016).

Chlamydomonas reinhardtii (Chlamydomonas) is a unicellular, ciliated, fresh-water green alga that has been utilized for studies of the cell cycle, photosynthesis, cilia biogenesis, and production of high-value molecules (Harris, 1989, 2001; Harris, 2009; Salome & Merchant, 2019; Stern & Harris, 2009; Witman & Harris, 2009). With availability of haploid genetics, a sequenced genome, mutant libraries, transformation system, gene editing capabilities, and established molecular toolkit (Baek et al., 2016; Blaby et al., 2014; Crozet et al., 2018; Dent, Haglund, Chin, Kobayashi, & Niyogi, 2005; Greiner et al., 2017; Jiang & Weeks, 2017; Kindle, Richards, & Stern, 1991; Kindle, Schnell, Fernandez, & Lefebvre, 1989; Li, Zhang, et al., 2016; Merchant et al., 2007; Picariello et al., 2020; Shin et al., 2016; Tulin & Cross, 2014; Zhang et al., 2014), Chlamydomonas has emerged as a highly valuable single-cell model system. Chlamydomonas also has a SUMOylation system and SUMO conjugation has been reported to regulate stress responses and the cell division cycle (Knoebb et al., 2015; Lin, Chung, Chen, Chen, & Fang, 2020; Wang et al., 2008). However, an updated comprehensive categorization of the Chlamydomonas SUMO system and its relationship to plant and animal systems remain limited. Here, we present a phylogenetic overview and annotation of the core components of the Chlamydomonas SUMO system and provide biochemical evidence of the SUMO-conjugating activity of SCE2 and SCE3 enzymes. In addition to SUMOylation and ubiquitination machineries, our analysis supports and extends the previous suggestion (Wang et al., 2008) that other ubiquitin-like posttranslational protein modifications may be present in Chlamydomonas.

2 | MATERIALS AND METHODS

2.1 | Sequence mining and collection

The full-length sequences of Arabidopsis SUMO-ACTIVATING ENZYME 1a (SAE1a, AT4G24940), SAE1b (AT5G50680), and SAE2 (AT2G21470) proteins were used as BLASTP queries and searched against the Chlamydomonas proteome (https://phytozome.jgi.doe.gov/pz/portal.html#info?alias=Org_Creinhardtii) to identify potential Chlamydomonas SAEs (E-value of 1E-25 was set as an arbitrary cutoff). Arabidopsis ubiquitin-activating enzyme (UBA1, AT2G30110), RUB1/NEDD8-activating enzyme (ECR1, AT5G19180), NEDD8-activating enzyme, (AXR1, AT1G05180), URM1-activating enzyme (CNX5, AT5G55130), UF1M-activating enzyme (UBA5, AT1G05350), and ATG12-activating enzyme (ATG7, AT5G45900) were also used as BLASTP queries and searched against Chlamydomonas ubiquitin or ubiquitin-like E1 candidates (E-value of 1E-40 was set as an arbitrary cutoff). Even though Cre03.g159400 and Cre14.g622550 contain the ThiF/MoeB domain (pfam00899), further analysis revealed that they encode tRNA threonylcarbamoyladenosine dehydratases (TCDs) and do not belong to the E1 enzymes, and were therefore, omitted from phylogenetic analysis. All of the other SUMO, ubiquitin or ubiquitin-like E1 candidates were included in phylogenetic analysis.

Similarly, the full-length protein sequence of Arabidopsis SCE1 (AT3G57870) was used as a BLASTP query and searched against the Chlamydomonas proteome to identify SCEs. Chlamydomonas ubiquitin or ubiquitin-like conjugating enzyme candidates were also identified using Arabidopsis UBC1 (AT1G14400), RCE1 (AT4G36880), RCE2 (AT2G18600), and UFC1 (AT1G27530) as the BLASTP queries and their homologs were searched for. The identified Chlamydomonas ubiquitin-like conjugating enzyme candidates were used as queries to search for more proteins containing the ubiquitin-conjugating enzyme domain (pfam00179).

As for SUMO E3 ligases, full-length sequences of Arabidopsis SIZ1 (AT5G60410), MM521 (AT3G15150), PIAL1 (AT1G08910), and PIAL2 (AT5G41580) proteins were used as a BLASTP query and their homologs were searched for in the Chlamydomonas proteome. An E-value of 1E-5 was set as an arbitrary cutoff. Only the candidates containing the SP-RING zinc finger domain (pfam02891) and a zinc finger of the MIZ type in NSE subunit (pfam11789) were kept for further analysis.

To identify Chlamydomonas SUMO proteases, the full-length protein sequences of Arabidopsis ESD4 (AT4G15880), ELS1 (AT3G06910), ELS2 (AT4G00690), OTS1 (AT1G60220), OTS2 (AT1G0570), FUG1 (AT3G48480), SPF1 (AT1G09730), and SPF2 (AT4G33620) were used as BLASTP queries and the ULP-type SUMO proteases were searched for in the Chlamydomonas proteome. Because the SUMO protease sequences are very divergent, E-value of 1E-4 was set as an arbitrary cutoff. Only the candidates containing the C48 cysteine protease domain (pfam02891) and their homologs were searched for in Chlamydomonas proteome (an E-value of 1E-2 was set as an arbitrary cutoff). Only candidates containing the C97 peptidase domain (pfam05903) were collected.
for further analysis. In addition, human USPL1 (Q5W0Q7) was used as a BLASTP query to search for Chlamydomonas homologs to identify the potential USPL1-type of SUMO proteases (C98 cysteine protease domain, pfam15499). However, no homolog was identified in Chlamydomonas. To identify deNEDDylating proteases in Chlamydomonas, the full-length protein sequence of Arabidopsis DFN1 (AT5G60190) was used as the BLASTP query and an E-value of 1E-25 was set as an arbitrary cutoff.

To validate the SUMOylation components of Chlamydomonas, reciprocal BLAST against The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/) and the Saccharomyces Genome Database (SGD; http://yeastgenome.org/) was also performed using the Chlamydomonas candidates to ensure that these subject hits most closely matched the corresponding SUMOylation components. Protein sequences were aligned by Clustal Omega program (Sievers et al., 2011) and the conserved/similar residues or conserved domains were highlighted.

2.2 Phylogenetic analyses

The accession numbers of proteins used for phylogenetic analysis are listed in DataS1. The protein sequences of Chlamydomonas reinhardtii, Arabidopsis thaliana, Homo sapiens, and Saccharomyces cerevisiae were retrieved from Phytozone version 12 (https://phytozone.jgi.doe.gov/pz/portal.html#info?alias=Org_Reinhardtii), the Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/), UniProt Knowledgebase (UniProtKB, https://www.uniprot.org/help/uniprot_kb), and the Saccharomyces Genome Database (SGD, https://www.yeastgenome.org/), respectively. Protein sequences were aligned by Clustal Omega program. Bootstrap values lower than 50% are not shown on the trees.

2.3 Culture conditions

Chlamydomonas strain 21gr (MT+) was maintained on Tris-acetate-phosphate (TAP) plates or cultured in TAP liquid medium under continuous cool white light (GE F40 CW) with illumination of 250 to 300 μmol photons m⁻² s⁻¹ at 24°C aerated under 0.5% (v/v) CO₂.

2.4 RNA isolation, RT-PCR, and plasmids construction

Cells were harvested from cultures growing in TAP liquid medium. Total RNA was isolated as described previously (Fang, Chung, Chen, Lopez-Paz, & Umen, 2014; Lin et al., 2020). The full-length cDNAs of Chlamydomonas SCE2, SCE3, SUMO1, SUMO2, SUMO3, E2F1, DP1, CDKG1, CYCD2, and CYCD3 were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using the gene-specific primers (Table S1). The PCR program was carried out as described previously (Lin et al., 2020). The SCE3 cDNA could not be amplified and was omitted from further study. The SCE2 cDNA (508 bp) fragment was digested by BglII and SalI and cloned into the BglII- and XhoI-digested pETDuet-SUMO4GG plasmid to generate pETDuet-SCE2-SUMO4GG. SUMO4GG encodes the mature SUMO4 with the diglycine at the C-terminus (Lin et al., 2020). The SCE3 cDNA (533 bp) fragment was digested by BglII and XhoI and cloned into the BglII- and XhoI-digested pETDuet-SUMO4GG plasmid to generate pETDuet-SCE3-t2-SUMO4GG. The pCDFDuet-SCE1-SUMO4GG, pRSFDuet-3XHA-PCNA, and pRSFDuet-3XHA-PCNA-SMT7709-972 plasmids were generated previously (Lin et al., 2020). SMT7 encodes the SUMO protease domain of SMT7 protein as previously described (Lin et al., 2020).

The amplified E2F1 (1557 bp), DP1 (1596 bp), and CDKG1 (1179 bp) cDNAs were digested with EcoRV and XhoI and cloned into the EcoRV- and XhoI-digested pRSFDuet-3XHA to generate pRSFDuet-3XHA-E2F1, pRSFDuet-3XHA-DP1, and pRSFDuet-3XHA-CDKG1, respectively. CYCD2 (1101 bp) and CYCD3 (1176 bp) cDNAs were digested with EcoRV and PacI and cloned into EcoRV- and PacI-digested pRSFDuet-3XHA to generate pRSFDuet-3XHA-CYCD2, pRSFDuet-3XHA-CYCD3, respectively. The SUMO protease domain of SMT7 cDNA, SMT7 (815 bp) was PCR amplified using the primers SMT7-972_fBamHI and SMT7-972_r_HindIII (Table S1) and cloned into BamHI- and HindIII-digested pRSFDuet-3XHA-E2F1, pRSFDuet-3XHA-DP1, pRSFDuet-3XHA-CDKG1, pRSFDuet-3XHA-CYCD2, and pRSFDuet-3XHA-CYCD3 to generate pRSFDuet-3XHA-E2F1_SMT7-972, pRSFDuet-3XHA-DP1-SMT7-972, pRSFDuet-3XHA-CDKG1-SMT7-972, pRSFDuet-3XHA-CYCD2-SMT7-972, and pRSFDuet-3XHA-CYCD3-SMT7-972, respectively.

To test the SUMO endopeptidase activity of SMT7, the SMT7 (811 bp) cDNA was PCR amplified using primers SMT7-972_fBamHI and SMT7-972_r_HindIII (Table S1) and cloned into BamHI- and XhoI-digested pET28a-6XHis-SMT3 (a gift from Dr. Jun-Yi Yang) to generate pET28a-6XHis-SMT3 (333 bp) and pET28a-6XHis-SMT3 (297 bp) were digested with NdeI and BamHI and cloned into NdeI- and BamHI-digested pET28a-6XHis-SMT7-972, respectively. The full-length cDNAs of SUMO1 (483 bp) and SUMO2 (309 bp) were digested with Nhel and BamHI and cloned into Nhel- and BamHI-digested pET28a-6XHis-SMT7-972, respectively. The full-length cDNAs of SUMO1 (483 bp) and SUMO2 (309 bp) were digested with NdeI and BamHI and cloned into NdeI- and BamHI-digested pET28a-6XHis-SMT7-972, respectively.
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2.5 | In vitro SUMOylation and SUMO endopeptidase assays

Ten nanograms of each pCDFDuet-SAE1-SAE2, pETDuet-SUMO4, pRSFDuet-targets (pRSFDuet-3XHA-PCNA, pRSFDuet-3XHA-EP300, pRSFDuet-3XHA-CDKG1, pRSFDuet-3XHA-CYCD2, and pRSFDuet-3XHA-CYCD3) were co-transformed into E. coli Rosetta (DE3) by electroporation and selected for the presence of all three plasmids. The in vitro SUMOylation assay was performed as described previously (Lin et al., 2020).

To examine SUMO endopeptidase activity of SMT709-972, one nanogram of pET28a-6XHis-SMT3, pET28a-6XHis-SUMO1-SMT709-972, pET28a-6XHis-SUMO2-SMT709-972, pET28a-6XHis-SUMO3-t1-SMT709-972, pET28a-6XHis-SUMO4-SMT709-972, pET28a-6XHis-SUMO1-SMT709-972/C928A, pET28a-6XHis-SUMO2-SMT709-972/C928A, pET28a-6XHis-SUMO3-t1-SMT709-972/C928A, pET28a-6XHis-SUMO4-SMT709-972/C928A, pET28a-6XHis-SUMO1-SMT709-972/C928A, pET28a-6XHis-SUMO2-SMT709-972/C928A, pET28a-6XHis-SUMO3-t1-SMT709-972/C928A, and pET28a-6XHis-SUMO4-SMT709-972/C928A were suspended in 400 μl of 1X SDS sampling buffer (10% [v/v] glycerol, 60 mM Tris/HCl pH 6.8, 2% [w/v] SDS, 0.01% [w/v] bromophenol blue, and 0.1 M DTT) and incubated at 95°C for 10 min. Protein samples were separated in a 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a poly(vinylidene fluoride) (PVDF) membrane (Immobilon-P, Merck Millipore). Blots were blocked in 1X TBST (20 mM Tris base, 150 mM NaCl, 0.1% [v/v] Tween 20) with 5% (w/v) nonfat milk at RT for 1 hr and incubated with primary antibodies diluted in 1X TBST with 5% (w/v) nonfat milk at 4°C overnight. Primary antibodies and dilution conditions used in this study were anti-HA (1:1000, 3F10 clone, Roche 11867423001), anti-polyHistidine (1:6000, Sigma-Aldrich H1029), anti-S (1:5000, Novagen 71549-3), and anti-SMT7 (1:3000). Blots were washed with 1X TBST 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-rabbit-IgG (1:20000, PerkinElmer NEF812) secondary antibodies for 1 hr at RT. After washing with 1X TBST three times for 15 min each, WesternBright ECL HRP substrate (Advansta K-12045-D50) was added to blots and chemiluminescence signals were captured by the BioRad ChemiDoc XRS + imaging System.

3 | RESULTS AND DISCUSSION

3.1 | SUMO proteins

SUMOs are around 100 amino acids in length and form a β-grasp fold that can be conjugated post-translationally to proteins through the E1-E2-E3 enzyme cascade (Geiss-Friedlander & Melchior, 2007). Initial studies (Wang et al., 2008) and our previous study (Lin et al., 2020) identified four Chlamydomonas SUMO genes designated by us as SUMO1 (Cre16.g662550), SUMO2 (Cre16.g675749), SUMO3 (Cre16.g675861), and SUMO4 (Cre16.g675637). The summarization of the SUMOylation components in the Chlamydomonas genome, the loci for each gene, and cataloging of the previously used names, proposed names, and current knowledge of activity is listed in Table 1. These Chlamydomonas SUMO proteins share the conserved diglycine residues that are required for processing by the SUMO proteases (Figure 2a). Similar to the previous report (Shin et al., 2010), SUMO1 contains four tandem IDAFVEQEGG repeats, which seemed to be lacking in SUMO2, SUMO3, and SUMO4 genes of Volvox carteri and Gonium pectoral, suggesting they are Chlamydomonas-specific.

All Chlamydomonas SUMO genes are located on chromosome 16 with SUMO2, SUMO3, and SUMO4 organized juxtaposed to each other. This suggests that SUMO2, SUMO3, and SUMO4 are likely to arise from a recent duplication event. Phylogenetic analysis revealed that Chlamydomonas SUMO genes formed a monophyletic clade (Figure 2b). SUMO2 and SUMO4 proteins share 74.3% sequence identity and are closely related. SUMO4 appears to be most highly expressed SUMO gene (Lin et al., 2020; Wang et al., 2008; Zones,
### TABLE 1  Components of the SUMOylation system present in the *Chlamydomonas reinhardtii* genome.

| SUMO system component | Phytozome locus ID (v5.5) | Previously NCBI gene ID | Current phytozome aliases or description | Previously used names | Proposed alias | Biochemical activity verified\(^g\) |
|-----------------------|---------------------------|-------------------------|------------------------------------------|-----------------------|---------------|-----------------------------------|
| **SUMO proteins**     |                           |                         |                                          |                       |               |                                   |
| Cre16.g675637         | XP_001695783\(^a\) GU126379\(^b\) | SUMO, SMT3              | CrSUMO9\(^g\), CreSMO1\(^c\)             | SUMO4                 | Yes\(^b\)     |                                   |
| Cre16.g675749         | XP_001695782\(^a\) GU126380\(^b\) | SUMO                    | CrSUMO9\(^g\), CreSMO2\(^c\)             | SUMO2                 | Yes\(^b\)     |                                   |
| Cre16.g662550         | XP_001697951\(^a\) XM_001697899\(^a\) | SUMO, SUM3, STM2        | CrSUMO14\(^d\), CreSMO4\(^c\)           | SUMO1                 | Yes\(^b\)     |                                   |
| Cre16.g675861         | None                      | SUMO                    | CreSMO3\(^c\)                            | SUMO3                 | Yes\(^f\)     |                                   |
| **SUMO fusions**      |                           |                         |                                          |                       |               |                                   |
| Cre17.g716251         | XP_001700385\(^a\)        | SUM5, SUMO89A           | CrSUMO\(_\text{like}_{89A}\)             | None                  | No            |                                   |
| Cre17.g716200         | EU553548\(^b\)            | SUM6, SUMO89B           | CrSUMO\(_\text{like}_{89B}\)             | None                  | No            |                                   |
| Cre17.g716150         | XP_00170386\(^b\)         | SUM4, SUMO90            | CrSUMO\(_\text{like}_{90}\)              | None                  | No            |                                   |
| Cre17.g716301         | None                      | None                    | CrSUMO\(_\text{like}_{90}\)              | None                  | No            |                                   |
| **SUMO-activating enzymes** |                       |                         |                                          |                       |               |                                   |
| Cre09.g408550         | XP_001690572\(^d\)        | SAE1                    | SAE1\(^d\)                               | SAE1                  | Yes\(^d\)     |                                   |
| Cre06.g296983         | XP_001690945\(^d\)        | SAE2, UBA2              | SAE2\(^d\)                               | SAE2                  | Yes\(^d\)     |                                   |
| **SUMO-conjugating enzymes** |                   |                         |                                          |                       |               |                                   |
| Cre02.g142000         | XP_001694849\(^c\)        | UBC4                    | CrUbcE2\(_1\), CrUbc9\(^c\), CreSCE1\(_a\) | SCE1                  | Yes\(^d,e,f\)|                                   |
| Cre03.g167000         | XP_001703521\(^a\)        | UBC3                    | CrUbcE2\(_2\), CrUBC3\(_a\), CreSCE1\(_b\) | SCE2                  | Yes\(^f\)     |                                   |
| Cre01.g019450         | None                      | UBE2I, UBC9             | CreSCE1\(_c\)                            | SCE3                  | Yes\(^f\)     |                                   |
| **SUMO ligases**      |                           |                         |                                          |                       |               |                                   |
| Cre12.g509090         | None                      | PIAS1                   | None                                    | SIZ1                  | No            |                                   |
| Cre17.g698200         | None                      | None                    | None                                    | MMS21                 | No            |                                   |
| **SUMO proteases/isopeptidases** |              |                         |                                          |                       |               |                                   |
| Cre16.g692600         | None                      | SMT7, ULP2, SENP1       | SMT7\(^d\)                              | SMT7/SENP1            | Yes\(^d\)     |                                   |
| Cre02.g142607         | None                      | None                    | None                                    | SENP2                 | No            |                                   |
| Cre02.g143467         | None                      | None                    | None                                    | SENP3                 | No            |                                   |
| Cre13.g563400         | None                      | None                    | None                                    | SENP4                 | No            |                                   |
| Cre06.g296350         | None                      | ULP1                    | None                                    | SENP5                 | No            |                                   |
| Cre06.g278197         | None                      | SENP7                   | None                                    | SENP6                 | No            |                                   |
| Cre12.g555850         | None                      | None                    | None                                    | DeSI-1                | No            |                                   |
| Cre02.g074600         | None                      | None                    | None                                    | DeSI-2                | No            |                                   |
| Cre17.g698100         | None                      | None                    | None                                    | DeSI-3                | No            |                                   |
| Cre16.g662450         | None                      | None                    | None                                    | DeSI-4                | No            |                                   |
| Cre09.g408350         | None                      | None                    | None                                    | DeSI-5                | No            |                                   |
| Cre12.g533600         | None                      | None                    | None                                    | DeSI-6                | No            |                                   |

\(^*\) marks incomplete protein sequence; \(^#\) marks misannotated proteins.

\(^a\) Wang, Y., Ladung, I., Miller, A.R., Horken, K.M., Plucinak, T., Weeks, D.P., and Bailey, C.P. (2008). The small ubiquitin-like modifier (SUMO) and SUMO-conjugating system of *Chlamydomonas reinhardtii*. Genetics 179, 177–192.

\(^b\) Shin, Y.C., Liu, B.Y., Tsai, J.Y., Wu, J.T., Chang, L.K., and Chang, S.C. (2010). Biochemical characterization of the small ubiquitin-like modifiers of *Chlamydomonas reinhardtii*. Planta 232, 649–662.

\(^c\) Augustine, R.C., York, S.L., Rytz, T.C., and Vierstra, R.D. (2016). Defining the SUMO system in Maize: SUMOylation is upregulated during endosperm development and rapidly induced by stress. Plant Physiology 171, 2191–2210.

\(^d\) Lin, Y.L., Chung, C.L., Chen, M.H., Chen, C.H., and Fang, S.C. (2020). SUMO protease SMT7 modulates ribosomal protein L30 and regulates cell-size checkpoint function. Plant Cell 32, 1283–1307.

\(^e\) Knobbe, A.R., Horken, K.M., Plucinak, T.M., Balassa, E., Cerutti, H., and Weeks, D.P. (2015). SUMOylation by a stress-specific small ubiquitin-like modifier E2 conjugase is essential for survival of *Chlamydomonas reinhardtii* under stress conditions. Plant Physiology 167, 753–765.

\(^f\) Present study.

\(^g\) Biochemical activity is defined as: Processing by a SUMO-specific protease/isopeptidase (SUMO proteins), Activation of SUMO Proteins in an in vitro SUMOylation system (SUMO-activating enzymes), Conjugation of SUMO Proteins in an in vitro SUMOylation system (SUMO-conjugating enzymes), Cleavage of SUMO precursor proteins or removal of SUMO conjugates from a protein (SUMO proteases/isopeptidases).
Consistent with the Phytozome annotation (Chlamydomonas reinhardtii v5.5), two SUMO3 splice variants were isolated and verified by RT-PCR. Similar to the previous study (Wang et al., 2008), all Chlamydomonas SUMO proteins including the newly identified SUMO3 revealed a conserved N-terminal SUMOylation site (Lin et al., 2020) that may be important for polymerization as demonstrated in vitro (Shin et al., 2010) and suggested in vivo (Lin et al., 2020).

In addition to four canonical SUMO genes, the initial description of Chlamydomonas SUMO proteins in Chlamydomonas identified three non-canonical SUMO fusion proteins, including Cre17.g716150, Cre17.g716200, and Cre17.g716251 (Wang et al., 2008). We have identified a fourth,
previously unidentified, SUMO fusion protein (Cre17.g716301, Table 1). These SUMO fusion proteins contain a SUMO domain (pfam11976) and a 2-oxoglutarate and Fe(II)-dependent (2OG-Fe(II)) oxygenase (pfam03171) domain (Figure 2c). The four SUMO-containing 2OG-Fe(II) oxygenase genes are organized in tandem on chromosome 17, suggesting that they are also likely to arise from a recent duplication event. Unlike the canonical SUMO proteins, the SUMO-containing 2OG-Fe(II) oxygenases lack the conserved diglycine residues and the second glycine is replaced by aspartagine (Figure 2c). The 2OG-Fe(II)) oxygenases are involved in diverse biological processes including DNA repairs, histone modification, biosynthesis and catabolism of plant hormones, and iron sensing (Farrow & Facchini, 2014). How the SUMO moiety affects the enzymatic functions of these 2OG-Fe(II) oxygenases remains to be studied. These types of non-canonical SUMO fusion proteins have been reported in maize and other land plants (Augustine, York, Rytz, & Vierstra, 2016).

3.2 | SUMO-activating enzymes and other E1 enzymes

Activation of mature SUMO requires the SUMO-specific E1-activating heterodimer enzyme (Desterro et al., 1999; Johnson et al., 1997; Okuma, Honda, Ichikawa, Tsumagari, & Yasuda, 1999; Tatham et al., 2001). Using Arabidopsis SAE1a (AT4G24940), SAE1b (AT5G50580), and SAE2 (AT2G21470) as BLAST queries searched against the Chlamydomonas proteome (see Materials and Methods), eight potential E1-activating enzymes were identified (Table 2). Because ubiquitin and ubiquitin-like E1-activating enzymes share considerable sequence similarity (Schulman & Harper, 2009), phylogenetic analysis was conducted to resolve these E1 enzymes. The analysis showed that only one Chlamydomonas SAE1 (SAE1, Cre09.g408550) and one SAE2 (SAE2, Cre06.g296983) are present in the Chlamydomonas genome (Figure 3a). The Chlamydomonas SAE1 and Arabidopsis SAE1a and SAE1b form a clad that is distantly related to SAE1 from humans and budding yeast. This discordance is not observed in SAE2 enzymes, where Chlamydomonas, Arabidopsis, humans, and yeast SAE2 form a monophyletic clad, as expected from vertical inheritance of SAE2 members. The presence of molybdopterin or thiamine biosynthesis adenylyltransferase domain (ThIF/MoeB, pfam00899) domain (Duda, Walden, Sfondouris, & Schulman, 2005; Lake, Wuebbens, Rajagopalan, & Schindelin, 2001; Lois & Lima, 2005) in both Chlamydomonas SAE1 and SAE2 proteins and the ubiquitin/SUMO-activating enzyme domain (UAEDub, pfam14732) domain (Lois & Lima, 2005) in SAE2 (Figure 3b and c) supports the notion that SAE1 and SAE2 belong to the canonical E1-activating enzymes (Schulman & Harper, 2009). In agreement with the phylogenetic analysis, Chlamydomonas heterodimeric SAE1-SEAE2 is able to activate SUMO (Lin et al., 2020).

Phylogenetical analysis also showed the presence of several putative ubiquitin and ubiquitin-like E1 enzymes in the Chlamydomonas genome (Figure 3a). Cre09.g386400 (UBA1) encodes a putative ubiquitin-activating (UBA) enzyme that forms a cluster with Arabidopsis UBA1 and UBA2, yeast UBA1, and human UBA1, UBA6, and UBA7. Similar to other E1 enzymes, Chlamydomonas UBA1 also contains the ubiquitin-activating enzyme active site (UBA_e1_thiol-Cys, pfam10585, Figure S1), suggesting that it is the potential ubiquitin-activating enzyme (Lee & Schindelin, 2008). Cre01.g002350, on the contrary, encodes a potential NEURAL PRECURSOR CELL EXPRESSED, DEVELOPMENTALLY DOWNREGULATED 8 (NEDD8)-RELATED to UBIQUITIN (RUB)-activating enzyme that is orthologous to Arabidopsis AUXIN RESISTANT 1 (AXR1), yeast UBQUTIN-LIKE PROTEIN ACTIVATION 1 (UL1), and human NEDD8-activating enzyme E1 subunit 1 (NAE1; Liakopoulos, Doenges, Matuschewski, & Jentsch, 1998; Merger, Heinzelmeir, Kuster, & Schwechheimer, 2015; Okuma et al., 1999), and is therefore, referred to as NAE1 (Figure 3a, Figure S2a, and Table 2). Chlamydomonas UBA3 (Cre12.g491500) is closely related to the Arabidopsis RUB-activating enzyme, E1 C-TERMINAL RELATED 1 (ECR1) and NEDD8-activating enzyme E1 catalytic subunit/UBA3 enzymes of yeast and humans (Figure 3a, Figure S2b, and Table 2). As described previously (Diaz-Troya, Perez-Perez, Florencio, & Crespo, 2008; Pérez-Pérez, Cusso, Heredia-Martinez, & Crespo, 2017), one potential Chlamydomonas autophagy-related protein B (ATG8) activating E1 enzyme (ATG7, Table 2 Ubiquitin and ubiquitin-like E1 and E2 enzymes identified in Chlamydomonas

| Ubiquitin/ubiquitin-like proteins | E1                                      | E2                                      |
|----------------------------------|-----------------------------------------|-----------------------------------------|
| RUB1/NEDD8                       | Cre01.g002350 (CrNae1)                  | Cre08.g370850 (CrRce1)                  |
|                                  | Cre12.g491500 (CrUBA3)                  |                                         |
| URM1                             | Cre09.g402034 (CrUBA4)                  | nf                                      |
| UFM1                             | Cre13.g582350 (CrUBA5)                  | Cre02.g142350 (CrUFC1)                 |
| ATG8                             | Cre03.g165215 (CrATG7)                  | Cre02.g102350 (CrATG3)                 |
| Ubiquitin                        | Cre09.g386400 (CrUBA1)                  | Cre06.g294850, Cre12.g515450, Cre08.g372400, Cre03.g202897, Cre05.g247600, Cre04.g212401, Cre12.g510300, Cre08.g383702, Cre06.g297650, Cre07.g342551, Cre10.g429000, Cre02.g141450, Cre06.g292800, Cre16.g693700, Cre01.g046850, Cre01.g026600, Cre01.g027200, Cre13.g570300, Cre10.g430050, Cre05.g240150, Cre12.g546650 |

Abbreviation: Cr, Chlamydomonas reinhardtii; nf, not found.
Cre03.g165215) was identified (Figure 3a, Figure S3a, and Table 2). In addition, one potential ubiquitin-related modifier 1 activating enzyme (UBA4, Cre09.g402034) that is involved in URMylation was found in the Chlamydomonas genome (Figure 3a, Figure S3b, and Table 2). Furthermore, the presence of a potential ubiquitin-fold modifier activating enzyme (UBA5, Cre13.g582350) suggests that UFM1 cascade is likely to be present in Chlamydomonas (Figure 3a, Figure S3c, and Table 2). Together, in addition to SUMOylation and ubiquitination, our phylogenetic analysis and those of Wang et al. (2008) suggests that Chlamydomonas may have NEDDylation-, URMylation-, and UFMylation-dependent post-translational modifications.

3.3 | SUMO-conjugating enzymes and other E2 enzymes

After activation by a SUMO-activating enzyme, SUMO is transferred from SAE2 to the E2-conjugating enzyme (SCE/UBC9) by forming a transient thioester bond between the active Cys residue of SCE/UBC9 and the C-terminal carboxy group before transferring SUMO to the target (Stewart, Ritterhoff, Klevit, & Brzovic, 2016; van Wijk & Timmers, 2010). Inspection of the Chlamydomonas genome for ubiquitin-conjugating enzyme (UBC, pfam00179) domain-containing proteins identified 25 proteins. To resolve these proteins used for phylogenetic analysis and sequence alignment are listed in DataS1.

In addition to SUMO E2-conjugating enzymes, many putative ubiquitin and ubiquitin-like E2-conjugating enzymes were identified in the Chlamydomonas genome. Based on classification of Arabidopsis UBC domain-containing E2 enzymes (Kraft et al., 2005), Chlamydomonas has at least one ortholog in 16 subgroups of E2 enzymes, except subgroup VIII, XIII, and XVI (Figure 4a). The presence of one potential Chlamydomonas NEDD8-conjugating enzyme (RCE1, Cre08.g370850, Figure 4a, Figure S4a, and Table 2), and one putative ubiquitin-fold modifier 1-conjugating enzyme 1 (UFC1, Cre02.g142350, Figure 4a, Figure S4b, and Table 2), supports that NEDDylation and UFMylation are present in Chlamydomonas. Consistent with the previous study (Pérez-Pérez et al., 2017), one autophagy E2 enzyme autophagy-related 3 (ATG3), Cre02.g102350, was identified (Figure 4c, and Table 2).

3.4 | Chlamydomonas SUMO E3 ligases

SUMO E3 ligases catalyze the transfer of SUMO from the charged E2 enzyme (E2-SUMO) to the target proteins. The SIZ1/PIAS-Really Interesting New Gene (SP-RING) SUMO E3 family is evolutionarily conserved in yeasts, animals, and plants (García-Dominguez, March-Díaz, & Reyes, 2008; Johnson & Gupta, 2001; Kahyo et al., 2001; Miura et al., 2005; Novatchkova, Tomanov, Hofmann, Stuible, & Bachmair, 2012; Park et al., 2010; Strunnikov, Aravind, & Koonin, 2001; Suzuki et al., 2009; Takahashi et al., 2001). Protein structure analysis reveals that SP-RING domain of SUMO E3 enzymes interacts with the E2-Ubl thioester to coordinate the closed active E2-SUMO conformation and stimulates conjugation by force-feeding a substrate lysine into a E2 active site (Streich & Lima, 2016). Although SUMO E1 and E2 enzymes are sufficient for in vitro SUMO conjugation at the lysine residues that are preferred in vivo, SUMO E3 ligases have been shown to be important for in vivo SUMOylation (Johnson & Gupta, 2001; Kahyo et al., 2001; Streich & Lima, 2016; Tomanov et al., 2014).

Siz/PIAS (SP-RING) E3 family proteins can be categorized into SIZ1, METHYL METHANESULFONATE-SENSITIVITY PROTEIN 21 (MMS21), and PROTEIN INHIBITOR OF ACTIVATED STAT-LIKE 1/2 (PIAL1/2) types of SUMO E3 ligases. Using the Arabidopsis SIZ1, MMS21, PIAL1, and PIAL2 as the BLAST queries and searching against the Chlamydomonas proteome, two potential SUMO E3 ligases were identified. Chlamydomonas SIZ1 (Cre12.g500900) belongs to SIZ1-related SUMO E3 ligase and is closely related to Arabidopsis SIZ1 (Figure S5). Similar to Arabidopsis SIZ1, Chlamydomonas SIZ1 has a plant-specific plant...
homeodomain (PHD, pfam00628, Figure 5b) in addition to the SP-RING zinc finger domain (pfam02891) and SAF-A/B, Acinus, and PIAS (SAP) DNA-binding domain (pfam02037). Chlamydomonas MMS21 (Cre17.g698200), on the contrary, contains a zinc finger of the MIZ type in NSE subunit (pfam11789, Figure 5c) and a potential SIM domain (Figure S6), and belongs to the MMS21 E3 ligases. Interestingly for Chlamydomonas MMS21, not all of the Zn-coordinating cysteine and histidine residues (Huang et al., 2009) conserved in Arabidopsis, humans, and yeast are present (Figure 5c). No PIAS-like E3 ligase was identified in the Chlamydomonas genome (Figure 5a).

### 3.5 Chlamydomonas SUMO proteases

After exhausting a BLAST search of the Chlamydomonas proteome using the Arabidopsis SUMO proteases/isopeptidases (Castro et al., 2018) as blasting queries, in addition to the previously
identified SUMO protease, SMT7/SENP1, six additional potential SUMO proteases ranging from 283 to 2,854 amino acids in length (Data S1) were identified (Figure 6a). Phylogenetic analysis conducted using the SUMO protease domain showed bifurcation of the Chlamydomonas SUMO proteases with Cre16.g692600 (SMT7/SENP1), Cre02.g142607 (SENP2), Cre02.g143467 (SENP3), Cre13.g563400 (SENP4), and Cre06.g296350 (SENP5) forms a cluster with the ULP1-type SUMO proteases, while Cre06.g278197 (SENP6) belongs to the ULP2-type SUMO proteases and is related to Arabidopsis SPF-type SUMO proteases (Figure 6b and Figure S7). Similar to SUMO proteases identified in other systems, the amino-terminal regions of the Chlamydomonas SUMO protease candidates are poorly conserved. They do contain a recognizable SUMO protease domain with the conserved catalytic triad at the C-terminal end (pfam02902, Figure 6b). Even so, alignment of sequences of seven SUMO protease domains of Chlamydomonas SUMO proteases share little conservation except the catalytic residues (Figure 6a). The SUMO protease activity of SMT7 (Cre16.g692600) has been demonstrated previously (Lin et al., 2020).

Even though Cre01.g055050 encodes a protein containing the SUMO protease domain (pfam02902, Figure 6a), it is clustered with the deNEDDylating enzymes of humans and Arabidopsis (Gane-Erdene et al., 2003; Mendoza et al., 2003; Mergner et al., 2015; Mergner, Kuster, & Schwechheimer, 2017; Shen et al., 2005; Wu et al., 2006), suggesting Cre01.g055050 encodes a deNEDDylating enzyme (Figure 6a). In summary, six ULP-type SUMO proteases and one deNEDDylating enzyme were identified in Chlamydomonas.

In addition to the canonical SUMO proteases, a new class of SUMO proteases, which belong to the C97 superfamily of cysteine proteases and contain a characteristic DeSUMOylating isopeptidase (DeSI) domain, have recently been reported in mammals and Arabidopsis (Orosa et al., 2018; Shin et al., 2012). Interestingly, DeSI SUMO protease have no representative in budding yeast but are found in other fungi including fission yeast Schizosaccharomyces pombe (Hickey et al., 2012). To search for the Chlamydomonas version of DeSI-type SUMO proteases, the DeSI domains of Arabidopsis DeSI-type SUMO proteases were used as BLASTP queries. Six DeSI-like SUMO protease candidates ranging from 167 to 880 amino acids in length (Data S1) were identified. Despite their sequence diversity, the DeSI-type SUMO proteases of Arabidopsis, humans, and Chlamydomonas share the conserved catalytic dyad (Suh et al., 2012) composed of histidine and cysteine (Figure 6c). We found that the conserved catalytic dyad is also present in AT4G31980 and AT1G80690 that have been reported to lack the key residues in their catalytic domain (Orosa et al., 2018). The SUMO protease activity of these Chlamydomonas DeSI-type proteins remains to be determined. In addition to DeSI-type SUMO proteases, ubiquitin-specific protease-like 1 (USPL1)-type SUMO protease has recently been characterized in humans (Schulz et al., 2012). Similar to Arabidopsis (Morrell & Sadanandom, 2019), no Chlamydomonas USPL1-type SUMO protease was identified.

It is intriguing that Chlamydomonas has an expanded number of SUMO proteases. Increased number of SUMO proteases in plants has been suggested to provide specificity for the SUMO system (Yates, Srivastava, & Sadanandom, 2016). Domestication selection pressure has been suggested to contribute to increased number of SUMO proteases in crop plants (Morrell & Sadanandom, 2019). Chlamydomonas, however, has not undergone the domestication process. Hence, we hypothesize that the increased number of SUMO proteases may be useful for microalgae to combat diverse environmental stresses.

### 3.6 | SUMO-conjugating activity of Chlamydomonas SCE2 and SCE3

Considering only one SCE is present in budding yeast, humans, and Arabidopsis, it is surprising that three SCE members are present in Chlamydomonas. To investigate how the sequence variations affect catalysis, we used SWISS-MODEL (Biasini et al., 2014) to predict the three-dimensional structures of Chlamydomonas SCE1, SCE2, and SCE3. The SCE3 splice variants, SCE3.t1 and SCE3.t2, were included in the modeling. The available Arabidopsis SCE1 model (Liu, Lois, & Reverter, 2019) was used as a template for modeling. The global model quality estimate (GQME) score with a number between 0 (low) and 1 (high) was used to evaluate the expected accuracy of a built model (Biasini et al., 2014). Chlamydomonas SCE1 protein is most similar to Arabidopsis SCE1 with GQME score at 0.87. The GQME scores of SCE2, and two splice variants SCE3.t1, and SCE3.t2 are 0.76, 0.73, and 0.76, respectively. Although the predictive backbone structures of Chlamydomonas SCEs were very similar, the major structural differences were found to be near the active cysteine residue where a thioester is formed with the activated SUMO (Figure 7a). For SCE2 and SCE3.t2, a relative long loop near the active cysteine sites was found. In addition to this loop, another loop proximal to the active cysteine was predicted from modeling...
The alignment of SUMO protease domain (pfam02902) of Chlamydomonas SUMOylation components. The catalytic triad histidine (H), aspartic acid (D), and cysteine (C) are in red font. (b) Unrooted phylogenetic relationship of SUMO proteases from Chlamydomonas, Arabidopsis, humans, and budding yeast. Bootstrap values of 50% or higher are shown for each clade. (c) The alignment of DesI-type SUMO protease domain (pfam05903) of Chlamydomonas SUMO proteases. The catalytic dyad histidine (H) and cysteine (C) are in red font. (d) Unrooted phylogenetic relationship of DeSI-type SUMO proteases from Chlamydomonas, Arabidopsis, human, and budding yeast. Bootstrap values of 50% or higher are shown for each clade. ** represents conserved residue. : represents residue showing strong similarity to residues at the same position in the listed proteins. . represents residue showing weak similarity to residues at the same position in the listed proteins. Abbreviations of genus and species names of the given locus numbers or gene identifiers are as follows: Chlamydomonas reinhardtii (Cr), Arabidopsis thaliana (At), Homo sapiens (Hs), and Saccharomyces cerevisiae (Sc). The accession numbers of proteins used for sequence alignment and phylogenetic analysis are listed in DataS1.
FIGURE 7 Characterization of Chlamydomonas SUMO-conjugating enzymes. (a) Chlamydomonas SCE1, SCE2, SCE3.t1, and SCE3.t2 are predicted to have similar three-dimensional folds. SCE proteins were threaded into the Arabidopsis SCE1 (AT3G57870) template using SWISS-MODEL (Biasini et al., 2014). The active Cys residue is labeled with a red asterisk. The models are displayed and colored coded by model quality estimates assigned by QMEAN to highlight regions of the model which has good quality (blue) or poor quality (red). (b) Chlamydomonas PCNA was conjugated with SUMO4 by SCE1, SCE2, and CrSCE3.t2. Expression of the SUMO component was confirmed by immunoblotting using the corresponding antibodies. (c) SUMOylated Chlamydomonas PCNA was removed by SMT7709-972. Expression vectors carrying the cDNAs encoding the individual SUMOylation components and SMT7709-972 were transformed into E. coli cells. Expression of the SUMO component was confirmed by immunoblotting. Two independent E. coli transformants carrying the indicated constructs were depicted as “1” and “2.” IB, immunoblot; α-HA, anti-hemagglutinin antibody; α-S, anti-S peptide antibody; α-His, anti-polyHistidine antibody; α-SMT7, anti-SMT7 antibody. Coomassie brilliant blue (CBB) staining showing equal loading of total protein amount. Total protein extracts were separated by a 10% or 12% SDS-PAGE. Black asterisks mark SUMO4-conjugated PCNA.
proteins because they are reported to regulate Chlamydomonas mitotic cell division (Fang, Reyes, & Umen, 2006; Li, Liu, Lopez-Paz, Olson, & Umen, 2016; Olson et al., 2010) and have at least one predicted SUMOylation site (Figure S10). MAT3 encodes a retinoblastoma-like tumor suppressor and negatively regulates heterodimeric transcription activator E2F1/DP1 to control cell cycle progression (Fang et al., 2006; Olson et al., 2010; Umen & Goodenough, 2001). CDKG1, on the contrary, interacts with CYCD2 or CYCD3 and acts as a positive cell cycle regulator by regulating activity of MAT3 protein (Li, Liu, et al., 2016). The full-length cDNA of E2F1, DP1, CDKG1, CYCD2, and CYCD3 were cloned into the pRSFDuet-3XHA vector (Lin et al., 2020) to make pRSFDuet-3XHA-E2F1, pRSFDuet-3XHA-DP1, pRSFDuet-3XHA-CDKG1, pRSFDuet-3XHA-CYCD2, and pRSFDuet-3XHA-CYCD3 plasmids (Figure 8a). Despite numerous attempts to amplify MAT3 cDNA, we failed to obtain the full-length cDNA. MAT3 was, therefore, omitted from further study. pRSFDuet-3XHA-E2F1, pRSFDuet-3XHA-DP1, pRSFDuet-3XHA-CDKG1, pRSFDuet-3XHA-CYCD2, or pRSFDuet-3XHA-CYCD3 was co-transformed with pCDFDuet-SAE1-SAE2 and pETDuet-SCE1-SUMO4GG into E. coli cells for in vitro SUMOylation tests. Co-expression of SAE1, SAE2, SCE1, SUMO4, and 3XHA-E2F1, 3XHA-DP1, 3XHA-CDKG1, 3XHA-CYCD2, and 3XHA-CYCD3 was verified by immunoblotting (Figure 8b). In addition to detecting a 3XHA-E2F1 (~58 kDa), 3XHA-DP1 (~74 kDa), 3XHA-CDKG1 (~50 kDa), 3XHA-CYCD2 (~43 kDa), and 3XHA-CYCD3 (~46 kDa) protein with the predicted size, low-mobility SUMO4-conjugated proteins were also detected when all of the SUMOylation components were present. E2F1 and DP1 were conjugated by multiple SUMO4s with two and one SUMO4 conjugates as the dominant ones, respectively (Figure 8b). CDKG1, CYCD2, and CYCD3 also had multiple SUMO4 conjugates (Figure 8b) with two and four SUMO4 conjugates being the most abundant SUMOylated CDKG1 and CYCD2 species, respectively. As a negative control, SUMO4 failed to be conjugated to the tested proteins when SAE1 and SAE2 were missing. Hence, we conclude that Chlamydomonas E2F1, DP1, CDKG1, CYCD2, and CYCD3 proteins are SUMOylated in vitro.

To test whether the SUMO-conjugated proteins can be removed by SUMO protease, the SUMO protease domain of SMT7 (SMT7709-972) was cloned into the pRSFDuet-3XHA-E2F1, pRSFDuet-3XHA-DP1, pRSFDuet-3XHA-CDKG1, pRSFDuet-3XHA-CYCD2, and pRSFDuet-3XHA-CYCD3 plasmids to make pRSFDuet-3XHA-E2F1-SMT7709-972, pRSFDuet-3XHA-DP1-SMT7709-972, pRSFDuet-3XHA-CDKG1-SMT76709-972, pRSFDuet-3XHA-CYCD2-SMT7709-972, and pRSFDuet-3XHA-CYCD3-SMT7709-972, respectively (Figure 8a). Expression of all the corresponding components were verified by immunoblotting (Figure 8b). When SMT7709-972 was co-expressed with all the SUMOylation components, only unconjugated proteins were detected, indicating that SMT7709-972 is capable of removing SUMO4 from conjugated tested proteins.

As important regulators of G1-S transition, E2F and DP proteins have been shown to be regulated post-translationally. For example, recent SUMO proteomics data indicated that E2Fs and DPs are SUMOylated in vivo (Hendriks et al., 2014; Lamoliatte et al., 2017; Miller, Barrett-Wilt, Hua, & Vierstra, 2010). Moreover, E2F1-interacting protein p14ARF has been shown to recruit SUMO E2-conjugating enzyme and promotes SUMOylation of E2F1 in human cells (Rizos, Woodruff, & Kefford, 2005). Additionally, SUMOylation of DPs facilitated by MMS21 negatively regulates cell cycle progression in Arabidopsis (Liu et al., 2016). Whether SUMOylation of E2F1 and/or DP1 modulates the activity of E2F1/DP1 transcription factor during the Chlamydomonas cell cycle remains to be investigated.

SUMOylation has been shown to regulate subcellular localization, activity, or stability of cyclin and CDK proteins. In humans, Ras oncogene is reported to induce SUMOylation of cyclin D1 leading to its nuclear localization that is required for oncogenic activity (Wang et al., 2011). In addition, SUMOylation of cyclin E is important for controlling origin firing at replication origins (Bonne-Andrea et al., 2013). In glioblastoma cells, stabilization of CDK6 by SUMOylation is important for cancer development and progression. (Bellali, Olson, & Hao, 2014). The present studies demonstrate that the key cell cycle regulators CDKG1, CYCD2, and CYCD3 are present in Chlamydomonas and can be SUMOylated in vitro. Whether SUMOylation regulates activity of the CDKG1, CYCD2, and CYCD3 proteins to control the Chlamydomonas cell cycle remains to be determined.

### 3.8 | SMT7 is able to process SUMO precursor proteins

SUMO proteases have dual enzymatic activity: the endopeptidase activity that processes the SUMO precursors to generate mature SUMO proteins and the isopeptidase activity that removes SUMO from SUMOylated proteins (deSUMOylation). The isopeptidase activity of SMT7 has been demonstrated previously (Lin et al., 2020). It is not clear whether SMT7 can process SUMO precursor proteins. Here, chimeric constructs that allowed translational fusion of the C-terminal 6XHis-tagged SUMO protease domain of SMT7 (SMT7709-972) and N-terminal 6XHis-tagged full length of yeast SUMO precursor (SMT3, YDR510W) or four full-length Chlamydomonas SUMO proteins were generated (Figure 9a). It is predicted that the endopeptidase activity of SMT7709-972 would process the SUMO precursors and generate the SMT7709-972 and SUMOGG peptides, which can be readily detected by immunoblotting. This approach has been used to confirm the endopeptidase activity of the human SENP1 (Xu & Au, 2005) and Arabidopsis SUMO proteases (Colby et al., 2006). As predicted, the SMT7709-972 processed the 6XHis-SMT3-SMT7709-972-6XHis chimeric protein (~45 kDa) and generated SMT7709-972-6XHis (~31 kDa) and 6XHis-SMT3 (~14 kDa) fragments with predicted size (Figure 9b). Even though SMT7709-972 was able to process yeast SMT3 precursor, it did not cleave it efficiently because a substantial amount of 6XHis-SMT3-SMT7709-972-6XHis chimeric protein remained intact. The SMT7709-972 also processed the 6XHis-SUMO1-SMT7709-972-6XHis (~50 kDa) chimeric protein and generated ~39 kDa SMT7709-972-6XHis and ~11 kDa 6XHis-SUMO1 fragments (Figure 9b). Interestingly, even though four
FIGURE 8  Key Chlamydomonas cell cycle regulators are SUMOylated. (a) E. coli transformants carrying the indicated plasmids containing the cDNAs encoding the indicated SUMOylation components. SAE1, and SCE1 are C-terminally fused to the S tag. The SAE2 and SUMO4GG are N-terminally fused to 6XHis. Tested cell cycle proteins are N-terminally fused to the 3XHA tag (3XHA-T). (b) In vitro SUMOylation assays of the indicated cell cycle regulators. Expression of the SUMO component was confirmed by immunoblotting using the corresponding antibodies. Arrowheads in CDKG1 panel indicate CDKG1 protein that migrates to the similar position as SAE1 protein. Two independent E. coli transformants carrying the indicated constructs were depicted as "1" and "2." IB, immunoblot; α-HA, anti-hemagglutinin antibody; α-S, anti-S peptide antibody; α-His, anti-polyHistidine antibody; α-SMT7, anti-SMT7 antibody. Coomassie brilliant blue (CBB) staining showing equal loading of total protein amount. Total protein extracts were separated by a 10% or 12% SDS-PAGE. Black asterisks mark SUMO4 conjugates
double glycine residues are present in the C-terminal region of SUMO1 protein (Figure 2a), SMT7709-972 appeared to recognize the double glycine residues are present in the C-terminal region of SUMO1 protein (Figure 2a), SMT7709-972 appeared to recognize

logenesis, and genome stability (Chymkowitch, Nguea, & Enserink, 2015; Enserink, 2015). Here, we provide an expanded categorization of the SUMO system for the unicellular model system Chlamydomonas reinhardtii and describe its core components in detail. To provide a more uniform and facile nomenclature, we also propose an updated and expanded annotation of the Chlamydomonas SUMOylation system (Table 1).

4 | CONCLUSION AND PERSPECTIVES

SUMOylation regulates many cellular processes including cell cycle progress, DNA repair, signaling, cellular stress response, transcription, chromatin modification, and genome stability (Chymkowitch, Nguea, & Enserink, 2015; Enserink, 2015). Here, we provide an expanded categorization of the SUMO system for the unicellular model system Chlamydomonas reinhardtii and describe its core components in detail. To provide a more uniform and facile nomenclature, we also propose an updated and expanded annotation of the Chlamydomonas SUMOylation system (Table 1).

Similar to budding yeast, Chlamydomonas has only one copy of each subunit (SAE1 and SAE2) of the SUMO-activating enzyme. For SUMO ligases, only one SIZ1 and one MMS21 were found in Chlamydomonas. Unlike budding yeast, however, many other Chlamydomonas SUMOylation components have multiple copies. In this study, 4 SUMO genes, 3 SCE genes, and 12 SUMO proteases were identified in Chlamydomonas. Among the 12 Chlamydomonas SUMO proteases, six belong to the canonical SUMO proteases that contain the C48 peptidase domain (pfam02902). Five of them (SENP1-5) belong to the ULP1-type SUMO proteases and SENP6 is the ULP2-type SUMO protease. Six other members, on the contrary, belong to a novel group of the C97 peptidase domain (pfam05903) SUMO proteases. In addition to the SUMO system, we also identified E1 enzymes of URMylation, NEDDylation, and UFMylation and E2 enzymes of NEDDylation, and UFMylation, suggesting these types of post-translational modifications may be present in Chlamydomonas. Lack of the UFM1 cascade in single-cell budding yeast leads to the hypothesis that it may play a role in the transition from unicellular to multicellular life (Komatsu et al., 2004). However, the presence of UFM activation enzyme (UBA5) and conjugating enzyme (UCF1) in unicellular Chlamydomonas argues that UFMylation may be already present in single-cell eukaryotic organisms but is missing in budding yeast. The functions of Chlamydomonas URMylation, NEDDylation, and UFMylation remain to be investigated.

Compared to other model systems, SUMOylation biology in Chlamydomonas is a relatively unexplored area. Information about many basic questions such as SUMO-dependent cellular processes and molecular consequences of SUMOylation is scarce. Even though SUMOylation has been shown to regulate the cell cycle and stress responses in Chlamydomonas (Knobbe et al., 2015; Lin et al., 2020; Wang et al., 2008), the underlying mechanisms remain unclear. Systemic identification of SUMO targets, elucidation of individual target specificities, and determination of the corresponding physiological roles will be important tasks for future studies.
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CONFLICT OF INTEREST
All authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
SCF conceived the idea and coordinated the study. SCF and YLL designed the experiments. SCF and YLL did phylogenetic analysis. YLL, CLC, PJH, and CHC did the DNA cloning. YLL, CLC, and PJH conducted in vitro SUMOylation assays. YLL and SCF analyzed the data and wrote the manuscript.

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REFERENCES
Armstrong, A. A., Mohideen, F., & Lima, C. D. (2012). Recognition of SUMO-modified PCNA requires tandem receptor motifs in Srs2. Nature, 483, 59–63. https://doi.org/10.1038/nature10883
Augustine, R. C., York, S. L., Rytz, T. C., & Vierstra, R. D. (2016). Defining the SUMO system in Maize: SUMOylation is up-regulated during endosperm development and rapidly induced by stress. Plant Physiology, 171, 2191–2210. https://doi.org/10.1104/pp.16.00353
Baek, K., Kim, D. H., Jeong, J., Sim, S. J., Melis, A., Kim, J. S., ... Bae, S. (2016). DNA-free two-gene knockout in Chlamydomonas reinhardtii via CRISPR-Cas9 ribonucleoproteins. Scientific Reports, 6, 30620. https://doi.org/10.1038/srep30620
Bellall, A. C., Olson, J. J., & Hao, C. (2014). SUMO1 modification stabilizes CDK6 protein and drives the cell cycle and glioblastoma progression. Nature Communications, 5, 4234. https://doi.org/10.1038/ncomms5234
Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., ... Schwede, T. (2014), SWISS-MODEL: Modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Research, 42, W252–W258. https://doi.org/10.1093/nar/gku340
Blaby, I. K., Blaby-Haas, C. E., Torsasse, N., Hom, E. F., Lopez, D., Aksoy, M., ... Prochnik, S. (2014). The Chlamydomonas genome project: A decade on. Trends in Plant Science, 19, 672–680. https://doi.org/10.1016/j.tipl.2014.05.008
Blomster, H. A., Hietakangas, V., Wu, J., Kouvonnen, P., Hautaniemi, S., & Sistonen, L. (2009). Novel proteomics strategy brings insights into the prevalence of SUMO-2 target sites. Molecular & Cellular Proteomics, 8, 1382–1390. https://doi.org/10.1074/mcp.M800551-MCP200
Bonne-Andrea, C., Kahil, M., Mechali, F., Lemaitre, J. M., Bossis, G., & Coux, O. (2013). SUMO2/3 modification of cyclin E contributes to the control of replication origin firing. Nature Communications, 4, 1850. https://doi.org/10.1038/ncomms2875
Castro, P. H., Santos, M. A., Freitas, S., Cana-Quijada, P., Lourenco, T., Rodrigues, M. A. A., ... Azevedo, H. (2018). Arabidopsis thaliana SPF1 and SPF2 are nuclear-located ULP2-like SUMO proteases that act downstream of SIZ1 in plant development. Journal of Experimental Botany, 69, 4633–4649. https://doi.org/10.1093/jxb/ery265
Castro, P. H., Tavares, R. M., Bejarano, E. R., & Azevedo, H. (2012). SUMO, a heavyweight player in plant abiotic stress responses. Cellular and Molecular Life Sciences, 69, 3269–3283. https://doi.org/10.1007/s00018-012-1094-2
Chymkowitch, P., Nguen, P. A., & Enserink, J. M. (2015). SUMO-regulated transcription: Challenging the dogma. BioEssays, 37, 1095–1105. https://doi.org/10.1002/bies.201500665
Colby, T., Matthai, A., Boeckelmann, A., & Stubille, H. P. (2006). SUMO-conjugating and SUMO-deconjugating enzymes from Arabidopsis. Plant Physiology, 142, 318–332. https://doi.org/10.1104/pp.106.085415
Cossec, J. C., Theurillat, I., Chica, C., Bua Aguini, S., Gaume, X., Andrieux, A., ... Dejean, A. (2018). SUMO safeguards somatic and pluripotent cell identities by enforcing distinct chromatin states. Cell Stem Cell, 23(7–8), e748. https://doi.org/10.1016/j.stem.2018.10.001
Crozet, P., Navarro, F. J., Willmunt, F., Mehrshahi, P., Bakowski, K., Lauersen, K. J., ... Lemaire, S. D. (2018). Birth of a photosynthetic chassis: A MoClo toolkit enabling synthetic biology in the microalga Chlamydomonas reinhardtii. ACS Synth Biol, 7, 2074–2086.
Dasso, M. (2008). Emerging roles of the SUMO pathway in mitosis. Cell Division, 3, 5. https://doi.org/10.1186/1747-1028-3-5
Dawlaty, M. M., Malureanu, L., Jeganathan, K. B., Kao, E., Sustmann, C., Tahk, S., ... van Deursen, J. M. (2008). Resolution of sister centromeres requires RanBP2-mediated SUMOylation of toposomerase IIa. Cell, 133, 103–115.
Dent, R. M., Haglund, C. M., Chin, B. L., Kobayashi, M. C., & Niyogi, K. K. (2005). Functional genomics of eukaryotic photosynthesis using insertional mutagenesis of Chlamydomonas reinhardtii. Plant Physiology, 137, 545–556.
Desterro, J. M., Rodriguez, M. S., Kemp, G. D., & Hay, R. T. (1999). Identification of the enzyme required for activation of the small ubiquitin-like protein SUMO-1. Journal of Biological Chemistry, 274, 10618–10624. https://doi.org/10.1074/jbc.274.15.10618
Desterro, J. M., Thomson, J., & Hay, R. T. (1997). Ubch9 conjugates SUMO but not ubiquitin. FEBS Letters, 417, 297–300. https://doi.org/10.1016/S0010-4716(97)01305-7
Di Bacco, A., & Gill, G. (2006). SUMO-specific proteases and the cell cycle. An essential role for SENPS in cell proliferation. Cell Cycle, 5, 2310–2313. https://doi.org/10.4161/cc.5.20.3367
Di Bacco, A., Ouyang, J., Lee, H. Y., Catic, A., Ploegh, H., & Gill, G. (2006). The SUMO-specific protease SENPs is required for cell division. Molecular and Cellular Biology, 26, 4489–4498. https://doi.org/10.1128/MCB.02301-05
Diaz-Troya, S., Perez-Perez, M. E., Florencio, F. J., & Crespo, J. L. (2008). The role of TOR in autophagy regulation from yeast to plants and mammals. Autophagy, 4, 851–865. https://doi.org/10.4161/aut.6555
Duda, D. M., Walden, H., Sfondouris, J., & Schulman, B. A. (2005). Structural analysis of Escherichia coli ThiF. Journal of Molecular Biology, 349, 774–786. https://doi.org/10.1016/j.jmb.2005.04.011
Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32, 1792–1797. https://doi.org/10.1093/nar/gkh340
Eifler, K., & Vertegaal, A. C. (2015). SUMOylation-mediated regulation of cell cycle progression and cancer. Trends in Biochemical Sciences, 40, 779–793. https://doi.org/10.1016/j.tibs.2015.09.006
Enserink, J. M. (2015). Sumo and the cellular stress response. Cell Division, 10, 4. https://doi.org/10.1186/s13008-015-0010-1
Fang, S. C., Chung, C. L., Chen, C. H., Lopez-Paz, C., & Umen, J. G. (2014). Defects in a new class of sulfate/anion transporter link sulfur acclimation responses to intracellular glutathione levels and cell cycle control. Plant Physiology, 166, 1852–1868. https://doi.org/10.1104/pp.114.251009
Fang, S.-C., Reyes, C. D. L., & Umen, J. G. (2006). Cell size checkpoint control by the retinoblastoma tumor suppressor pathway. PLoS Genetics, 2, e167. https://doi.org/10.1371/journal.pgen.0020167
Farrow, S. C., & Facchin, P. J. (2014). Functional diversity of 2-oxoglutarate/Fe(II)-dependent dioxygenases in plant metabolism. Frontiers in Plant Science, 5, 524. https://doi.org/10.3389/fpls.2014.00524

Gan-Erdene, T., Nagalalleswari, K., Yin, L., Wu, K., Pan, Z. Q., & Wilkinson, K. D. (2003). Identification and characterization of DEN1, a dedydylase of the ULP family. Journal of Biological Chemistry, 278, 28892–28900. https://doi.org/10.1074/jbc.M30289020

Garcia-Dominguez, M., March-Diaz, R., & Reyes, J. C. (2008). The PHD domain of plant PIAS proteins mediates sumoylation of bromodomain GTE proteins. Journal of Biological Chemistry, 283, 21469–21477. https://doi.org/10.1074/jbc.M708176200

Geiss-Friedlander, R., & Melchior, F. (2007). Concepts in sumoylation: A decade on. Nature Reviews Molecular Cell Biology, 8, 947–956. https://doi.org/10.1038/nrm2293

Gong, L., Li, B., Millas, S., & Yeh, E. T. (1999). Molecular cloning and characterization of human AOS1 and UBA2, components of the sentinel-activating enzyme complex. FEBS Letters, 448, 185–189. https://doi.org/10.1016/S0014-5793(99)00367-1

Gong, L., & Yeh, E. T. (2006). Characterization of a family of nucleolar SUMO-specific proteases with preference for SUMO-2 or SUMO-3. Journal of Biological Chemistry, 281, 15869–15877. https://doi.org/10.1074/jbc.M51158200

Greiner, A., Kelterborn, S., Evers, H., Kreimer, G., Sizova, I., & Hegemann, P. (2017). Targeting of photoreceptor genes in Chlamydomonas reinhardtii via zinc-finger nucleases and CRISPR/Cas9. The Plant Cell, 29, 2498–2518.

Harris, E. H. (1989). Chlamydomonas Sourcebook - a comprehensive guide to biology and laboratory use. San DiegoAdademic Press.

Harris, E. H. (2001). Chlamydomonas as a model organism. Annual Review of Plant Physiology and Plant Molecular Biology, 52, 363–406.

Harris, E. H. (2009). The Chlamydomonas Sourcebook; Second Edition; Introduction to Chlamydomonas and its Laboratory Use; Volume 1. Elsevier Ltd.

Hendriks, I. A., D’Souza, R. C., Yang, B., Verlaan-de Vries, M., Mann, M., & Vertegaal, A. C. (2014). Uncovering global SUMOylation signaling networks in a site-specific manner. Nature Structural & Molecular Biology, 21, 927–936. https://doi.org/10.1038/nsmb.2890

Hendriks, I. A., & Vertegaal, A. C. (2016). A comprehensive compilation of SUMO proteomics. Nature Reviews Molecular Cell Biology, 17, 581–595. https://doi.org/10.1038/nrm.2016.81

Hickey, C. M., Wilson, N. R., & Hochstrasser, M. (2012). Function and regulation of SUMO proteases. Nature Reviews Molecular Cell Biology, 13, 755–766. https://doi.org/10.1038/nrm3478

Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation, Annual Review of Genetics, 30, 405–439. https://doi.org/10.1146/annurev.genet.30.1.405

Huang, L., Yang, S., Zhang, S., Liu, M., Lai, J., Qi, Y., … Yang, C. (2009). The Arabidopsis SUMO E3 ligase AtMMS21, a homologue of NSE2/MSM21, regulates cell proliferation in the root. The Plant Journal, 60, 666–678. https://doi.org/10.1111/j.1365-313X.2009.03992.x

Jackson, S. P., & Durocher, D. (2003). Identification and characterization of DEN1, a dedydylase of the ULP family. Journal of Biological Chemistry, 278, 28892–28900. https://doi.org/10.1074/jbc.M30289020

Jonikas, M. C. (2016). An indexed, mapped mutant library enables advanced genetic analysis version 7.0 for bigger datasets. Molecular and Developmental Biology, 23, 1870–1874. https://doi.org/10.1038/sj.molbev.850054

Li, S. J., & Hochstrasser, M. (1999). A new protease specific for the ubiquitin-like Smt3 protein. EMBO Journal, 18, 2367–2377. https://doi.org/10.1016/j.molcel.2014.05.046

Li, S. J., & Hochstrasser, M. (2000). The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. Molecular and Cellular Biology, 20, 2367–2377. https://doi.org/10.1128/MCB.20.7.2367-2377.2000

Li, Y., Zhang, R., Patena, W., Gang, S. S., Blum, S. R., Ivanova, N., … Jonikas, M. C. (2016). An indexed, mapped mutant library enables reverse genetics studies of biological processes in Chlamydomonas reinhardtii. The Plant Cell, 28, 367–387.

Li, Y., Liu, D., Lopez-Paz, C., Olson, B. J., & Umen, J. G. (2016). A new class of cyclin dependent kinase in Chlamydomonas is required for coupling cell size to cell division. Elife, 5, e10767. https://doi.org/10.7554/eLife.10767

Liakopoulos, D., Doenges, G., Matuschewski, K., & Jentsch, S. (1998). A novel protein modification pathway related to the ubiquitin system. EMBO Journal, 17, 2208–2214. https://doi.org/10.1093/emboj/17.8.2208
Shen, L. N., Liu, H., Dong, C., Xirodimas, D., Naismith, J. H., & Hay, R. T. (2005). Structural basis of NEDD8 ubiquitin discrimination by the deNEDDylating enzyme NEDP1. *EMBO Journal*, 24, 1341–1351. https://doi.org/10.1038/sj.emboj.7600628

Shin, E. J., Shin, H. M., Nam, E., Kim, W. S., Kim, J. H., Oh, B. H., & Yun, Y. (2012). DeSUMOylating isopeptidase: A second class of SUMO protease. *EMBO Reports*, 13, 339–346. https://doi.org/10.1038/embor.2012.3

Shin, S. E., Lim, J. M., Koh, H. G., Kim, E. K., Kang, N. K., Jeon, S., … Jeong, Y. C., Liu, B. Y., Tsai, J. Y., Wu, J. T., Chang, L. K., & Chang, S. C. (2016). Capturing a substrate in an actin-binding protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, 7, 539. https://doi.org/10.1038/msb.2015.177

Sriramachandran, A. M., Meyer-Teschendorf, K., Pabst, S., Ulrich, H. D., Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W. Z., … van den Burg, H. A., Kini, R. K., Schuurink, R. C., & Takken, F. L. (2010). Arabidopsis small ubiquitin-like modifier paralogs have distinct functions in development and defense. *The Plant Cell*, 22, 1998–2016.

Tomanov, K., Zeschmann, A., Hermkes, R., Eiffer, K., Ziba, I., Greico, M., … Bachmair, A. (2014). Arabidopsis PIA1 and 2 promote SUMO chain formation as E4-type SUMO ligases and are involved in stress responses and sulfur metabolism. *The Plant Cell*, 26, 4547–4560.

Tulin, F., & Cross, F. R. (2014). A microbial avenue to cell cycle control in the plant superkingdom. *The Plant Cell*, 26, 4019–4038. https://doi.org/10.1105/tpc.114.129312

Umen, J. G., & Goodenough, U. W. (2001). Control of cell division by a retinoblastoma protein homolog in Chlamydomonas. *Genes & Development*, 15, 1652–1661. https://doi.org/10.1101/gad.892101

van den Burg, H. A., Kini, R. K., Schuurink, R. C., & Takken, F. L. (2010). Arabidopsis small ubiquitin-like modifier paralogs have distinct functions in development and defense. *The Plant Cell*, 22, 1998–2016.

Wang, X. D., Lapi, E., Sullivan, A., Ratnayaka, I., Goldin, R., Hay, R., & Lu, X. (2011). SUMO-modified nuclear cyclin D1 bypasses Ras-induced senescence. *Cell Death and Differentiation*, 18, 304–314. https://doi.org/10.1038/cdd.2010.101

Wang, Y., Ladunga, I., Miller, A. R., Horken, K. M., Plucinak, T., Weeks, D. P., & Bailey, C. P. (2008). The small ubiquitin-like modifier (SUMO) and SUMO-conjugating system of *Chlamydomonas reinhardtii*. *Nature Communications*, 179, 177–192.

Witman, G. B., Harris, E. H. (2009). The Chlamydomonas Sourcebook; Second Edition; Cell Motility and Behavior; Volume 3. Elsevier Ltd.

Wotton, D., Pemberton, L. F., & Merrill-Schools, J. (2017). SUMO and chromatin remodeling. *Advances in Experimental Medicine and Biology*, 963, 35–50.

Wu, K., Yamoah, K., Dolios, G., Gan-Erdene, T., Tan, P., Chen, A., … Pan, Z. Q. (2003). DEN1 is a dual function protease capable of processing the C terminus of Nedd8 and deconjugating hyper-neddylated CUL1. *Journal of Biological Chemistry*, 278, 28882–28891. https://doi.org/10.1074/jbc.M302888200

Xu, Z., & Au, S. W. (2005). Mapping residues of SUMO precursors essential in differential maturation by SUMO-specific protease, SENP1. *The Biochemical Journal*, 386, 325–330. https://doi.org/10.1042/BJ20041210

Yates, G., Srivastava, A. K., & Sadanandom, A. (2016). SUMO proteases: Uncovering the roles of deSUMOylation in plants. *Journal of Experimental Botany*, 67, 2541–2548. https://doi.org/10.1093/jxb/erw092

Yunus, A. A., & Lima, C. D. (2009). Structure of the Siz/PIAS SUMO E3 ligase Siz1 and determinants required for SUMO modification of PCNA. *Molecular Cell*, 35, 669–682. https://doi.org/10.1016/j.molcel.2009.07.013

Zhang, R., Patena, W., Armbruster, U., Gang, S. S., Blum, S. R., & Jonikas, M. C. (2014). High-throughput genotyping of green algal mutants reveals random distribution of mutagenic insertion sites and endonucleolytic cleavage of transforming DNA. *The Plant Cell*, 26, 1398–1409. https://doi.org/10.1105/tpc.114.124099

Zones, J. M., Blaby, I. K., Merchant, S. S., & Umen, J. G. (2015). High-resolution profiling of a synchronized diurnal transcriptome from *Chlamydomonas reinhardtii* reveals continuous cell and metabolic differentiation. *The Plant Cell*, 27, 2743–2769.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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