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Studies on the Small Ubiquitin-Like Modifier (SUMO) E2 Conjugases of the SUMOylation System in *Chlamydomonas reinhardtii* and their Role in Stress Physiology

Amy R. Knobbe

*University of Nebraska-Lincoln, amiller@huskers.unl.edu*

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STUDIES ON THE SMALL UBIQUITIN-LIKE MODIFIER (SUMO) E2 CONJUGASES OF THE SUMOYLATION SYSTEM IN *Chlamydomonas reinhardii* AND THEIR ROLE IN STRESS PHYSIOLOGY

by

Amy Rose Ann Knobbe

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STUDIES ON THE SMALL UBIQUITIN-LIKE MODIFIER (SUMO) E2
CONJUGASES OF THE SUMOYLATION SYSTEM IN Chlamydomonas reinhardtii
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Amy Rose Ann Knobbe, Ph.D

University of Nebraska, 2012

Advisor: Donald P. Weeks

The eukaryotic protein post-translational modification by SUMOylation is involved in a diverse array of cellular processes, including various stress responses. A fully functional SUMOylation system is present in the unicellular green alga Chlamydomonas reinhardtii, and SUMOylation of multiple high molecular weight proteins is induced in response to abiotic stress in this organism. We report here the characterization of a SUMO E2 conjugase deletion mutant in C. reinhardtii, mut5. SUMO E2 conjugase enzymes are responsible for the conjugation of the protein SUMO to a lysine residue within a target protein. C. reinhardtii mutants in which the SUMO E2 conjugase CrUBC9 has been deleted (mut5) fail to modify proteins with SUMO in response to multiple stress conditions, and this failure to SUMOylate generally results in a reduced tolerance to a given stress. Complementation of CrUBC9 mutants with the deleted gene demonstrates that CrUBC9 is solely responsible for SUMOylation under stress conditions, and that it predominantly localizes to the nucleus.

In addition, we identify the likely presence of two distinct SUMO E2 conjugase proteins in the C. reinhardtii genome. This is in marked contrast to virtually all other organisms studied to date, in which a single essential SUMO E2 conjugase has been
identified. Bioinformatic analyses allowed the identification of the likeliest candidate for this second SUMO E2 conjugase, CrUBC3. Comparison of CrUBC3 and CrUBC9 reveals that they have distinct sequence features that distinguish them from other known SUMO E2 conjugases. In addition, we show that the transcripts encoding these proteins are regulated in a distinct and opposite manner in response to stress, consistent with separate functions for these proteins within the cell. Expression of these proteins in a heterologous yeast system allowed the examination of the functionality of these two proteins as SUMO E2 conjugases, and suggests that the two likely function in distinct, non-overlapping capacities within *C. reinhardtii*. 
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CHAPTER 1

Literature Review
LITERATURE REVIEW

History of SUMO

The protein Small Ubiquitin-like Modifier (SUMO) was originally identified in yeast, Saccharomyces cerevisiae, as a high copy suppressor of a temperature-sensitive mutation in a centromeric protein (MIF2) and given the name SMT3 (Meluh and Koshland, 1995). Homologs for SMT3 were identified in other eukaryotic organisms and given various names, including PIC1 (mouse) and UBL1 (human) (Boddy et al., 1996; Howe et al., 1998). However, it wasn’t until just over a year later that this protein would be identified as a post-translational modification.

The first protein identified as a target for SUMOylation was the RanGTPase activating protein RanGAP1 (Matunis et al., 1996; Mahajan et al., 1997). Because the protein modified was RanGAP1, the initial report describing SUMO as a post-translational modification named the protein Gap Modifying Protein 1 (GMP1) (Matunis et al., 1996), but the second publication named the protein Small-ubiquitin-like-modifier (SUMO) based on its modest sequence similarity to ubiquitin (Mahajan et al., 1997). Likely because the protein was subsequently shown to modify multiple additional proteins, the name SUMO became the conventional name for all subsequent homologs. The revelation that unmodified RanGAP1 localized to the cytoplasm and SUMO-modified RanGAP1 localized at the nuclear envelope demonstrated that SUMO was a post-translational modification and provided an initial putative function for SUMOylation (Matunis et al., 1996; Mahajan et al., 1997). Shortly after the initial identification that SUMO modified the protein RanGAP1, it was shown that multiple proteins within the
cell are targeted for SUMOylation, and that the majority of SUMOylated proteins localize to the nucleus (Kamitani et al., 1997).

The number of SUMO proteins for a given organism can vary widely. While yeast contains a single SUMO gene (Meluh and Koshland, 1995), human cells are known to express three separate SUMO proteins that all become covalently attached to target proteins (Kamitani et al., 1998), while Arabidopsis is predicted to encode eight different SUMO proteins (Kurepa et al., 2003).

Crystal structures of SUMO proteins revealed that despite the relatively low similarity of sequence between SUMO and ubiquitin, the structures are highly similar and consist of five β-sheets and an α-helix consistent with the ubiquitin superfold (Bayer et al., 1998). There are several notable exceptions to this similarity, however. SUMO contains a long, flexible N-terminal extension that extends away from the rest of the protein, and also has several differences in surface charge distribution (Bayer et al., 1998). Importantly, in several SUMO proteins this N-terminal extension contains a lysine residue that can be SUMOylated resulting in the formation of poly-SUMO chains on a target protein (Tatham et al., 2001).

SUMOylation

SUMO shares similarity to ubiquitin not only in its three-dimensional structure, but also in the enzymatic cascade that results in its conjugation to target proteins (Figure 1-1). This was suggested early on when the molecular nature of the post-translational modification was revealed as an isopeptide bond between the carboxyl-group of a C-terminal glycine and the ε-amino group of a target lysine residue, just as was shown for ubiquitin (Matunis et al., 1996). A consensus motif for SUMOylation based on initial
**Figure 1-1. The SUMOylation pathway.** The enzymatic cascade that results in SUMOylation of a target protein begins with the proteolytic cleavage of a newly translated SUMO protein by a SUMO protease (ULP1, SENP), after a conserved double glycine motif near the C-terminus of the SUMO protein. Cleaved SUMO, in an ATP-dependent reaction, forms a thioester with a catalytic cysteine residue within an E1 activase heterodimer (UBA2/AOS1), followed by the transfer of SUMO to an E2 conjugase (UBC9). The E2 conjugase catalyzes the formation of an isopeptide bond between SUMO and the ε-amino group of a lysine residue within a target protein, usually with the help of an E3 ligase. SUMO proteases can also cleave this isopeptide bond, making the post-translational modification reversible.
targets was identified as ΨKXE, where Ψ was any large, hydrophobic amino acid, K was the target lysine, X was any amino acid, and E was a glutamic acid (Rodriguez et al., 2001). In vitro this consensus was shown to promote SUMOylation, however in vivo a nuclear localization signal was also required for SUMOylation, suggesting that the combination of these two might direct a protein to be modified by SUMO (Rodriguez et al., 2001). Subsequently, proteins such as PCNA were identified as targets for SUMO modification on lysine residues that did not conform to the identified consensus (Hoege et al., 2002). In addition, the identification of plasma membrane proteins that are SUMOylated is highly suggestive that nuclear localization is not required for SUMOylation in all circumstances (Dai et al., 2009).

A second motif has been identified in target proteins for SUMOylation known as Phosphorylation Dependent SUMO Modification (PDSM). The motif is defined as ΨKXEXXSP in which the SUMO consensus sequence is separated by two amino acids from a phosphorylatable serine followed by a proline (Hietakangas et al., 2006). Serine to alanine mutations of this motif block SUMOylation at the target lysine, while serine to aspartic acid mutations result in constitutive SUMOylation, suggesting that phosphorylation of a PDSM directs the SUMOylation status of the protein (Hietakangas et al., 2006).

**SUMO Proteases**

The first paper identifying SUMO as a post-translational modification also showed that the process was reversible, suggesting there must be an enzymatic activity that could de-SUMOylate target proteins (Matunis et al., 1996). Shortly thereafter, the protein ULP1 was identified in yeast and shown to act as a protease that could cleave
both the peptide bond after the conserved double glycine (-GG) motif at the C-terminus of a newly translated SUMO pro-protein, as well as isopeptide bonds between SUMO and a target protein (Figure 1-1) (Li and Hochstrasser, 1999). Importantly, the NEM sensitivity of ULP1 revealed it was likely a cysteine protease (Li and Hochstrasser, 1999). While ULP1 is required for advancement through the cell cycle, a second SUMO protease was identified, ULP2, that had weak sequence similarity to ULP1 but was not essential (Li and Hochstrasser, 2000). SUMO proteases show no homology to known de-ubiquitinating enzymes (Li and Hochstrasser, 2000). Until recently, SUMO proteases were identified in other eukaryotic organisms based on their homology to ULP1, although a novel de-SUMOylating enzyme potentially representing a new class of SUMO proteases that can de-SUMOylate target proteins but not process SUMO pro-proteins was recently identified (Shin et al., 2012).

**E1 Enzymes**

After cleavage of the SUMO pro-protein to expose the conserved double-Glycine (-GG) motif at the C-terminus, SUMO becomes attached to a SUMO E1 activase through a thioester bond at a catalytic cysteine residue within the E1 (Figure 1-1). In ubiquitination, this step is carried out by a single polypeptide known as UBA1 (McGrath et al., 1991). However, in the SUMOylation pathway, this enzymatic activity is carried out by two separate proteins that form a heterodimer. In yeast, these proteins are known as AOS1 and UBA2 which have homology to the N-terminal and C-terminal domains of UBA1, respectively (Johnson et al., 1997). The human homolog of AOS1 is known as SUMO Activating Enzyme 1 (SAE1) and the human homolog of UBA2 is known as SUMO Activating Enzyme 2 (SAE2) (Lois and Lima, 2005). The E1 enzyme adenylates
SUMO at its C-terminus in an ATP dependent reaction and then transfers the SUMO to the catalytic cysteine in SAE2 forming the thioester bond (Lois and Lima, 2005).

E2 Enzymes

The E2 conjugase, UBC9, is a critical component in the SUMOylation pathway as it interacts with the SUMO protein, E1 activase, E3 ligases (discussed below), as well as proteins targeted for modification by SUMO (Figure 1-1). UBC9 was originally identified as an essential putative ubiquitin conjugase involved in cell cycle regulation based on its sequence similarity to other known ubiquitin conjugases (Seufert et al., 1995). *Saccharomyces cerevisiae* ScUBC9 is approximately 35% identical to known ubiquitin conjugating (UBC) proteins in the same organism (Seufert et al., 1995). However, shortly after the discovery of SUMO as a novel post-translational modification, several groups independently identified UBC9 as the enzyme responsible for conjugation of SUMO, not ubiquitin (Desterro et al., 1997; Johnson and Blobel, 1997; Schwarz et al., 1998). Importantly, these reports established that there is no overlap between the ubiquitination and SUMOylation machinery in the cell, as ubiquitin was incapable of forming a thioester with UBC9, and SUMO was incapable of forming a thioester at the catalytic cysteine residue of the known ubiquitin conjugase UBC5 (Desterro et al., 1997; Johnson and Blobel, 1997).

Several structures have been solved for UBC9 proteins from a variety of organisms which has led to information about how SUMO E2 conjugases interact with various components in the SUMOylation pathway. The N-terminal region of UBC9 is likely involved in binding of the E1 activase, SUMO, and various E3 ligases, while residues in the C-terminal region are implicated in binding to residues surrounding the
target lysine in substrate proteins (van Waardenburg et al., 2006). NMR analysis of the binding between UBC9 and SUMO proteins revealed one feature that distinguishes these proteins from their ubiquitin counterparts. UBC9 has a strong positive electrostatic potential that allows it to interact with the negatively charged surfaces of the SUMO protein (Liu et al., 1999). This may provide a method of substrate discrimination for UBC9 interacting with SUMO instead of ubiquitin. Ubiquitin has a positive surface charge in the analogous surface region which would likely lead to electrostatic repulsion between ubiquitin and UBC9 (Liu et al., 1999).

**E3 Enzymes**

Because only a single protein, UBC9, is responsible for conjugating SUMO to all of the target proteins in a cell, E3 ligases are thought to be required to obtain substrate specificity in vivo (Figure 1-1). SUMO E3 ligases are characterized by their ability to increase SUMOylation of a target protein in vitro, and are generally required for SUMOylation in vivo (Johnson and Gupta, 2001). The first E3 enzymes identified for the SUMOylation pathway were homologous to Protein Inhibitor of Activated Stat (PIAS) proteins and contained a RING domain similar to known ubiquitin E3 ligases (Johnson and Gupta, 2001; Sachdev et al., 2001; Takahashi et al., 2001). In yeast and Arabidopsis these E3 ligases are known as SIZ proteins (Takahashi et al., 2001; Miura et al., 2005). SUMO E3 ligases can be classified into two categories, the aforementioned RING-type ligases and non-RING-type ligases. One such example of a non-RING domain E3 ligase is the polycomb protein Pc2, which acts to recruit UBC9 and its target protein CtBP together in a subnuclear structure known as the PcG complex in order to enhance SUMOylation (Kagey et al., 2003). In addition, the original SUMO target, RanGAP1, is
targeted for SUMOylation by the non-RING E3 ligase RanBP2 and crystallographic data revealed that these non-RING domain E3 ligases bind to a region of the UBC9 protein that is distinct from the binding domain for RING E3 ligases (Pichler et al., 2002; Tatham et al., 2005). Three different E3 ligases have been shown to be capable of enhancing in vitro SUMOylation of the protein Mdm2, including two PIAS (RING-type) E3 ligases and RanBP2 (a non-RING) E3 ligase (Miyauchi et al., 2002). This demonstrates that a single target can likely be acted upon by multiple E3 ligases.

**Functions of SUMOylation**

Initial suggestions as to the function of SUMO dealt largely with subcellular localization. The aforementioned relocalization of the protein RanGAP1 from the cytoplasm to the nuclear pore complex upon modification by SUMO was the first example of subcellular re-localization of a protein directed by SUMOylation (Matunis et al., 1996; Mahajan et al., 1998). SUMO also was shown to co-localize with the protein PML to large multi-protein complexes in the nucleus called PML nuclear bodies, and subsequent identification of PML as a target for SUMOylation led to the suggestion that SUMOylation of proteins might play a role in sub-nuclear localization of target proteins (Howe et al., 1998; Kamitani et al., 1998; Muller et al., 1998). This view was further strengthened by the revelation that another protein associated with “nuclear dots”, Sp100, might also be a target for SUMOylation (Sternsdorf et al., 1997).

Interestingly, SUMOylation has also been shown to be involved in subcellular localization outside of the nucleus. In the soil amoeba, *Dictyostelium discoideum*, SUMOylation of the MAP kinase MEK1 leads to its translocation from the nucleus to the plasma membrane (Sobko et al., 2002).
While initial reports on SUMOylation indicated a predominantly nuclear localization of SUMOylated targets, further work has revealed that the role of SUMO in the cell is far more complex, and that substrates for SUMOylation are found throughout the cell. Shortly after RanGAP1, PML, and Sp100 were identified as targets for SUMOylation, the NF-κB inhibitor, IκBα, was identified as a target for SUMOylation (Desterro et al., 1998). IκBα sequesters the transcription factor NF-κB to the cytoplasm until ubiquitination of IκBα targets it for degradation by the proteasome, releasing NF-κB to translocate to the nucleus. Lysine 21, the target for ubiquitination in IκBα, was shown to also be a target for SUMOylation. SUMOylation at this residue blocked ubiquitination, therefore preventing the degradation of IκBα and functioning to localize NF-κB to the cytoplasm (Desterro et al., 1998).

In the temperature-sensitive ubc9 mutant of S. cerevisiae, a defect in the ScUBC9 protein causes cells to stop dividing after a few cell divisions at the restrictive temperature of 37°C, indicating a role for SUMO modification in cell cycle progression (Seufert et al., 1995). The mutant also showed increased stability in a variety of cyclin proteins, and it was erroneously hypothesized that UBC9 was a ubiquitin conjugase that targeted these cyclins for degradation in normal cell cycle progression (Seufert et al., 1995). Since then, however, it has been established that SUMO modification of at least one yeast cyclin, Cdc3, antagonizes ubiquitin-dependent protein degradation in a manner analogous to IκBα SUMO modification (Takahashi et al., 1999).

SUMO modification also acts as an antagonist to ubiquitin modification of PCNA, but does not act on its protein stability. Ubiquitination of PCNA on lysine K164 does not target the protein for degradation, but instead targets the protein to act in DNA
repair pathways instead of DNA synthesis (Hoege et al., 2002). PCNA is SUMOylated under normal conditions, preventing ubiquitination, but becomes de-SUMOylated under sublethal levels of DNA damage, allowing the protein to become ubiquitinated and targeted for DNA repair pathways (Hoege et al., 2002).

In addition to a role in subcellular localization, SUMO modification has also been shown to regulate enzyme activity. The histone deactylase HDAC4 is a target for SUMOylation, and mutation of the target lysine residue in HDAC4 results in decreased deactylase activity of the enzyme (Kirsh et al., 2002).

The identification of SUMOylated proteins throughout the eukaryotic cell, combined with the various effects this modification exerts on a variety of proteins (Geiss-Friedlander and Melchior, 2007), raises the important point that identifying a protein as a substrate for SUMOylation does not answer any questions about what role that modification might play with regards to the function of the protein. Indeed, often the role of SUMOylation for a target protein can be rather complex and multi-layered. In the case of Glycogen Synthase Kinase 3β (GSK3β), mutants that cannot be SUMOylated show incorrect sub-cellular localization, reduced phosphorylation, reduced kinase activity, and decreased stability (Lee et al., 2008).

**Cross-talk between SUMOylation and other post-translational modifications**

There appears to be significant cross-talk and overlap between SUMOylation and other post-translational modifications, particularly phosphorylation. The SUMOylation of IκBα occurs on the same lysine residue as targeted for ubiquitination and acts to prevent ubiquitination of this protein as described above (Desterro et al., 1998). In addition, IκBα is also a substrate for phosphorylation and this modification appears to act
to control whether or not IκBα is SUMOylated or ubiquitinated. Phosphorylation of IκBα marks it for ubiquitination, and phosphorylated IκBα cannot be SUMOylated (Desterro et al., 1998). Negative regulation of SUMOylation by phosphorylation occurs for other proteins as well, as phosphorylation appears to negatively regulate the SUMOylation of PML (Muller et al., 1998). The MEK1 protein from *D. discoideum* that is translocated from the nucleus to the plasma membrane upon SUMOylation is both itself a protein kinase and a substrate for phosphorylation. It was shown that phosphorylation of MEK1 regulates SUMOylation, as a non-phosphorylatable mutant cannot be SUMOylated. Furthermore, a constitutively active mutant in which the phosphorylation sites were mutated to glutamate residues was constitutively SUMOylated (Sobko et al., 2002).

Several SUMO E3 ligases are phosphorylated, although the role of this phosphorylation is not yet clear (Johnson and Gupta, 2001; Takahashi et al., 2001).

A clear example of the cross-talk between SUMO and phosphorylation is the PDSM motif described above in which a nearby serine residue is phosphorylated to direct SUMOylation within a canonical SUMO motif. However, there are other examples of phosphorylated residues that enhance SUMOylation of a target protein that do not belong to the PDSM motif (Vanhatupa S et al., 2008), suggesting that, depending on the target protein and context, phosphorylation can act to both positively and negatively regulate SUMOylation of proteins.

**Role for SUMOylation in abiotic stress**

The notion that SUMOylation played a role in stress response came from experiments aimed at determining the roles of the various SUMO proteins in mammalian cells. SUMO-1 is predominantly conjugated to proteins under non-stress conditions,
whereas SUMO-2 and SUMO-3 are conjugated to proteins in response to a variety of abiotic stresses, including heat stress, salt stress, and oxidative stress (Saitoh and Hinchey, 2000; Šramko et al., 2006). Similar observations have been made in regard to the preferential use of the proteins AtSUMO1 and AtSUMO2 for SUMOylation in response to a variety of stresses in Arabidopsis (Kurepa et al., 2003).

The role of SUMO during stress has perhaps, best been studied in response to heat stress. The heat shock factors, HSF1, HSF2, and HSF4b, are modified by SUMO in mammalian cells (Goodson et al., 2001; Hong et al., 2001; Hietakangas et al., 2006). SUMOylation increases the DNA binding activity of these transcription factors (Goodson et al., 2001; Hong et al., 2001). In addition, HSF1, which modulates the induction of heat shock protein (HSP) expression in response to elevated stress, is SUMOylated upon heat stress and this SUMOylation correlates with a localization of HSF1 to nuclear granules (Hong et al., 2001).

In Arabidopsis HSFA2 is targeted for SUMOylation during extended exposure to heat stress and during recovery (Cohen-Peer et al., 2010). In this case, SUMOylation negatively regulates the activity of this transcription factor. Another Arabidopsis transcription factor, c-Myb, is modified by SUMO in response to various stress conditions, including heat stress, although the role of this modification is still unclear (Šramko et al., 2006).

Several proteomic studies have attempted to identify what proteins globally are changed in response to various stress treatments. One of the most comprehensive and quantitative reports to date identified several hundred proteins that changed
SUMOylation state in response to heat stress (Golebiowski et al., 2009). Proteins directly involved in stress response, such as HSF1 and HSP proteins (HSP40, -60, and -70), were identified as SUMO targets in response to heat stress (Golebiowski et al., 2009). Proteins involved in transcription, translation, DNA repair, and cell cycle regulation are also frequently identified targets for SUMOylation in response to heat stress (Zhou et al., 2004; Golebiowski et al., 2009; Miller et al., 2010; Bruderer et al., 2011).

**SUMO in Chlamydomonas reinhardtii**

Much is still unknown about the SUMOylation pathway in the green microalga *C. reinhardtii*. Despite a fully sequenced genome, there has been no definitive identification of the E1 activase enzymes for the SUMOylation pathway, and neither has an E3 ligase been identified. Several putative SUMO proteins were identified in the genome, CrSUMO96, CrSUMO97, and CrSUMO148 (Wang et al., 2008). The high degree of similarity between CrSUMO96 and CrSUMO97, combined with the failure to detect CrSUMO97 transcripts, suggests it is a pseudogene. CrSUMO148 is a unique SUMO protein in that it has a C-terminal extension that contains five double glycine (-GG-) motifs (Wang et al., 2008). A functional UBC9, CrUBC9, has been identified and shown to have SUMO E2 conjugase activity in an in vitro assay (Wang et al., 2008). A putative SUMO protease has been identified in the genome as a result of a screen for suppressors of the cell cycle mutant MAT3 which contains a deletion of the cell cycle regulator retinoblastoma (RB) (Fang and Umen, 2008). As with other organisms, abiotic stress has been shown to induce SUMOylation in *C. reinhardtii* (Wang et al., 2008).
Stress in *Chlamydomonas reinhardtii*

The unicellular green alga *C. reinhardtii* has classically been used as a model organism for photosynthetic and flagellar research (Merchant *et al.*, 2007). More recently, it has been utilized as a model organism for potentially improving production of algal biofuels. *C. reinhardtii* accumulates triacylglycerols (TAGs) in response to both nitrogen deprivation and salt stress (Siaut *et al.*, 2011; Msanne *et al.*, 2012). Attempts to utilize *Chlamydomonas* as a strain for biofuel production include genetic manipulations both to increase biofuel production as well as to enhance traits that make *C. reinhardtii* more amenable to growth in a photo-bioreactor (Bonente *et al.*, 2011). As stress conditions used to induce TAG production may affect several cellular functions from growth rate, to cell viability, to the types of TAG produced in *C. reinhardtii*, understanding the stress physiology of this organism will be of both academic and potential commercial importance.

**Heat stress**

The response of *C. reinhardtii* cells to heat stress involves both changes in post-translational modifications, such as the dephosphorylation of the chaperone BiP (Díaz-Troya *et al.*, 2011), as well as *de novo* protein synthesis (Nicholson and Howe, 1989). Heat stress also results in the conjugation of ubiquitin to multiple proteins in the cell (Wettern *et al.*, 1990).

Of critical importance to cellular adaptation to heat stress are the heat shock proteins (HSPs). Heat shock proteins bind unfolded proteins and act in several capacities inside the cell, including aiding in the correct folding of proteins and preventing the aggregation of denatured proteins (Schroda *et al.*, 1999). *Chlamydomonas* has several
HSPs located throughout the cell. HSP70A localizes to the cytoplasm, while HSP70B localizes to the chloroplast (Schroda et al., 1999). The overexpression of a heterologous HSP70 from the alga *Porphyra seriata* increased the thermal tolerance of *Chlamydomonas*, highlighting the importance these proteins can have on the viability of a cell in elevated temperatures (Park et al., 2012). The induction of HSPs is controlled by transcriptional regulators known as heat shock factors. The *C. reinhardtii* genome encodes two heat shock factors, HSF1 and HSF2 (Schulz-Raffelt et al., 2007). HSF1 is synthesized under non-stress conditions, but its expression increases in response to elevated temperatures (Schulz-Raffelt et al., 2007). In response to heat shock, the HSF1 protein becomes hyper-phosphorylated, increasing its transcriptional activation activity (Schulz-Raffelt et al., 2007). HSF1 plays a key role in maintaining cell viability in response to heat stress, as RNAi targeting of the HSF1 transcript resulted in a loss of cell viability after exposure to 40°C (Schulz-Raffelt et al., 2007).

Understanding how *C. reinhardtii* cells identify and respond to stress conditions will need to include identification of proteins and post-translational modifications involved in this complex response. The work presented here provides a detailed characterization of a mutant of *C. reinhardtii*, mut5, that lacks the SUMO E2 conjugase, CrUBC9 (Wang et al., 2008), and fails to SUMOylate in response to various abiotic stress conditions. We show complementation of this mutant and subcellular localization of CrUBC9 primarily to the nucleus. In addition, discovery of *bona fide* SUMOylated proteins in the mut5 mutant under non-stress conditions led to the proposal of a second functional SUMO E2 conjugase encoded in *C. reinhardtii*. Experiments leading to the tentative identification of this second functional E2 enzyme are also described.
CHAPTER 2

The SUMO E2 conjugating enzyme CrUBC9 of *Chlamydomonas reinhardtii* is required for SUMOylation under abiotic stress conditions
INTRODUCTION

The presumed role of the post-translational modification Small Ubiquitin Like Modifier (SUMO) inside the cell has expanded greatly from its initial identification as a signal for nuclear pore localization for the protein RanGAP1 (Matunis et al., 1996; Mahajan et al., 1997; Geiss-Friedlander and Melchior, 2007). The attachment of SUMO to the lysine residue of a target protein, termed SUMOylation, has since been shown to be involved in a variety of other processes including subcellular localization of proteins (Sternsdorf et al., 1997; Howe et al., 1998; Kamitani et al., 1998; Muller et al., 1998; Sobko et al., 2002), regulation of protein turnover (Desterro et al., 1998), changes in enzymatic activity (Kirsh et al., 2002), and cell cycle progression (Seufert et al., 1995).

In addition to a shared overall three-dimensional structure with ubiquitin (Bayer et al., 1998), the process by which a target protein is modified by SUMO is also similar to that of ubiquitin. SUMO is translated as an inactive precursor that is cleaved by a protease after a conserved double glycine (-GG) motif within the C-terminus of the SUMO protein (Li and Hochstrasser, 1999; Geiss-Friedlander and Melchior, 2007). The mature SUMO protein is then conjugated to the ε-amino group of a lysine residue within a target protein through an enzymatic cascade involving an E1 activase, E2 conjugase, and E3 ligase (Gareau and Lima, 2010). Although the E1 and E2 enzymes are usually capable of SUMOylating a target protein in vitro, within the cell an E3 ligase is generally required (Johnson and Gupta, 2001; Gareau and Lima, 2010). In addition to cleaving the immature SUMO pro-protein, SUMO proteases can deSUMOylate target proteins, making this post-translational modification reversible (Li and Hochstrasser, 1999; Gareau and Lima, 2010). Within the SUMOylation enzymatic cascade, the E2 conjugase is the...
enzyme responsible for catalyzing the formation of an isopeptide bond between SUMO and a target protein (Johnson and Blobel, 1997; Schwarz et al., 1998). It is also of particular importance as it interacts with SUMO, E1, and E3 enzymes within the SUMOylation cascade. One point of distinction between SUMOylation and ubiquitination with regards to E2 conjugase proteins is the sheer number of ubiquitin E2 conjugases (UBCs) compared to their SUMO counterparts. The yeast genome encodes 11 ubiquitin conjugating enzymes, while both human and Arabidopsis genomes contain several dozen (Bachmair et al., 2001; Kraft et al., 2005; Ye and Rape, 2009). In contrast, each of these organisms encodes a single, essential SUMO E2 conjugase (Seufert et al., 1995; Saitoh and Hinchey, 2000; Nacerddine et al., 2005; Geiss-Friedlander and Melchior, 2007; Saracco et al., 2007). In yeast this protein is named ScUBC9, as it was originally proposed to be a ubiquitin conjugating enzyme based on its homology to other known ubiquitin conjugases, and the human homolog was given the same name (hUbc9) (Seufert et al., 1995; Watanabe et al., 1996; Yasugi and Howley, 1996). The Arabidopsis homolog is called Sumo Conjugating Enzyme 1 (SCE1) (Saracco et al., 2007). The rice genome is the only genome known to encode more than a single SUMO E2 conjugase, as it is predicted to encode two nearly identical (93% identity) SUMO conjugating enzymes that appear to be the result of a recent gene duplication event (Nigam et al., 2008).

A temperature-sensitive mutation in the yeast ScUBC9 gene, ubc9, showed an arrest of the cell cycle at the restrictive temperature of 37°C, therefore implicating ScUBC9 and SUMOylation in cell cycle progression (Seufert et al., 1995). However, SUMOylation has also been shown to play an important role in a variety of cellular processes, including stress response. Human cells encode at least three, possibly four
SUMO proteins, and characterization of these SUMO isoforms led to the discovery that under abiotic stress such as elevated temperature, increased osmotic pressure, and oxidative stress conditions SUMO2 and SUMO3 are preferentially conjugated to target proteins instead of the SUMO1 isoform (Saitoh and Hinchey, 2000). An analogous situation has since been identified in Arabidopsis in which the AtSUMO1 and AtSUMO2 proteins are conjugated to target proteins in response to stress (Kurepa et al., 2003).

One indication of the major role that SUMOylation plays in response to abiotic stress is the gross changes in protein SUMOylation occurring in response to abiotic stress conditions. In human cells, proteomic analysis after heat shock revealed that the SUMOylation status of several hundred proteins changed (Golebiowski et al., 2009; Bruderer et al., 2011). Much of what we know about the role of SUMOylation in stress response in plants comes from E3 and SUMO protease mutants that show distinct new phenotypes when exposed to various stresses. Mutants of one such E3 ligase, SIZ1, from Arabidopsis have been shown to have increased sensitivity to phosphate deprivation, excess copper, drought stress, and both cold and elevated temperatures (Miura et al., 2005; Yoo et al., 2006; Catala et al., 2007; Miura et al., 2007; Miura and Hasegawa, 2008; Chen et al., 2011). Interestingly, siz1 mutants in Arabidopsis show increased tolerance, rather than sensitivity, to salt stress (Miura et al., 2011). In contrast, mutants of the SUMO proteases Overly Tolerant to Salt 1 and 2 (OTS1 and OTS2) in Arabidopsis show extreme sensitivity to salt, suggesting that the role of SUMOylation is likely unique to various abiotic stresses (Conti et al., 2008).

The unicellular green alga Chlamydomonas reinhardtii has long been a model organism for studies of photosynthesis and flagellar structure and function (Merchant et
More recently, it is being examined as a potential source for biofuel production (Bonente et al., 2011; Msanne et al.). Both nitrogen deprivation and salt stress have been shown to induce the accumulation of triacylglycerols (TAGs) in *C. reinhardtii* (Siaut et al., 2011).

*C. reinhardtii* possesses a functional SUMOylation system that induces the modification of proteins by SUMO in response to various abiotic stress conditions similar to that observed in other eukaryotic organisms (Wang et al., 2008). Although a functional SUMO system exists in *C. reinhardtii*, little is known about the components of the SUMOylation cascade in this organism. *C. reinhardtii* encodes one canonical SUMO protein (CrSUMO96) and likely a second uniquely longer SUMO protein (CrSUMO148) which possesses a longer C-terminal extension with multiple double glycine motifs (Wang et al., 2008). Although no genes encoding the SUMO pathway E1 or E3 enzymes for SUMO have been definitively identified in *C. reinhardtii*, multiple candidate genes for an E2 conjugase are present in the genome and one of these, designated CrUBC9, was shown to have weak SUMO E2 conjugase activity in an *in vitro* assay (Wang et al., 2008). None of the candidate CrUBC9 enzymes are close homologs, such as is the case for rice (Nigam et al., 2008; Wang et al., 2008). In addition, a mutant in a putative SUMO protease, *smt*-7, has been identified and appears to be involved in cell cycle regulation due to its effect on cell size (Fang and Umen, 2008).

We report here the characterization of the mutant *mut*5 which has a complete deletion of the CrUbc9 gene, the E2 conjugase previously reported to have weak *in vitro* SUMO E2 conjugase activity. We show that *mut*5 cells lack SUMOylation in response to various stress conditions including multiple abiotic stress treatments and carbon
deprivation. In addition, mutant cells exhibit growth phenotypes under elevated temperature, salt, and osmotic pressure. Complementation of mut5 with CrUBC9 cDNA restores both SUMOylation under abiotic stress and growth under elevated temperature and osmotic pressure, indicating that CrUBC9 is a functional E2 conjugase in C. reinhardtii essential for response and adaptation to stress. The viability of mut5 under standard non-stress laboratory conditions raises the intriguing possibility that C. reinhardtii may be unique from all other eukaryotic organisms studied to date in that multiple, distinct functional SUMO E2 conjugases might be present and active under non-stress conditions.

**RESULTS**

*mut5 is defective in SUMOylation in response to abiotic stress*

It was previously demonstrated that in response to a heat shock of 42°C, multiple high molecular weight proteins are SUMOylated in *C. reinhardtii* (Wang et al., 2008). To determine whether or not *CrUBC9* is responsible for this stress-induced SUMOylation, wild-type and *mut5* cultures were shifted to 42°C for one hour and the pattern of SUMOylation in these cultures was analyzed by immunoblotting with anti-SUMO antibodies (Figure 2-1). Wild-type cells (CC124) grown at 25°C show one major protein at ~130kD but little to no other high molecular weight SUMOylated proteins. In contrast, wild-type cells exposed to 42°C show many high molecular weight SUMOylated proteins (>65kD), indicating these proteins were SUMOylated in response to heat stress, as has been previously observed (Wang et al., 2008). These newly modified proteins are detectable after at least 30 minutes at 42°C and persist at 60
Figure 2-1. *mut5* fails to accumulate high molecular weight SUMOylated proteins in response to heat shock at 42°C. A starting sample was prepared from CC124 and *mut5* cultures grown at 25°C prior to exposure to 42°C. Both cultures were shifted to 42°C and whole cell extracts were prepared from samples taken at 30 and 60 minutes. SUMOylated proteins in the samples were separated on bis-Tris SDS-PAGE gels and detected by immunoblot analysis (IB) with anti-SUMO antibodies (upper panel). Cell densities were used to normalize loading so that protein from the same number of cells was loaded in each lane. Staining of the protein blot with Reactive Brown stain demonstrated similar loading between lanes (lower panel).
IB: Anti-SUMO

Reactive Brown
minutes. *mut5* cells grown at 25°C show a similar pattern of SUMOylation compared to wild-type cells grown at 25°C. However, *mut5* cells shifted to 42°C show a SUMOylation pattern that appears identical to non-stressed cells and no evidence of protein modification by SUMO in response to 42°C as was observed for wild-type cells. These results demonstrate that *mut5* is incapable of SUMOylating proteins in response to a shift from 25°C to 42°C and therefore suggest that the CrUBC9 protein is responsible for heat-stress induced SUMOylation.

Similar to SUMOylation patterns observed during the 42°C heat treatment, *C. reinhardtii* also modifies high molecular weight proteins with SUMO in response to salt, osmotic, and 37°C stress conditions (Wang *et al.*, 2008). To determine if the SUMOylation deficiency observed in *mut5* is specific for the 42°C treatment, or if the same deficiency can be observed for additional abiotic stress treatments, wild-type and *mut5* cells were exposed to various other stress conditions and analyzed by immunoblotting with anti-SUMO antibodies. In response to heat stress at 37°C, salinity stress with 175 mM NaCl, osmotic stress with 300 mM sorbitol, and reactive oxygen species (ROS) stress with 2 mM H₂O₂, wild-type cells showed SUMOylation of high molecular weight proteins in a pattern similar to what was observed for 42°C (Figure 2-2). In contrast, *mut5* cells showed no change in SUMOylation patterns between non-stressed and stressed cells under all four additional abiotic stress conditions tested. These results suggest that CrUBC9 is responsible for the SUMOylation patterns observed in *C. reinhardtii* in response to diverse abiotic stresses.
Figure 2-2. *mut5* fails to SUMOylate in response to diverse abiotic stresses.

Immunoblot analysis of wild-type and *mut5* cells after exposure to (A) 37°C, (B) 175 mM NaCl, (C) 300 mM sorbitol, (D) 2 mM H₂O₂. Whole cell samples were prepared from cells grown in TAP at 25°C, and then at 30 minutes and 60 minutes after the start of each stress treatment. Cell densities were used to normalize loading so that protein from the same number of cells was loaded in each lane. Reactive Brown staining of the protein blot shows similar loading between lanes (lower panel).
Constitutively SUMOylated proteins are likely present in mut5

The pattern of proteins detected with anti-SUMO antibodies in mut5 extracts appeared identical to the pattern of proteins detected in non-stressed wild-type cells. Because mut5 has a complete deletion of the CrUBC9 gene, the presence of any SUMOylated proteins in mut5 would indicate the presence of at least one additional SUMO E2 conjugase in the C. reinhardtii genome. To confirm that the bands detected in these immunoblots are indeed SUMOylated proteins, a competition assay was performed by pre-incubating the anti-SUMO antibodies with excess recombinant SUMO to sequester SUMO antibodies and thus diminish the signal obtained from any bona fide SUMO proteins on protein blots of cell extracts. Recombinant CrSUMO96, the protein against which the anti-SUMO antibodies were generated, was preincubated with anti-SUMO antibodies before application to a protein blot of wild-type and mut5 extracts isolated at both 25°C and 42°C. As a negative control anti-SUMO antibodies were also preincubated with either a non-fat dry milk-based blocking reagent alone or with recombinant CrUBIQ1A (ubiquitin). These mixtures were then applied independently to identical protein blots (Figure 2-3).

In wild-type extracts, recombinant CrSUMO96 competed away the signal of all the high molecular weight proteins (>65 kD) in extracts resolved at 42°C, indicating the detection of SUMOylation in response to heat stress. In addition, in both non-stressed wild-type and mut5 cell extracts, signals from several bands were competed away, including the prominent band at approximately 130 kD (Figure 2-3, middle panel). This strongly suggests that those proteins are in fact bona fide SUMOylated proteins present in both wild-type and mut5 cells. In addition, preincubation of the anti-SUMO antibodies
**Figure 2-3.** *mut5 extracts contain authentic SUMOylated proteins.* Proteins in extracts of wild-type and *mut5* cells previously incubated at 25°C or for one hour at 42°C [along with recombinant SUMO96 (S) and UBIQ1A (U)] were separated by electrophoresis on bis-Tris SDS-PAGE gels and used to prepare three identical immunoblots. The protein blot shown in the upper panel was incubated with anti-SUMO antibodies alone. Protein blots shown in the middle and lower panels were incubated with anti-SUMO antibodies pre-incubated with either saturating amounts of recombinant CrSUMO96 (middle panel) or CrUBIQ1A (lower panel).
IB: Anti-SUMO

Reactive Brown

Lane 1 2 3 4 5 6

IB: Anti-SUMO + rSUMO96

Reactive Brown

IB: Anti-SUMO + rUBIQ1A

Reactive Brown
with recombinant ubiquitin (CrUBIQ1A) failed to compete away the signal from those same proteins, suggesting that the anti-SUMO antibodies are specific for SUMO (Figure 2-3, lower panel). Furthermore, the anti-SUMO antibodies failed to recognize recombinant CrUBIQ1A on those same blots suggesting that our antibodies do not significantly cross react with ubiquitin (Figure 2-3, lane 6). Together, these data suggest that at least one SUMO E2 conjugase in addition to CrUBC9 is present in *C. reinhardtii* cells, but is incapable of acting to modify proteins with SUMO in response to abiotic stress.

**CrUBC9 is the only SUMO E2 conjugase in C. reinhardtii that SUMOylates proteins in response to abiotic stress**

The failure of *mut5* to SUMOylate proteins in response to abiotic stress suggests that the SUMO E2 conjugase that SUMOylates those proteins constitutively modified by SUMO in *mut5* cells is incapable of acting on proteins SUMOylated in response to abiotic stress. To confirm that *mut5* is completely deficient in SUMOylation response to abiotic stress treatment, the pattern of SUMOylation in wild-type and *mut5* cells after exposure to 42ºC was examined over a longer period of time. Wild-type and *mut5* cultures were shifted to 42ºC as above and whole cell extracts were prepared after one hour, as well as after every three hours for a total of nine hours. SUMOylation patterns were then analyzed by immunoblot analysis with anti-SUMO antibodies (Figure 2-4). Wild-type cells shifted to 42ºC showed SUMOylation of high molecular weight proteins after one hour at 42ºC, as was observed above. This pattern of SUMOylation persisted over nine hours, although a slight decrease in SUMOylation by six to nine hours appeared to occur. In contrast, *mut5* cells failed to induce any detectable SUMOylation
Figure 2-4. *mut5* is unable to SUMOylate stress-related proteins during prolonged heat stress at 42ºC. Whole cell extracts were isolated from wild-type and mut5 cultures grown at 25ºC prior to shifting cultures to 42ºC. Whole cell extracts were isolated after one, three, six, and nine hours at 42ºC. Upper panel is an immunoblot with anti-SUMO antibodies, lower panel is a Reactive Brown stain of the blot showing equivalent loading of proteins.
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![Image of gel electrophoresis](image)
in response to heat stress over the course of nine hours. In addition to the failure to induce SUMOylation in response to heat stress, the signal for several of the constitutively SUMOylated proteins observed in *mut5* appeared to decrease slightly by 6 hours at 42°C and showed a more dramatic decrease in signal by 9 hours at 42°C, suggesting they were either being de-SUMOylated in response to heat stress, or that they were being degraded as a result of cell damage and/or death in response to the prolonged incubation at the elevated temperature. Collectively, these results strongly suggest that CrUBC9 is required for SUMO modification in response to abiotic stress in *C. reinhardtii*, and that if additional SUMO E2 conjugases are expressed in *mut5* cells, they are completely incapable of SUMOylation in response to abiotic stress.

**CrUBC9 is required for cell viability and growth at 37°C**

Because all other organisms studied to date have a single, essential UBC9 gene, no knockouts have been available in these organisms for examination of phenotypes generated in response to abiotic stresses. This makes *mut5* unique in that it contains a complete *CrUBC9* deletion and fails to SUMOylate in response to abiotic stress. This allows the examination of the phenotypes associated with an inability to SUMOylate in response to abiotic stress. It has been reported that wild-type *C. reinhardtii* strains are usually capable of growth between 15°C and 35°C (Harris, 2009). Because both elevated temperatures tested for SUMOylation in this study (Figures 2-1 and 2-2A) were outside this range, the lower temperature was used to determine if there was a difference in cell viability between wild-type and *mut5*. To test this, the growth of wild-type and *mut5* cultures in liquid media was compared at 20°C and 37°C. Dilute cultures of wild-type
and mut5 were maintained at both 20°C and 37°C and their OD660 was monitored over the course of 72 hours (Figure 2-5A).

Growth curves for both wild-type and mut5 cells showed similar growth rates at 20°C, indicating that there appears to be no deleterious effect on cell growth and division in mut5 under non-stress conditions. Comparative OD660 values for wild-type and mut5 cultures increased by 91- and 106-fold, respectively, by the end of 72 hours. Wild-type cells incubated at 37°C lagged behind both wild-type and mut5 cultures grown at 20°C but still showed growth over the course of 72 hours, resulting in an OD660 at 72 hours that was 43 times higher than the initial OD660. In contrast, mut5 failed to show any significant growth at 37°C, only reaching an OD660 6 times higher than its starting OD660 by the end of 72 hours. The lack of significant growth observed for mut5 at 37°C raised the possibility that extended exposure to elevated temperatures could prove lethal. To test this, cultures normalized for cell densities were spotted in a 1:4 dilution series on multiple TAP plates and incubated at 37°C. Individual plates were removed to room temperature after 1, 2, and 3 days at 37°C (Figure 2-5B) and allowed to incubate for an additional 4 days at room temperature after the last plate was removed from 37°C to assess cell viability after incubation at 37°C for the indicated time period. These spot tests revealed that wild-type cells indeed maintained their viability during extended exposure to 37°C. A TAP plate maintained at 37°C for 72 hours showed no apparent loss in cell viability in CC124 cells when compared to a control plate that was maintained at 25°C. The same control plate showed similar cell growth between CC124 and mut5 cells, indicating again that under non-stress conditions there appears to be no major difference in cell viability between wild-type and mut5 cells. However, after just 24 hours at 37°C a
Figure 2-5. *mut5* is not viable at 37°C. (A) The OD$_{660}$ of wild-type and *mut5* cultures diluted to an initial OD$_{660}$ between 0.005-0.022 was monitored every 24 hours for 72 hours. Data points are the average of three independent cultures, and error bars represent the standard deviation. (B) A 1:4 dilution series of wild-type and *mut5* cells was spotted in duplicate on four individual TAP plates. One was incubated at 25°C (top panel) while the remaining three were incubated at 37°C in the light. Plates were shifted from 37°C to 25°C after 1-3 days. (C) Wild-type and *mut5* cultures were diluted to a concentration of 400 cells/ml. Aliquots of 250µl were plated from initial dilutions and then every 24 hours for 48 hours after shifting to 37°C. Data points reflect the average number of viable cells relative to the original dilution and are the average of three independent experiments. Error bars represent the standard deviation.
A

![Graph showing Relative OD (660nm) vs Time (Hours) for CC124 and mut5 at 20ºC and 37ºC.]

B

![Images of cells for CC124 and mut5 over 3 days at 37ºC, with relative numbers of viable cells indicated.]

C

![Graph showing Relative Number of Viable Cells vs Time (hours) at 37ºC for CC124 and mut5.]

Days at 37ºC

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Relative Number of Viable Cells

Time (hours) at 37ºC

CC124

mut5
loss of cell viability was apparent in mut5 cells. The loss in viability increased in severity over the course of 72 hours to the point at which virtually no viable mut5 cells remained after 72 hours (Figure 2-5B, lower panel). This demonstrates that mut5 cells are not only incapable of growth at 37°C, but that mut5 cells are not viable at this temperature. In an attempt to quantify how quickly mut5 cells die at 37°C, very dilute (400 cells/ml) cultures of CC124 and mut5 were again shifted to 37°C and 250 µl aliquots were plated at 24 and 48 hours after shifting (Figure 2-5C). Wild-type cells again showed strong growth at 37°C, reaching a cell density approximately three times greater than the initial culture by the end of 48 hours at 37°C. Within 24 hours at 37°C, however, the percentage of viable cells in the mut5 culture had decreased to ~50%, and no viable cells were detected at 48 hours. The sensitivity of mut5 cells to incubation at 37°C, combined with its failure to SUMOylate high molecular weight proteins in response to heat stress, strongly suggests that the ability of wild-type cells to survive at elevated temperatures is dependent upon SUMOylation of target proteins by CrUBC9.

The growth of wild-type and mut5 cells in the presence of either sorbitol or NaCl (Figures 2-2B, C) was tested to determine if SUMOylation played a role in the ability of cells to grow under osmotic or salinity stress, respectively. Normalized cultures were spotted on TAP plates and TAP plates supplemented with 300 mM sorbitol or 175 mM NaCl. When a 1:4 dilution series of wild-type and mut5 cells was spotted on control TAP plates, similar growth could be observed between the two, consistent with what was previously observed on both solid and liquid media, above (Figure 2-6A, B). However, when these same cells were spotted on TAP+300 mM sorbitol, both wild-type and mut5
Figure 2-6. Stress phenotypes of *mut5* under osmotic and salt stress. (A) Wild-type and *mut5* cultures were diluted in a 1:4 dilution series and spotted on TAP and TAP+300 mM sorbitol plates. Plates were incubated at 25°C to assess growth. (B) Wild-type and *mut5* cultures were diluted in a 1:4 dilution series and spotted on TAP and TAP+175 mM NaCl plates. Plates were incubated at 25°C to assess growth.
cells were capable of growth, but *mut5* exhibited a reduced growth rate compared to wild-type cells (Figure 2-6A).

When wild-type and *mut5* cells were spotted on medium containing 175 mM NaCl, wild-type cells showed strongly reduced growth compared to control cells spotted on a TAP plate. In contrast, *mut5* cells were incapable of growth on plates containing 175 mM NaCl (Figure 2-6B). These data suggest that the SUMOylation of proteins by CrUBC9 enables *C. reinhardtii* cells to tolerate fluctuations in their osmotic and ionic environments, and without these protein modifications the ability to survive becomes severely compromised.

**CrUBC9 targets proteins for SUMO modification during carbon (acetate) deprivation**

In addition to abiotic stress, cells can encounter stress when deprived of nutrients. To determine if CrUBC9 is also involved in SUMOylation in response to nutrient deprivation, SUMOylation patterns in wild-type and *mut5* cells in response to nutrient deprivation were tested. Deprivation of phosphate, nitrogen, and sulfur over a period of 48 hours caused no apparent change in SUMOylation patterns in wild-type cells compared to cells grown on TAP medium (data not shown). Attempts to deprive cells of all carbon by placing them in TP medium (i.e., TAP medium containing no acetate) and incubating in the dark proved rapidly lethal for cells. Therefore, to slow the rate of cell death and slow the rate of carbon deprivation, cells were pelleted, resuspended in a medium with half the normal concentration of acetate, and incubated in the dark. To ensure any changes in SUMOylation pattern observed were the result of carbon
deprivation and not just the result of dark incubation, a control culture incubated in full strength TAP in the dark was also tested. Whole cell extracts were prepared from wild-type and mut5 cells after 24 and 48 hours of carbon deprivation and SUMOylation patterns analyzed by immunoblot analysis with anti-SUMO antibodies. In carbon-deprived cells, the pattern of SUMOylation in wild-type cells after 48 hours of carbon deprivation compared to initial cells, or even cells at 24 hours after deprivation, showed a novel repertoire of SUMOylated proteins (Figure 2-7). This new pattern of SUMOylation was distinct from both the patterns observed under non-stress conditions as well as the pattern observed under abiotic stress. Wild-type cells deprived of carbon for 48 hours produced a new, prominent ~75 kDa protein, as well as several other more minor bands of both larger and smaller proteins. This was distinct from the pattern observed under non-stress and abiotic stress conditions, the latter of which produces a large number of proteins of high molecular weight that are modified by SUMO, resulting in a “smear” of proteins in this molecular weight range. mut5 cells deprived of carbon for up to 48 hours showed no change in SUMOylation pattern compared to the initial culture, suggesting that mut5 is incapable of mounting a SUMOylation response as a result of carbon deprivation. In addition, wild-type cells incubated in the dark in full strength TAP medium also failed to SUMOylate after 48 hours in the dark, suggesting that the change in pattern observed in wild-type samples with half the normal amount of acetate is indeed the result of carbon deprivation rather than light deprivation. These data suggest that under carbon deprivation, CrUBC9 SUMOylates multiple proteins in a pattern distinct from abiotic stress. Combined with the observations above, this indicates that CrUBC9 may be responsible for all SUMOylation that occurs in C. reinhardtii in
**Figure 2-7.** *mut5* fails to SUMOylate in response to carbon-source deprivation.

Control extracts of wild-type and *mut5* cultures were prepared from cells grown in TAP medium prior to carbon deprivation. Wild-type and *mut5* cultures were washed twice in water, resuspended in TAP medium with 8.7 mM acetate instead of the usual 17.4 mM acetate, and incubated in the dark to slowly deprive them of carbon. Whole cell samples were prepared after 24 and 48 hours of carbon deprivation. Control wild-type cells were incubated in full strength TAP medium (17.4 mM acetate) in the dark (last two lanes). The OD$_{660nm}$ was monitored every 24 hours and used to ensure loading of protein from equivalent numbers of cells into each lane of a bis-Tris SDS polyacrylamide gel. Reactive Brown stain of the protein blot demonstrated similar loading between lanes (lower panel).
<table>
<thead>
<tr>
<th>CC124</th>
<th>mut5</th>
<th>CC124</th>
</tr>
</thead>
<tbody>
<tr>
<td>-C</td>
<td>-C</td>
<td>dark</td>
</tr>
</tbody>
</table>

TAP 2hr. 4hr. TAP 2hr. 4hr. TAP 2hr. 4hr.

IB: Anti-SUMO

Reactive Brown
response to a diverse range of stress conditions. Based on the suggestion from the competition assay above that at least one other SUMO E2 conjugase may be present in *C. reinhardtii* extracts (Figure 2-3), these data also suggest that there may be a division of labor between CrUBC9 and a second SUMO E2 conjugase. That is, CrUBC9 is responsible for SUMOylation under stress conditions, while another, likely essential E2 conjugase, SUMOylates proteins as part of normal cell growth and division in *C. reinhardtii*.

**Complementation of *mut5* with CrUbc9**

The genetic lesion produced by insertional mutagenesis with a paromomycin resistance gene in *mut5* involves deletion of nucleotides 9166813-9188745 on chromosome 2 of the *C. reinhardtii* genome, based on the sequence available at Phytozome v. 8 (http://www.phytozome.net/). This includes a complete deletion of the *CrUBC9* gene as well as the complete deletion of four neighboring genes and the deletion of a portion of two genes at the borders of the insertion. Therefore, to document that the lack of SUMOylation and the resulting phenotypes observed in *mut5* are specifically the result of the *CrUBC9* deletion, complementation of *mut5* with a *CrUBC9* transgene was attempted. To achieve this, the inability of *mut5* cells to grow at 37°C was used as a screen for complemented cells. *mut5* cells were co-transformed with plasmids containing the *CrUBC9* transgene (Figure 2-8A) and the *Streptomyces hygroscopicus* aminoglycoside phosphotransferase gene (aph7”) which confers resistance to Hygromycin B. Hygromycin B resistant transformants were spotted on TAP plates and incubated at 37°C to assess their growth. Lines that appeared to grow at 37°C were re-screened in comparison to wild-type and *mut5* controls to confirm their phenotypes.
Figure 2-8. Complementation of mut5 with CrUBC9. (A) Diagram of pGenD-Ubc9.int2 expression cassette. NdeI and EcoRI sites used for cloning CrUbc9 cDNA into pGenD as well as endogenous NcoI and SacII sites used for adding the second intron of the CrUBC9 gene are shown. (B) mut5 lines 1, 4, and 7 putatively complemented with the CrUBC9 gene construct pGenD-Ubc9.int2 were screened for their ability to grow at 37°C. Normalized cell cultures of wild-type, mut5, and complemented lines were spotted on two TAP plates, one of which was incubated at 25°C as a control, the other at 37°C for three days before shifting to 25°C to assess growth. (C) Putatively complemented lines were tested for the ability to SUMOylate proteins in response to 42°C. Cell cultures were shifted to 42°C for one hour and whole cell samples analyzed for SUMOylation by immunoblot analysis with anti-SUMO antibodies. (D) Confirmation of the expression of CrUBC9 in complemented lines by RT-PCR analysis. RNA was isolated from wild-type, mut5, and complemented lines. RT-PCR using CrUbc9 specific primers was used to check for the expression of CrUbc9 transcripts (upper panel). The next to last lane (Ubc9) contains RT-PCR product from a cDNA clone of CrUBC9. Expression of another predicted UBC enzyme, CrUbc3, was used as a control (lower panel). Lanes marked H2O are negative controls with no template RNA added.
A

PsaDp → Ub → c9 → PsaDt

B

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C

<table>
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D

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<th>Ubc9</th>
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<td>Ubc3</td>
<td>H2O</td>
</tr>
</tbody>
</table>
Figure 2-8B shows one such re-screen of three lines transformed with the *CrUBC9* gene construct. On a control plate maintained at 25°C, wild-type, *mut5*, and transformed lines all grow similarly. However, while the vast majority of *mut5* cells die after three days at 37°C, transformed lines showed a restored ability to survive at 37°C, approaching that of wild-type. Lines confirmed positive for growth at 37°C were then screened for their ability to SUMOylate proteins in response to temperature stress (Figure 2-8C). When putatively complemented lines were shifted to 42°C for one hour, immunoblot analysis confirmed the modification of proteins by SUMO in a pattern similar to that of wild-type cells exposed to 42°C. RT-PCR analysis of complemented lines confirmed the expression of *CrUbc9* mRNA (Figure 2-8D), indicating that the transgene is correctly spliced and expressed. Using primers specific for the *CrUbc9* transcript, a 500 bp product was identified in wild-type cells as well as complemented lines, but not in *mut5*. An intron was included in the *CrUbc9* transgene used for transformation to improve expression and enable distinction of RT-PCR products originating from potential genomic DNA contamination and from spliced transgene mRNA template. These results demonstrating complementation of the *mut5* mutant phenotype with a wild type copy of the *CrUBC9* cDNA confirm that CrUBC9 is responsible for SUMOylation of proteins in response to stress, and that this SUMOylation is necessary for survival at 37°C.

Given the fact that complementation of *mut5* with *CrUBC9* largely restored the wild-type phenotype at 37°C, complemented lines were tested to determine if they restored a wild-type phenotype for the other observed phenotypes. When complemented lines were spotted on TAP plates supplemented with 300 mM sorbitol, they showed an increased ability to grow compared to the parental line, *mut5* (Figure 2-9A). This
Figure 2-9. Phenotypes of complemented mut5. (A) Normalized cell cultures were spotted in a 1:4 dilution series on TAP and TAP+300 mM sorbitol plates to assess growth under osmotic stress. (B) Cultures normalized for cell density were spotted in a 1:4 dilution series on TAP and TAP+175 mM NaCl plates to assess growth under high salt stress. (C) Cultures were shifted from TAP to TAP with 8.7mM acetate (-C) for 48 hours and whole cell extracts were analyzed by immunoblot analysis with anti-SUMO antibodies. Reactive Brown stain (lower panel) shows similar loading per lane.
indicates that SUMOylation of proteins in response to sorbitol stress results in an enhanced ability to grow under these conditions. Interestingly, complemented lines showed little to no change in their ability to grow on 175 mM NaCl when compared to mut5 (Figure 2-9B). One complemented line, line #1, showed no growth on TAP+175 mM NaCl, akin to mut5. Lines #4 and #7 showed really weak growth on TAP+175 mM NaCl, nowhere near the growth levels observed for wild-type cells. These results suggest that while SUMOylation is likely partially responsible for the growth of wild-type cells in media with elevated salt, this may reflect differing, non-optimal CrUBC9 expression levels in the transgenic lines or that additional factors are still missing in mut5 lines complemented with the CrUBC9 gene construct that are responsible for growth at elevated salt.

The ability of complemented lines to restore the SUMOylation of proteins in response to carbon (acetate) deprivation was also tested. Two complemented lines, #1 and #4, were selected for carbon deprivation. Whole cell extracts were analyzed for SUMOylation patterns after 48 hours in carbon deprivation. In both lines, the samples showed a SUMOylation pattern similar to that observed in wild-type cells at the same time point (Figure 2-9C). These results confirm that CrUBC9 SUMOylates proteins in response to carbon deprivation.

Localization of the CrUBC9 protein

The ability to complement mut5 provided an opportunity to generate cells expressing the CrUBC9 protein tagged with the red fluorescence protein, mCherry. Initially, the CrUBC9 transgene used for previous complementation studies was fused at
its 3’ end to an mCherry cDNA, but this construct produced no complemented lines, suggesting that the mCherry protein was interfering with the ability of CrUBC9 to SUMOylate target proteins at 37°C. Therefore, a linker region designed to allow both proteins to fold and act independently was introduced. The amino acid sequence glutamic acid-alanine-alanine-alanine-arginine (E-A-A-A-R) repeated four times has been shown to allow the flexible linkage of two proteins (Elrouby and Coupland, 2010). When DNA coding for four tandem repeats of this amino acid sequence (4 X EAAAR) was introduced in-between the CrUBC9 and mCherry coding regions (Figure 2-10A), lines capable of growth at 37°C were recovered but wild-type growth rate was not completely restored, suggesting the mCherry protein might still be interfering with CrUBC9 activity to some degree (Figure 2-10B). In addition to partially restored growth at 37°C, the putatively complemented line #14 showed SUMOylation in response to 42°C (Figure 2-10C), indicating that the fusion protein can function to SUMOylate proteins in response to elevated temperature. Fluorescence microscopy revealed that the mCherry signal for Line #14 localized predominantly to the nucleus, indicating that the functional UBC9-4XEAAAR-mCherry fusion is largely a nuclear protein (Figure 2-11). Under the 42°C stress condition, the localization of the fusion protein did not change, suggesting that the majority of SUMOylation that occurs under abiotic stress may occur in the nucleus. If the mCherry signal was amplified to saturation, a diffuse mCherry signal could be detected outside of the nucleus in an area that did not coincide with chloroplast autofluorescence, suggesting that a small proportion of the Ubc9-4XEAAAR-mCherry protein may reside in the cytoplasm under all stress conditions tested (Figure 2-12).
Figure 2-10. Complementation of mut5 with a CrUBC9-4XEAAAR-mCherry fusion protein. (A) Diagram of the Ubc9-4XEAAAR-mCherry expression cassette. The thin line indicates the intron of CrUBC9. (B) Growth at 37°C of line #14 complemented with the UBC9-4XEAAAR-mCherry fusion at 37°C. Normalized cell cultures were spotted on two identical TAP plates, one of which was incubated at 37°C for three days, the other at 25°C. (C) Immunoblot analysis of UBC9-4XEAAAR-mCherry transformant for SUMOylation under elevated temperature stress. Wild-type, mut5, and line #14 were shifted from 25°C to 42°C for one hour. Whole cell extracts were analyzed by immunoblotting with anti-SUMO antibodies (upper panel). Reactive brown stain (lower panel) shows equivalent loading.
Figure 2-11. CrUBC9 localizes to the nucleus. Fluorescence microscopy of *mut5* cells complemented with CrUBC9-4XEAAAR-mCherry and incubated at 25ºC or 42ºC. Line 14 was shifted to 42ºC and samples analyzed by fluorescence microscopy after 10, 30 and 60 minutes at 42ºC. Row 1 shows *mut5* cells that have not been complemented. Row 2 shows Line 14 cells prior to the shift to 42ºC. Rows 3-5 show Line 14 cells at 10, 30, and 60 minutes after shifting to 42ºC, respectively. Each row shows (from left to right) transmitted light, chloroplast autofluorescence (in false green color), mCherry fluorescence, and a merged image of the chloroplast and mCherry signals. Scale bar shown in the upper right panel is the same as for all panels shown.
<table>
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<th>Transmitted Light</th>
<th>Autofluorescence</th>
<th>mCherry</th>
<th>Merged</th>
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<td>42°C – 30 min</td>
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<td>UBC9-4XEAAAR-mCherry</td>
<td>42°C – 60 min</td>
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</table>

5µm
Figure 2-12. A small portion of the UBC9-4XEAAAR-mCherry fusion protein localizes outside the nucleus. Image is from amplifying the mCherry signal to saturation to detect any weak signal. Arrows indicate mCherry signal that resides diffusely outside the nucleus but does not merge with autofluorescence from the chloroplast, suggesting a cytoplasmic localization. Images were taken from transformant #14 (Figure 2-11) exposed to 42°C stress for 60 minutes.
To confirm that the mCherry signal detected by fluorescence microscopy was indeed functional CrUBC9 fused to mCherry, a second construct was generated in which the mCherry protein fused to CrUBC9 was tagged with the last two exons encoding the C-terminal portion of the CIA5 protein (Figure 2-13A). The latter tag was used because high-titer antibody to the C-terminal domain of CIA5 was available that would allow detection of the chimeric protein on immunoblots of cell extracts. In addition, the strong tandem HSP70/RBCS promoter was used in an attempt to increase protein expression. A single transformant, Line #61, capable of modest growth at 37ºC was obtained after transformation with this construct, but again wild-type growth rate was not completely restored (Figure 2-13B). To confirm that the fusion was both expressed and functional, cell extracts were analyzed by immunoblotting to detect the expression of both the fusion protein (using antibodies against CIA5) and SUMOylation patterns under stress conditions. When immunoblots containing proteins extracted from wild-type, mut5, and Line #61 cells grown 25ºC and 42ºC were probed with anti-Cia5 antibodies, extracts of all three cell types showed a band at ~100 kD corresponding to the endogenous CIA5 protein. However, in Line #61 extracts an additional CIA5 epitope-containing protein was detected, consistent with the predicted size of the 51 kD for the fusion protein (Figure 2-13C, upper panel). Furthermore, immunoblot analysis with anti-SUMO antibodies revealed that Line #61 SUMOylated proteins in a pattern similar to wild-type cells in response to 42ºC heat shock for one hour (Figure 2-13C, lower panel). These results suggest that the UBC9-4XEAAAR-mCherry fusion is both expressed as a full length fusion and is functional. Fluorescence microscopy of Line #61 revealed a weaker, but similarly localized mCherry signal than in Line #14, namely a predominantly nuclear
Figure 2-13. Expression of Ubc9-4XEAAAR-mCherry fusion tagged with the CIA5 epitope. (A) Diagram of the Ubc9-4XEAAAR-mCherry-CIA5ep expression cassette, including the HSP70/RbcSp tandem promoter. Thin line indicates the second intron of the CrUBC9 gene. (B) Growth at 37ºC of complemented cell line 61 transformed with the UBC9-4XEAAAR-mCherry-Cia5ep construct. Cultures normalized for cell density were spotted on two identical TAP plates, one of which was incubated at 37ºC for three days, the other at 25ºC. (C) Immunoblot analysis of UBC9-4XEAAAR-mCherry-Cia5ep transformant #61 for expression of UBC9-mCherry fusion protein and SUMOylation under elevated temperature stress. Wild-type, mut5, and line #61 were shifted from 25 to 42ºC for one hour. Whole cell extracts were analyzed by immunoblotting with anti-Cia5 antibodies (upper immunoblot) and anti-SUMO antibodies (lower immunoblot). (D) Fluorescence microscopy of UBC9-4XEAAAR-mCherry transformant #61. Panels from top to bottom show: Transmitted light, chloroplast autofluorescence (in false green color), mCherry fluorescence, and a merged image of autofluorescence and mCherry. The scale bar shown in the upper right hand corner of the second panel is the same size for all panels.
Ub c9 PsaDtmCherry
HSP70/RbcSp 4XEAAAR Cia5ep
A
B
CC124 mu5 61

25ºC 37ºC

11 2.8 0.7 0.2

Cells (x10^3)

25ºC 37ºC

C

CC124 mu5 61

IB: Anti-Cia5

Reactive Brown

IB: Anti-SUMO

Reactive Brown

D

Transmitted Light

Auto-fluorescence

mCherry

Merge
localization (Figure 2-13D). Together, these data suggest that the bulk of the CrUBC9 protein localizes to the nucleus in *C. reinhardtii* under both stress and non-stress conditions.

**DISCUSSION**

The prior report from this laboratory of a SUMOylation system in *C. reinhardtii* included the identification of a putative SUMO E2 conjugase (CrUBC9) that showed weak *in vitro* SUMOylation activity (Wang et al., 2008). This protein is 63% identical to the yeast ScUBC9, which is essential for cell viability. Therefore, the viability of the *CrUBC9* deletion mutant, *mut5*, described here, raises several intriguing possibilities. Could SUMOylation be dispensible in *C. reinhardtii*? This is unlikely as this post-translational modification has been implicated in many important cellular processes, and in a variety of diverse taxonomic groups SUMO or SUMO pathway mutants are lethal (Seufert et al., 1995; Johnson et al., 1997; Li and Hochstrasser, 1999; Melchior, 2000; Nacerddine et al., 2005; Saracco et al., 2007). There are two reports of viable SUMO mutants in *Schizosaccharomyces pombe* and *Aspergillus nidulans*, however they both show severe growth defects, including reduced cell growth and abnormal cell morphology (al-Khodairy et al., 1995; Wong et al., 2008). The fact that *mut5* cells show no obvious growth differences compared to wild-type cells under standard laboratory conditions at 20°C (Figure 2-5A) suggests they are likely not SUMOylation deficient and raises the more likely possibility that a second, unique SUMO E2 conjugase may exist in *C. reinhardtii* cells.
Heretofore, rice was the only eukaryotic organism known to encode more than one SUMO E2 conjugase: two nearly identical (93%) SCE1 enzymes (Nigam et al., 2008). The original description of the SUMO system in *C. reinhardtii* raised the possibility that the genome could contain as many as four SUMO E2 conjugase enzymes, including CrUBC9, but none of the proposed enzymes is more than 54% identical to CrUBC9 (Wang et al., 2008). In addition, if a second SUMO E2 conjugase is encoded in the *C. reinhardtii* genome, it is obviously not a case of functional redundancy because it was shown here that only CrUBC9 was capable of SUMOylating proteins in response to various abiotic stresses. *mut5* cells exposed to elevated temperature, high salt, high osmotic pressure, and oxidative stress, showed no ability to mount the SUMOylation response to these stresses (Figures 2-1, 2-2, 2-4).

Competition assays provided additional evidence that a second SUMO E2 conjugase likely exists in *C. reinhardtii*, as *mut5* cell extracts were demonstrated to contain constitutively SUMOylated proteins that could be competed away in an immunoblot analysis (Figure 2-3). Therefore, it is likely that SUMOylation as part of normal cell growth and division is fulfilled by a second functional SUMO E2 conjugase in *C. reinhardtii* that allows the constitutive SUMOylation required under normal growth and development in the mutant *mut5*. The ability of *mut5* cells to SUMOylate in a pattern essentially identical to that of wild-type cells under non-stress conditions permitted the discovery and characterization of this conditionally lethal mutation in *C. reinhardtii*.

Exposure of cells to 37°C resulted in the induction of protein modification by SUMO, and the importance of this response was shown by the reduced viability of *mut5* cells at this temperature. In both liquid culture and on solid plates, wild-type cells show
an ability not only to survive, but grow at 37°C (Figure 2-5). In stark contrast, mut5 cells were intolerant to this temperature, with approximately 50% of the cells dying within twenty-four hours after a shift to 37°C (Figure 2-5C). Complementation of mut5 cells with CrUBC9 cDNA restored the ability of cells to grow at 37°C, which confirms that CrUBC9 per se is solely responsible for restoring cell viability at elevated temperatures in this conditionally lethal mutant (Figure 2-8B).

Other phenotypes observed for mut5 cells exposed to abiotic stress included reduced growth on TAP+300 mM sorbitol (Figure 2-6A), and an inability to grow on TAP+175 mM NaCl (Figure 2-6B). Complementation of mut5 cells also nearly restored cells to a wild-type growth capability on 300 mM sorbitol (Figure 2-9A). Interestingly, complemented lines did not show a restored wild-type phenotype on TAP+175 mM NaCl (Figure 2-9B). There are several possible reasons for this observation. One possibility is that the mut5 mutant contains a second site mutation elsewhere in its genome in a gene required for growth at elevated salt conditions. If this were the case, complementation with CrUBC9 would not restore the growth of cells to wild-type levels. A second possible explanation is that our transgene, expressed via the PsaD promoter, is not expressed at a sufficient level to complement this phenotype. Our RT-PCR analysis to assess expression of CrUBC9 mRNA in wild-type and complemented lines actually showed a stronger signal for the complemented lines than for wild-type cells (Figure 2-8D). Although this assay was not quantitative, the amount of RNA used for each RT-PCR reaction was the same, so the increased signal may suggest that CrUBC9 mRNA is more abundant in the complemented. We cannot, however, exclude the possibility that differences may exist in the ability to translate the mRNAs derived from the transgene.
and the endogenous gene due to differing non-coding regions. A third possibility is suggested from salt-sensitive and salt-tolerant mutants isolated in *Arabidopsis*. Mutants of the SUMO proteases OTS1 and OTS2 show increased salt sensitivity in *Arabidopsis* (Conti et al., 2008; Conti et al., 2009) while mutants of the SUMO E3 ligase SIZ1 show increased tolerance to salt stress (Miura et al., 2011). Combined, these data suggest that reduced SUMOylation and/or de-SUMOylation may be important for cellular response to salt stress in plants. If, indeed, as our RT-PCR results suggest, the transgenic *CrUbc9* mRNA is more abundant in our complemented lines than in wild-type cells, this could uncouple the balance between SUMOylation and de-SUMOylation, leading to a phenotype similar to what is observed for the OTS1 and OTS2 protease mutants of *Arabidopsis*.

In addition to abiotic stress, *mut5* cells lacked the SUMOylation response to carbon deprivation (Figure 2-7). SUMOylation in response to nutrient deprivation has been observed before in plants. The *Arabidopsis* SUMO E3 ligase mutant *siz1* shows increased sensitivity to phosphate deprivation (Miura et al., 2005). It has been suggested that the transcription factor essential for the phosphate starvation response, *PHR1*, is SUMOylated, a reaction demonstrated to occur *in vitro* (Miura et al., 2005). While we did not observe changes in SUMOylation under short-term phosphate deprivation in *C. reinhardtii* cells, we did observe a unique SUMOylation pattern that appears in response to carbon deprivation (Figure 2-7). To our knowledge, this is the first identification of carbon deprivation as a stress that induces SUMO modification in a photosynthetic cell. Somewhat analogous SUMOylation responses have also been observed in animals during hibernation torpor, a state of decreased physiological activity in which organs are
exposed to reduced levels of oxygen due to decreased blood flow, and this results in
 dramatic increases in SUMOylation in brain, liver, and kidney tissues (Lee et al., 2006).

The ability to complement mut5 allowed the expression of tagged CrUBC9
constructs to localize the CrUBC9 protein. Complementation of mut5 cells with
constructs expressing C-terminal mCherry fusions to CrUBC9 resulted in cells with a
restored ability to SUMOylate in response to abiotic stress, indicating that the CrUBC9
protein is functional when fused to mCherry in this manner (Figures 2-10, 2-13).
Fluorescence microscopy localized the fusion protein predominantly to the nucleus,
although a very small portion of the tagged protein appeared to localize diffusely to the
cytoplasm (Figures 2-11, 2-12). Shifting cells to 42°C did not change the localization of
CrUBC9, which is consistent with the notion that the majority of SUMO targets in the
cell are nuclear, and this holds true for the SUMOylation activity induced response to
stress. Previously, anti-SUMO antibodies were used to detect SUMO proteins in C.
reinhardtii cells and a predominantly nuclear localization was observed (Wang et al.,
2008). This is consistent with our data that documents the enzyme responsible for
conjugating SUMO to target proteins also localizes predominantly to the nucleus. UBC9
homologs identified in other organisms also localize to the nucleus, and targets for
SUMOylation in response to heat stress are largely nuclear-localized (Rodriguez et al.,
2001; Bruderer et al., 2011).

In summary, CrUBC9 has been shown to be dispensible for cell viability, as
evidenced by the fact that cells with a complete deletion of the CrUBC9 gene(mut5) are
viable under non-stress conditions. However, this E2 enzyme is absolutely essential for
SUMOylation and growth under various stress conditions. Future studies aimed at
identification of the target proteins subjected to SUMO modification under these stress conditions will elucidate more precisely how this ability to survive is achieved.

**MATERIALS AND METHODS**

*Chlamydomonas reinhardtii* strains, growth conditions

Wild-type strain CC124 was obtained from the Chlamydomonas Genetics Center at Duke University (Durham, NC). *mut5* (Δ*ubc9*) is a derivative of CC124 and was the kind gift of Dr. Heriberto Cerutti. Cultures were grown in Tris-acetate phosphate (TAP) media (Harris, 2009) unless otherwise stated.

**Stress treatments**

For heat shock experiments in liquid cultures, 25ml of mid-log phase cells were transferred to a pre-warmed 125ml flask at either 37°C or 42°C as indicated in a pre-warmed rotary shaker with continuous light (30-40μmol s⁻¹ m⁻²). For high salt treatment in liquid, TAP supplemented with 1.75M NaCl was added to liquid culture to a final concentration of 175mM NaCl. For high osmotic pressure treatment in liquid, TAP supplemented with 3M sorbitol was added to liquid cultures to a final concentration of 300mM. For H₂O₂ treatment, H₂O₂ was added to liquid cultures to a final concentration of 2mM.

For growth curves at 20°C and 37°C, wild-type and *mut5* cultures were diluted in TAP media to an OD₆₆₀ between 0.005-0.022 and incubated at either 20°C or 37°C under continuous light (30-40μmol s⁻¹ m⁻²). OD₆₆₀ readings were taken every 24 hours for 72 hours. For growth at 37°C on plates, cultures with normalized cell counts were spotted in
a 1:4 dilution series on TAP plates. After incubation at 37°C for the indicated time, plates were shifted to 25°C for several days to assess growth. Growth was compared to a control plate that was maintained at 25°C. For quantification of cell viability at 37°C, mid-log phase cells were diluted to a density of 400 cells/ml, so that a 250µl aliquot of cells would contain approximately 100 cells. 250µl aliquots of cells from the original dilution, as well as after 24 and 48 hours at 37°C were plated on TAP plates and incubated at 25°C. The number of colonies arising from each 250µl aliquot was counted as a measure of the number of viable cells in each 250µl aliquot. All growth on plates under non-stress (25°C) conditions was in continuous light (100µmol s\(^{-1}\) m\(^{-2}\)).

For cell viability under salt and osmotic stress, normalized cell cultures were spotted in a 1:4 dilution series on TAP and TAP + 175mM NaCl plates, or TAP and TAP+300mM sorbitol plates, respectively. Plates were incubated at 25°C (100 µmol s\(^{-1}\) m\(^{-2}\)) to assess growth.

For carbon deprivation, cultures were harvested at 2,000xg for 5 minutes and washed twice with sterile water. Final cell pellets were resuspended in equal parts TP (TAP media with no acetate) and TAP liquid media resulting in a final concentration of 8.7mM acetate instead of the usual 17.4mM acetate found in full strength TAP media. Cultures were wrapped in aluminum foil to prevent photosynthesis. Control cultures of wild-type cells were resuspended in full strength TAP media and also wrapped in aluminum foil to eliminate light. The density of starting cultures was determined by counting cells with a hemacytometer and by measuring the OD\(_{660}\). The OD\(_{660}\) of each culture was also determined at 24 and 48 hours of nutrient deprivation. The fold increase or decrease in the OD\(_{660}\) compared to that of the initial culture was used to load
equivalent numbers of cells per lane on a bis-Tris SDS-PAGE gel used for immunoblot analysis.

**Immunoblot analysis**

Whole cell extracts were prepared by lysing cells directly in loading buffer. Cell pellets were resuspended in 1/10\textsuperscript{th} volume of loading buffer (50mM Tris-Cl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 0.1% Bromophenol Blue (w/v), 100mM β-mercaptoethanol) and boiled for 4 minutes. Proteins were separated on an 8.5% bis-Tris SDS-PAGE gel in 1X MOPS Buffer (50mM MOPS, 50mM Tris, 1mM EDTA, 0.1% SDS (w/v), 5mM sodium bisulfite). After separation, proteins were transferred to a nitrocellulose membrane using a Trans-Blot® SD Semi-Dry Transfer Cell (20V for one hour) (Bio-Rad, Herculus, CA). After transfer, blots were stained with a solution of 0.05% Reactive Brown in 5% acetic acid to visualize the proteins transferred to the membrane and assess the loading between lanes. The membrane then was treated with 3% (w/v) milk powder in TBS+Tween-20 (500mM NaCl, 100mM Tris-Cl, pH 7.5, 0.05% Tween-20 (v/v) ) for 30 minutes at room temperature prior to incubation in primary antibody overnight at 4°C. After exposure to primary antibody, blots were washed twice in TBS+Tween-20 (TBST) before incubation in secondary horseradish peroxidase (HRP) tagged donkey anti-rabbit antibody (GE Healthcare, Piscataway, NJ) in 1X TBST for one hour at room temperature. After incubation with secondary antibody, blots were washed twice with TBST and once with TBS. Protein bands bound by primary antibodies and adherent horseradish peroxidase (HRP)-labeled antibodies were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The anti-SUMO primary antibody is a polyclonal antibody directed against the
CrSUMO96 protein of *C. reinhardtii* and has been previously described (Wang *et al*., 2008). A 1:1000 dilution of the 3\(^{rd}\) bleed was used for detection of SUMO proteins. Anti-Cia5 antibodies have been previously described (Wang *et al*., 2005) and were used at a 1:10,000 dilution. Secondary antibody for anti-SUMO and anti-Cia5 primary antibodies was HRP-linked donkey anti-rabbit immunoglobulins used at a 1:10,000 dilution.

**Competition assay**

Recombinant CrSUMO96 was overexpressed in *E. coli* and purified as previously described (Wang *et al*., 2008). For production of Chlamydomonas UBIQ1A, total RNA was isolated from CC124 cells using Trizol LS (Invitrogen, Grand Island, NY) according to the manufacturer’s recommendations. UBIQ1A mRNA was amplified from CC124 total RNA using the following primers: (5’- CCCCCATATGCAGATTTTCGTGAAGACCC – 3’), NdeI site underlined and (5’- CCCAGAATTTCAGCCAACGTCCTTCAGC - 3’), BamHI site underlined. UBIQ1A cDNA was cloned into pET-28b using NdeI and BamHI restriction sites. UBIQ1A was overexpressed and purified using the same conditions as noted above for CrSUMO96. Proteins were quantified using Bio-Rad Protein Quantification Kit (Bio-Rad, Hercules, CA).

For assays testing for the specificity or non-specificity of SUMO antibody binding to proteins on immunoblots, proteins in equivalent amounts of extracts of CC124 and *mut5* from both 25°C and one hour at 42°C were separated by electrophoresis on three 8.5% bis-Tris SDS-PAGE gels along with 0.5ng of CrSUMO96 and CrUBIQ1A. Anti-SUMO primary antibodies were pre-incubated in a dry milk blocking solution
alone, blocking solution with 50µg of recombinant CrSUMO96, or blocking solution with 50µg of recombinant CrUBIQ1A for one hour at room temperature prior to applying to one of the blots. After incubation at 4°C overnight in primary antibody solutions, blots were processed as described above for other types of immunoblots.

**Cloning of CrUbc9 and complementation of mut5**

The *UBC9* cDNA (500bp) was amplified by RT-PCR from *C. reinhardtii* RNA using the following primers: (5’– TAAAACATATGATCTGGCGTCGCA –3’) and (5’-AAATGAATTCTCAGGAGGTGC -3’) which added NdeI and EcoRI sites (underlined), respectively. The cDNA was then cloned into the pGenD expression cassette using the NdeI and EcoRI restriction sites (Fischer and Rochaix, 2001). This placed the *UBC9* cDNA under the control of the PsaD promoter. Subsequently, the second intron plus some of the flanking coding sequence of the *CrUBC9* gene was amplified from *C. reinhardtii* genomic DNA using the following primers: (5’ – GAAC-CTGATGAAGTGGAAGTCC –3’) and (5 – TAGATGTTGGGTGGAAGAAGC -3’). The resulting genomic DNA fragment contained an endogenous NcoI site in the coding region flanking the intron at the 5’ end, and an endogenous SacII site in the coding region flanking the intron at the 3’ end. These restriction sites were used to clone the intron into the *CrUBC9* cDNA. The resulting plasmid was named pGenD-Ubc9.int2.

For complementation, mut5 cells were co-transformed with pPsaD-Ubc9.int2 and pHyg3, which confers resistance to Hygromycin B (Berthold *et al.*, 2002), using standard electroporation conditions (Shimogawara *et al.*, 1998). Transformants were selected on medium containing 30µg/ml Hygromycin B. Individual transformants were picked into
100µl of TAP media in the first and fourth rows a 96-well plate and grown 24-48 hours at 25ºC. The second through third and fifth through eighth rows of the same plate were used to generate a 1:4 dilution series of each transformant which was subsequently spotted on two individual TAP plates. One plate was incubated at 25ºC, while the other was incubated at 37ºC for three days. Colonies that showed growth at 37ºC after three days were re-screened in a similar dilution series assay to assess growth of putative complemented cells at 37ºC compared to wild-type and mut5 cells.

Expression of CrUbc9 mRNA was confirmed in complemented lines by RT-PCR using the SuperScript III One-Step RT-PCR System (Invitrogen, Grand Island, NY) and the same primers that were used for the cloning of the CrUBC9 cDNA (above). As a control for the presence of mut5 RNA, a portion of a second predicted UBC transcript, CrUbc3, was amplified using the following primers: (5’- TACGGCCTTGTGGGGAAA-CCATTGACC -3’) and (5’- ACGATCTGCACGATGGGATGCTGG -3’).

**CrUBC9-4XEAAAR-mCherry fusion**

For expression of a CrUBC9-4XEAAAR-mCherry fusion, CrUBC9 cDNA from the pGenD-Ubc9.int2 plasmid was amplified with the following primers: 5’ – GAACCT-GATGAAGTGGAAGTGCC – 3’ and 5’ – TAAAAAGATCTCGAGGGTGTCGC-GGG – 3’ (BamHI site underlined). A segment of DNA encoding four repeats of glutamic acid, alanine, alanine, alanine and arginine (4XEAAAR) was generated by annealing the following primers and filling in with Phusion DNA Polymerase (Thermofisher Scientific) 5’ – TCAAGATCTGGAGGCCGC TGCCCCGCGAGGTGGCTGGCCTGCGGAGGCGGGCTGCGC – 3’ and
5’ – CTGCAG GTGACACTCTAGCGCGGCGCCGACGCTCGCG-GGCAGCCGCCTC – 3’. The resulting double stranded fragment encoded four E-A-A-A-R repeats flanked by in frame BamHI and SaI restriction sites. mCherry cDNA was amplified using the following primers: 5’ – TAAAGTGACGTGAGCAAGGCGAGG – 3’ (SaI site underlined), and 5 – GATGAATTCTTAGTACGTGCTGCTAGCCT-TGTACAGCTCGTC-CATG – 3’ (EcoRI and NheI sites underlined.) The corresponding BamHI sites in the amplified CrUBC9 cDNA and 4XEAAAR fragment and SaI sites in the 4XEAAAR fragment and mCherry cDNA were used to ligate the three fragments together in order to generate the CrUBC9-4XEAAAR-mCherry fusion. The endogenous NeoI site of the CrUBC9 cDNA and EcoRI site of the mCherry cDNA were used to ligate the fusion into pGenD-Ubc9.int2. The resulting plasmid was named pUxM.

The CIA5 epitope tagged version of CrUBC9-4XEAAAR-mCherry was generated as follows. A BspHI (blunted) – KpnI fragment that included the last two exons of CIA5 and the PsaD terminator from the plasmid pGenD-Cia5 was cloned into NheI (blunted) – KpnI cut pUxM. The resulting plasmid was called pUxMC. To hopefully increase the expression of the fusion protein, an HSP70/RBCS tandem promoter was generated by cloning a PsiI – Ascl HSP70p fragment from the plasmid pBS-AphVIII into PsiI-Ascl cut pJR40 (Neupert et al., 2009). The resulting plasmid, pHSP70-JR40 contained an expression cassette with the HSP70/RBCS tandem promoter and the PsaD terminator. The CIA5 epitope tagged Ubc9.int2-4XEAAAR-mCherry transgene from pUxMC was cloned into NdeI-EcoRI cut pHSP70-JR40. The resulting plasmid was called pHUxMC. mut5 cells transformed with either pUxM or pHUxMC
were screened as above for pGenD-Ubc9.int2 using growth at 37°C to initially identify potential complemented lines.

**Confocal Microscopy**

Live images of *C. reinhardtii* were captured using a Nikon A1 confocal imaging system mounted on a Nikon Eclipse 90i microscope with a 100x objective. mCherry and chloroplast signal were acquired sequentially with a 561.5nm excitation and 570-620nm emission and 641nm excitation and 662-737nm emission respectively and pseudocolored for visualization.
CHAPTER 3

Identification of CrUBC3, a possible second, functional SUMO E2 conjugase in

*Chlamydomonas reinhardtii*
INTRODUCTION

The modification of proteins by Small ubiquitin-like modifier (SUMO) in eukaryotes is considered essential for normal cell growth and development (Geiss-Friedlander and Melchior, 2007). In Saccharomyces cerevisiae, mammalian cells, and Arabidopsis thaliana a single gene has been identified that encodes for a SUMO E2 conjugase, the enzyme that catalyzes the formation of an isopeptide bond between SUMO and a lysine residue within a target protein. Deletion mutants in any of these SUMO E2 conjugase genes is lethal, underscoring the fact that SUMOylation is essential for cell viability (Seufert et al., 1995; Yasugi and Howley, 1996; Desterro et al., 1997; Johnson and Blobel, 1997; Nacerddine et al., 2005; Geiss-Friedlander and Melchior, 2007; Saracco et al., 2007). In the fission yeast, Schizosaccharomyces pombe, deletion of the HUS5 SUMO E2 conjugase gene results in a hus5 mutant that is viable but shows severe growth defects (al-Khodairy et al., 1995), which is again consistent with a central role for SUMO modification in normal cell growth. SUMO E2 conjugases are similar in sequence to ubiquitin conjugase enzymes, and initial reports of these genes, prior to the identification of SUMO as a post-translational modification, led to their prediction as ubiquitin conjugating enzymes (UBCs) (al-Khodairy et al., 1995; Seufert et al., 1995).

Translated SUMO protein must be processed by a SUMO protease after a conserved double-glycine (-GG-) motif near the C-terminus of the protein (Li and Hochstrasser, 1999). After this, SUMO is activated in an ATP-dependent reaction and forms a thioester bond with a cysteine residue in a SUMO E1 activase (Johnson et al., 1997). This same first step exists in the ubiquitin conjugation pathway as well. In yeast, a single polypeptide UBA1 forms the entire ubiquitin E1 activase enzyme (McGrath et
In the SUMOylation pathway, two separate polypeptides (AOS1 and UBA2) form a heterodimer to act as an E1 activase (Johnson et al., 1997). AOS1 shows homology to the N-terminal region of UBA1 while UBA2 shows homology to the C-terminal region of UBA1 (Johnson et al., 1997). The E1 activase then transfers the SUMO or ubiquitin to its corresponding E2 conjugase. E2 conjugase enzymes are responsible for catalyzing the conjugation of a SUMO or ubiquitin to a target protein, although in both cases this usually involves the assistance of an E3 ligase (Geiss-Friedlander and Melchior, 2007). E3 ligases act either by stimulating the catalytic activity of the SUMO E2 conjugase or by acting as an adaptor that brings together the target protein and SUMO-bound E2 conjugase (Johnson and Gupta, 2001; Takahashi et al., 2001; Tatham et al., 2005). There is some degree of similarity between ubiquitin and SUMO E3 ligases, although there is one class of ubiquitin E3 ligases, HECT, for which there is no SUMO counterpart. This class of E3 ligases contains a catalytic cysteine which forms an intermediate thioester bond with ubiquitin prior to transfer to the target protein (Dye and Schulman, 2007). RING ligases, however, have been identified for both SUMO and ubiquitin. These include the PIAS-type and SIZ1 SUMO E3 ligases (Johnson and Gupta, 2001; Sachdev et al., 2001; Takahashi et al., 2001; Miura et al., 2005). In addition, a non-RING, non-HECT class of SUMO E3 ligases has been identified, and RanBP2, the E3 ligase for the first discovered SUMO target, RanGAP1, falls into this category (Pichler et al., 2002; Tatham et al., 2005). It is important to note that although there is analogy or even homology between the given components of the two pathways, the SUMOylation and ubiquitination cascades are parallel but distinct. The E2 conjugase enzymes for SUMO cannot form a thioester with ubiquitin, and
similarly ubiquitin E2 conjugases are incapable of forming thioesters with SUMO (Desterro et al., 1997).

Another important distinction between the ubiquitin and SUMO pathways is the number of genes identified for each component in the pathway. Given the vast number of proteins targeted by SUMO or ubiquitin, regulating what proteins are targeted for modification and under what conditions likely requires a great deal of regulation and specificity. The ubiquitin pathway accomplishes this through the use of multiple E2 conjugases and E3 ligases in various combinations. As an example, as many as 25 ubiquitin E2 conjugases have been identified in Arabidopsis thaliana, along with hundreds of potential E3 ligases (Bachmair et al., 2001; Kraft et al., 2005). In stark contrast, only a single SUMO E2 enzyme has been identified in virtually every eukaryotic organism for which the SUMOylation pathway has been characterized (Seufert et al., 1995; Yasugi and Howley, 1996; Saracco et al., 2007). Additionally, in comparison to the hundreds of ubiquitin E3 ligases in Arabidopsis, only three SUMO E3 ligases have been identified in this same organism (Miura et al., 2005; Huang et al., 2009; Ishida et al., 2009). The one exception to the rule of a single SUMO E2 conjugase is in rice, which is predicted to encode two E2 conjugase enzymes (OsSCE1 and OsSCE2) (Nigam et al., 2008). However, the predicted proteins for these genes are 93% identical and are the result of a recent gene duplication (Nigam et al., 2008).

Understanding the specificity and regulation of SUMO E2 conjugases will be critical in beginning to unravel how cells use so few enzymes to modulate such varied responses throughout the cell (Geiss-Friedlander and Melchior, 2007).
The unicellular green alga *Chlamydomonas reinhardtii* appears unique from all other organisms studied to date in that a deletion mutant in the closest SUMO E2 conjugase homolog in this organism (*CrUBC9*) is viable under standard, non-stress laboratory conditions with no obvious growth defects. This mutant, *mut5* (described in Chapter 2), fails to SUMOylate in response to various abiotic stress treatments and shows increased sensitivity to many of those same stress treatments, suggesting that *CrUBC9* is essential for SUMOylation in response to abiotic stress treatment. In particular, the ability of wild-type *C. reinhardtii* cells to grow at 37°C compared to the rapid death of *mut5* cells at this same temperature highlights the critical nature of *CrUBC9* in cell response and survival under stress conditions.

When the SUMOylation system of *C. reinhardtii* was first investigated, four potential SUMO E2 conjugase proteins were proposed, and *CrUBC9* was identified as the best candidate based on its similarity to other known SUMO E2 conjugases as well as demonstration of its weak *in vitro* SUMOylation activity (Wang *et al.*, 2008). It was shown in Chapter 2 that the *C. reinhardtii* mutant *mut5* fails to SUMOylate in response to a number of abiotic stresses, and that this is the direct result of the deletion of the SUMO E2 conjugase gene *CrUBC9*. However, *mut5* grows at a similar rate to wild-type cells under standard laboratory conditions, and immunoblot analysis with anti-SUMO antibodies suggests that there are authentic SUMOylated proteins in *mut5* (Figure 2-3). Collectively, these data suggest that at least one additional SUMO E2 conjugase may exist in the *C. reinhardtii* genome. However, unlike the rice genome, no nearly identical protein to *CrUBC9* can be found in the genome.
One complication of bioinformatic analysis to identify potential SUMO E2 conjugases is the degree of similarity between SUMO- and ubiquitin-pathway components. To identify which other proteins in the genome might be additional SUMO E2 conjugases, a bioinformatics approach was carried out to identify distinguishing features between SUMO and ubiquitin conjugase amino acid sequences.

We show here that a single additional gene, *CrUBC3*, likely codes for a second SUMO E2 conjugase in *C. reinhardtii*. *CrUBC3* is one of the three additional genes initially proposed as potential SUMO E2 conjugase enzymes (Wang *et al.*, 2008), but our present analysis indicates that the other two are more likely ubiquitin conjugases. *CrUbc3* and *CrUbc9* mRNAs are transcriptionally regulated in a distinct and opposite manner in response to abiotic stress, with *CrUbc9* transcript levels increasing upon a shift from 25 to 42°C, and *CrUbc3* transcript levels decreasing. This is consistent with our observations that CrUBC9 plays an important role in stress response, for which CrUBC3 cannot functionally substitute. While the SUMO E2 conjugase enzymes from many other organisms have been shown to functionally complement for the ScUBC9 protein in *S. cerevisiae*, neither CrUBC9 nor CrUBC3 could functionally complement for ScUBC9 in this yeast. Attempts to knockout ScUBC9 protein when either CrUBC9 or CrUBC3 were expressed in wild-type yeast cells resulted in what appear to be major chromosomal rearrangements to maintain the endogenous ScUbc9. This inability to functionally substitute for ScUBC9 suggests that both CrUBC9 and CrUBC3 act in a specialized manner within the cell compared to the SUMO E2 conjugase enzymes of other organisms, and that CrUBC9 and CrUBC3 likely perform unique, non-overlapping functions in *C. reinhardtii*. Finally, a search for CrUBC3 homologs to identify if this
phenomenon of distinct SUMO E2 enzymes is conserved in other organisms identified a putative homolog only in the genome of *Volvox carteri*, a green alga, suggesting this might be a highly specialized adaptation for members of the Volvocales.

**RESULTS**

**Identification of distinguishing features between SUMO and Ubiquitin E2 conjugases**

Earlier analysis of the *C. reinhardtii* genome suggested there may be as many as four SUMO E2 conjugases encoded in the genome (Wang *et al.*, 2008). Problematic to such an analysis is that SUMO and ubiquitin E2 conjugase enzymes can be similar in sequence, and, importantly, in all other organisms studied to date only a single essential SUMO E2 conjugase exists (Melchior, 2000), with the exception of two nearly identical genes encoding SUMO E2 conjugases in the rice genome (Nigam *et al.*, 2008). However, no deduced protein with such high similarity to CrUBC9 can be found in the *C. reinhardtii* genome. In an effort to identify the best possible candidate for a second SUMO E2 conjugase in *C. reinhardtii*, additional bioinformatic analysis was undertaken to potentially identify features that could distinguish a SUMO E2 conjugase from an ubiquitin E2 conjugase.

First, a multiple sequence alignment with confirmed SUMO and ubiquitin E2 conjugase enzymes across multiple phylogenetic groups was generated to identify those residues conserved uniquely in SUMO or ubiquitin conjugases versus those shared by both SUMO and ubiquitin conjugases. Three categories of residues within the alignment were identified: (1) those residues that are strictly conserved among both SUMO E2 conjugases AND ubiquitin conjugases; (2) those residues that are 100% conserved among
ubiquitin conjugases and for which no SUMO E2 conjugase has the same amino acid at that position; and (3) those residues that are 100% conserved among SUMO E2 conjugases and for which no ubiquitin conjugases share the same amino acid at that position. The reasoning behind identifying these three categories of residues was that the residues belonging to the first category would be residues important for the common enzymatic activity between SUMO and ubiquitin E2 conjugases, namely formation of an isopeptide bond between the C-terminus of a SUMO or ubiquitin protein and the ε-amino group of a target lysine residue. This includes the catalytic cysteine residue that forms a thioester bond with either SUMO or ubiquitin (Wu et al., 2003). Those residues belonging to categories 2 and 3 would reflect amino acids important for either recognizing or reacting specifically with ubiquitin or SUMO, respectively.

This original alignment (Figure 3-1) produced 17 residues that were strictly conserved across all SUMO and ubiquitin E2 conjugases (Category 1 residues, shown in green), seven residues that were uniquely and 100% conserved across all ubiquitin conjugases (Category 2 residues, shown in blue), and 25 residues that were uniquely and 100% conserved across all SUMO E2 conjugases (Category 3 residues, shown in red). Uniquely and strictly conserved residues were defined as those residues for which all of the residues at that position for one type of E2 conjugase were 100% conserved in the alignment, and for which none of the residues at that same position for the other type of E2 conjugase shared the same residue.

The amino acid sequence for the *S. cerevisiae* ScUBC9 protein was used in a BLASTp search of the *C. reinhardtii* genome to look for potential SUMO E2 conjugase homologs. Twenty-two homologs to ScUBC9 were identified and aligned
Figure 3-1. Alignment used for determining strictly conserved residues in SUMO
and/or ubiquitin E2 conjugases. Protein sequences were aligned using ClustalW. Red
highlighted residues are uniquely and 100% shared by SUMO E2 conjugase enzymes.
Blue highlighted residues are uniquely and 100% shared by ubiquitin conjugases. Green
highlighted residues are shared by both SUMO and ubiquitin conjugases.
with the sequences from the original alignment to determine how many residues they contained from each category. The results of these analyses are shown in Table 3-1. CrUBC9 (Cre02.g142000.t1.1), which was previously demonstrated to be the SUMO E2 conjugase responsible for stress-dependent SUMOylation in *C. reinhardtii*, and the gene deleted in *mut5*, was most consistent with the SUMO consensus that had been identified (23 of the 25 Category 3 residues identified were present in CrUBC9). In addition, CrUBC9 contained all of the Category 1 residues that are 100% conserved across all SUMO and ubiquitin conjugases, and none of the ubiquitin conjugase consensus residues that had been identified (Category 2 residues), which would again be consistent with CrUBC9 being a functional SUMO E2 conjugase. Only one additional protein identified in the BLASTp results, Cre03.g167000.t1.1, had more than half of the 25 SUMO consensus residues (Category 3), all of the 17 shared consensus residues (Category 1), and none of the seven ubiquitin consensus residues (Category 2). This protein, Cre03.g167000.t1.1, therefore is the likeliest candidate to be the other functional SUMO E2 conjugase present in *C. reinhardtii*.

Previously, four potential SUMO E2 conjugases had been identified in the genome of *C. reinhardtii* (Wang *et al.*, 2008). All four of these proteins were identified in the BLASTp search of the *C. reinhardtii* genome with the ScUBC9 amino acid sequence (Table 3-1), including both CrUBC9 (Cre02.g142000.t1.1) and Cre03.g167000.t1.1 described above. In addition, the other two previously proposed SUMO E2 conjugases included Cre16.g693700 and Cre06.g292800 (Table 3-1). Cre02.g142000 and Cre03.g167000 are the corresponding transcript names for CrUBC9 and the best SUMO E2 conjugase candidate identified above. An expanded alignment...
Table 3-1. Comparison of putative SUMO E2 conjugases in *C. reinhardtii* to consensus residues uniquely identified in other SUMO E2 conjugases. Protein sequences coded by the transcripts shown were individually aligned with the proteins shown in the alignment in Figure 3-1 containing multiple SUMO and ubiquitin conjugase enzyme sequences to identify what residues it contained at consensus sites. The E-value corresponds to the E-value given for each transcript when the ScUbc9 sequence was used as a query in a BLASTp search of the *C. reinhardtii* genome. Category 1 refers to those residues that are 100% conserved across all E2 enzymes (both SUMO and ubiquitin). Seventeen residues belonging to this category were identified in the original alignment. Category 2 refers to those residues that are 100% conserved in ubiquitin E2 conjugases only, and for which no SUMO E2 conjugase has the same residue at that position. Seven residues were identified belonging to this category in the original alignment. Category 3 residues refers to those residues that are 100% conserved in SUMO E2 conjugases only, and for which no ubiquitin conjugase has the same residue at that position. Twenty-five residues belonging to this category were identified in the initial alignment.
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<th>Category 2 (Ubiquitin-only Residues)</th>
<th>Category 3 (SUMO-only Residues)</th>
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</tr>
<tr>
<td>Cre01.g027200.t1.1</td>
<td>2.2e-10</td>
<td>17</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Cre12.g510300.t1.2</td>
<td>3.6e-9</td>
<td>11</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Cre08.g372400.t1.1</td>
<td>6.2e-8</td>
<td>9</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Cre19.g753050.t1.1</td>
<td>2.0e-7</td>
<td>7</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Cre04.g212401.t1.1</td>
<td>1.3e-6</td>
<td>12</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Cre07.g342506.t1.1</td>
<td>1.36e-4</td>
<td>9</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Cre16.g693150.t1.2</td>
<td>1.44e-4</td>
<td>10</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
that includes the 11 sequences from the original alignment used to define the shared, ubiquitin-only, and SUMO-only consensus residues (Figure 3-1) as well as the four previously predicted SUMO E2 conjugases from *C. reinhardtii* is shown in Figure 3-2, and the results of this alignment are summarized in Table 3-2. Again, Cre02.g142000 (CrUBC9) and Cre03.g167000 have 23 and 17 of the SUMO consensus residues, respectively, in the expected alignment, and none of the ubiquitin consensus residues. Conversely, the other two previously suggested E2 conjugases have either none (Cre16.g693700) or just one (Cre06.g292800) of the SUMO consensus residues identified in the alignment. In addition, these latter two proteins have six and five of the seven ubiquitin consensus residues, respectively, identified in the alignment. Three of the four predicted proteins (Cre02.g142000 (CrUBC9), Cre03.g167000, and Cre16.g693700) have all 17 of the shared consensus residues in the alignment, while Cre06.g292800 has 15 of the 17. Combined, these results provide strong suggestive evidence that while Cre03.g167000 is likely a second SUMO E2 conjugase in *C. reinhardtii*, Cre16.g693700 and Cre06.g292800 likely code for ubiquitin conjugases or perhaps for other E2 conjugases that conjugate additional ubiquitin-like proteins (Miura and Hasegawa, 2010). In version 4 of the *Chlamydomonas* genome (http://genome.jgi-psf.org/Chlre4/Chlre4.info.html), Cre02.g142000 is annotated as UBC9, and Cre03.g167000 as UBC3. Hereafter, these proteins will be referred to by these two annotated names.

**Real-time RT-PCR analysis of CrUBC9 and CrUBC3**

Because a deletion mutant (*mut5*) of *CrUBC9* fails to accumulate SUMOylated proteins in response to stress, it would appear that if CrUBC3 is also a functional SUMO
Figure 3-2. Alignment of four predicted SUMO E2 conjugases in *C. reinhardtii* with 11 known ubiquitin and SUMO E2 conjugases from other organisms. Protein sequences were aligned using ClustalW. Red highlighted residues are uniquely and 100% shared by SUMO E2 conjugase enzymes. Blue highlighted residues are uniquely and 100% shared by ubiquitin conjugases. Green highlighted residues are shared by both SUMO and ubiquitin conjugases.
Table 3-2. Summary of shared residues between known SUMO and ubiquitin conjugases and the four previously proposed SUMO E2 conjugases in *Chlamydomonas*. Total number of residues for the canonical ubiquitin and SUMO E2 conjugase patterns are shown in the first two rows. The number of residues for each consensus was determined based on the alignment shown in Figure 3-2.
<table>
<thead>
<tr>
<th>Locus Name</th>
<th>Shared Consensus</th>
<th>Ubiquitin-only Consensus</th>
<th>SUMO-only Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin Consensus</td>
<td>17</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>SUMO Consensus</td>
<td>17</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Cre02.g142000.t1.1 (CrUBC9)</td>
<td>17</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Cre03.g167000.t1.1 (CrUBC3)</td>
<td>17</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>Cre16.g693700.t1.1</td>
<td>17</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Cre06.g292800.t1.1</td>
<td>15</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>
E2 conjugase it fails to operate under stress conditions. For an initial comparison of CrUBC9 and CrUBC3, the relative expression levels of their corresponding transcripts were analyzed in wild-type cells under stress and non-stress conditions. Real-time qRT-PCR of cDNA isolated from wild-type CC124 at 25°C and after shifting to 42°C for one hour revealed that under non-stress conditions, CrUbc9 transcript is more abundant than CrUbc3 transcript levels (Figure 3-3). When equivalent amounts of cDNA were used in qPCR reactions (technical triplicate of biological triplicate) the average Ct value for CrUbc9 transcripts under non-stress conditions was 27.7±0.269 while the average Ct value for CrUbc3 was 32.4±0.467. As a reference, the normalizing gene used in these reactions was the G-Protein transcript which had an average Ct value of 20.9±0.328. If these Ct values are used to estimate the relative transcript abundance of CrUbc3 compared to that of CrUbc9, it suggests that under normal growth conditions, CrUbc3 transcript is likely expressed at approximately 6% of the level that CrUbc9 transcript is expressed (Figure 3-3). A shift to 42°C resulted in a modest increase in the expression level of CrUbc9, such that when normalized to G-Protein, an approximately 2.7-fold increase in CrUbc9 transcript levels was detected. In contrast, the shift to 42°C resulted in a marked down-regulation of CrUbc3 transcripts to approximately 2% of their levels under non-stress conditions (Figure 3-3). One caveat here is that the starting Ct values for CrUbc3 were already quite high, so the average Ct value of CrUbc3 transcripts was 37.5±0.738 which is nearing the maximum number of cycles, 40. However, these data are consistent with the hypothesis that CrUBC9 is involved in stress response and adaptation, whereas CrUBC3 is not. In addition, the fact that the two transcripts have
Figure 3-3. qRT-PCR analysis of *CrUbc9* and *CrUbc3*. RNA isolated from wild-type *C. reinhardtii* cultures before and after a shift to 42ºC was used as template for a qRT-PCR analysis using primers specific for *CrUbc3* and *CrUbc9*. The reference gene *G-Protein* was used, and its expression level did not change in response to 42ºC. Expression levels for each transcript are normalized to the levels of *CrUbc9* in one technical replicate in order to compare the relative transcript abundance under non-stress (25º) conditions among transcript levels based on their near identical PCR amplification efficiencies. Error bars indicate the standard deviation among biological replicates.
separate, opposite regulation in response to stress also suggests they may carry out distinct functions within the cell.

Neither CrUBC9 nor CrUBC3 cannot functionally substitute for ScUBC9 in *S. cerevisiae*.

The possibility that two distinct proteins might code for SUMO E2 conjugase enzymes in *C. reinhardtii* is intriguing, as the presence of two unique SUMO E2 conjugases in the same organism has not been previously reported. CrUBC3 was identified as the likeliest possible candidate for a second E2 conjugase in *C. reinhardtii* (see above), and the functionality of this enzyme as a SUMO E2 conjugase was investigated by testing its expression in *S. cerevisiae*. A temperature-sensitive mutant of the ScUBC9 gene in yeast, Y0174 (*ube9*), fails to grow at 37°C, and complementation of this phenotype by the heterologous expression of UBC9 homologs from other organisms has been used to identify functional SUMO E2 conjugase enzymes (Yasugi and Howley, 1996; Ohsako and Takamatsu, 1999). In an attempt to confirm the SUMO E2 conjugase activity of both CrUBC9 and CrUBC3, the ability of these proteins to complement the temperature-sensitive defect of Y0174 was tested. Expression of either CrUBC9 or CrUBC3 failed to complement the 37°C growth defect in Y0174 (data not shown).

Because normal growth temperature for *S. cerevisiae* cells is 30°C, a method to test the ability of the putative SUMO E2 conjugases from *C. reinhardtii* to functionally substitute for ScUbc9 at this lower temperature was designed. *CrUBC9* and *CrUBC3* cDNAs under the control of the yeast GPD promoter were introduced into wild-type yeast on autonomously replicating plasmids. After introduction of either 6XHIS-tagged CrUBC9 or 6XHIS-tagged CrUBC3, disruption of the endogenous *ScUBC9* gene was attempted.
using the LEU2 selectable marker flanked by sequence homologous to the endogenous gene. Since \textit{ScUBC9} is an essential gene in yeast (Seufert \textit{et al.}, 1995), knockout should only be feasible when another functional SUMO E2 conjugase is expressed. As shown in Figure 3-4, introduction of 6XHIS-CrUBC9 appeared to allow the endogenous \textit{ScUBC9} gene to be disrupted. The disruption of the \textit{ScUBC9} gene by homologous recombination with an LEU2 marker was demonstrated by PCR with primers that flanked either side of the LEU2 insertion site into the yeast \textit{ScUBC9} gene. Each set of primers (HR PCR#1 and #2) included one primer that annealed upstream or downstream of the \textit{ScUBC9} gene and a corresponding primer in the reverse orientation that annealed within the LEU2 marker. True disruption lines should show PCR products of 1.2kb and 1.3kb for the upstream and downstream primer sets, respectively (Figure 3-4, middle and lower panels). A control PCR of the endogenous \textit{ScUBC9} gene (from start codon to stop codon) was performed to look for the absence of the endogenous \textit{ScUBC9} gene (Figure 3-4, upper panel). When 6XHIS-CrUBC9 was expressed in Y0002 cells, a disruption line with apparently no detectable endogenous \textit{ScUBC9} gene was identified, and the corresponding PCR products for the homologous recombination event were amplified, suggesting that the \textit{ScUBC9} gene had, indeed been knocked out (Figure 3-4, left panels). It should be noted that this line was identified in a screen of dozens of transformants, suggesting that if successful homologous recombination occurred, it was with very low frequency. When 6XHIS-CrUBC3 was expressed in Y0002 cells, lines that amplified PCR products consistent with homologous recombination that would disrupt the \textit{ScUBC9} gene could be identified (Figure 3-4, right panels). However, a product for the endogenous \textit{ScUBC9} could still be detected as well. As these cells should be haploid, the
Figure 3-4. PCR analysis of possible *ScUBC9* disruptions. DNA isolated from potential disruption lines as well as wild-type cells and parental Y0002 lines expressing either 6XHIS-CrUBC9 or 6XHIS-CrUBC3 was used as template for PCR reactions using primers designed to amplify either the endogenous *ScUBC9* gene (upper panels), the left border after homologous recombination between the LEU2 marker and *ScUBC9* genomic DNA (middle panels), or the right border after homologous recombination between the LEU2 marker and *ScUBC9* genomic DNA (lower panels).
Y0002
6XHIS-CrUBC9
6XHIS-CrUBC9:Δubc9
Y0002
6XHIS-CrUBC3
6XHIS-CrUBC3:Δubc9 #1
6XHIS-CrUBC3:Δubc9 #2
H₂O

ScUBC9
HR PCR#1
HR PCR#2
DNA giving rise to this product cannot be in its endogenous location, as the PCR results using primers to detect homologous recombination clearly demonstrate that the LEU2 marker inserted into the genome at the \textit{ScUBC9} gene (Figure 3-4, right panel).

Therefore, the disruption of \textit{ScUBC9} could not be confirmed in these lines. As neither \textit{CrUBC9} nor \textit{CrUBC3} could complement the temperature-sensitive \textit{ubc9} mutant in Y0174, it was hypothesized that disruption lines would be incapable of growth at 37°C. To that end, 1:4 dilutions of potential disruptions were spotted on YPD plates and incubated at 25, 30, or 37°C to assess their ability to grow. The \textit{ScUBC9} disruption line in which 6XHIS-\textit{CrUBC9} was expressed (6XHIS-\textit{CrUBC9}:\textit{Δubc9}) showed similar growth at 25°C and 30°C, but failed to grow at 37°C, consistent with our hypothesis (Figure 3-5). Interestingly, the \textit{ScUBC9} disruption lines generated when 6XHIS-\textit{CrUBC3} was expressed, which still show an amplification product consistent with the \textit{ScUBC9} gene, also fail to grow at 37°C. In order to confirm whether or not true disruption lines were achieved, Southern Blot analysis of disruption lines was carried out after digesting genomic DNA of wild-type, parental, and disruption lines with enzymes that should produce a unique pattern of bands for the endogenous \textit{ScUBC9} gene and the \textit{ScUBC9} gene after disruption with the LEU2 marker when probed with \textit{ScUBC9} cDNA (Figure 3-6). Surprisingly, Southern analysis indicated that not only was a fragment of DNA consistent with the endogenous \textit{ScUBC9} gene present in potential disruption lines expressing \textit{CrUBC3}, DNA consistent with the endogenous \textit{ScUBC9} gene was also present in the potential disruption line expressing \textit{CrUBC9}. Specifically, digestion of genomic DNA with the enzyme KpnI should produce a band of size 3069 bp that hybridizes to \textit{ScUBC9} cDNA probe after homologous recombination to disrupt the
Figure 3-5. Growth tests of potential ScUBC9 disruption lines. Yeast cells were spotted in a 1:4 dilution series on YPD plates and incubated at 25, 30, or 37°C to assess their ability to grow at these temperatures.
Figure 3-6. Southern analysis of potential ScUBC9 disruption lines. (A) The predicted cut sites relative to the endogenous wild-type gene or post-homologous recombination event (post-HR) are shown. Black boxes indicate ScUBC9 sequence to which the ScUBC9 DIG-labeled probe should hybridize. (B) DNA isolated from potential knockout lines as well as wild-type cells and parental Y0002 lines expressing either 6XHIS-CrUBC9 or 6XHIS-CrUBC3 were digested with KpnI and ScaI and separated on a 0.8% 0.5X TBE gel to separate DNA fragments. After transfer to a positively charged nylon membrane, DNA fragments were probed with DIG-labeled full length ScUBC9 cDNA.
A

3047bp

Scal KpnI Scal
Wild-type

3069bp

Scal KpnI Scal

5691bp

KpnI ScaI

Post-HR

LEU2

B

Y0002 6XHIS-CrUBC9 6XHIS-CrUBC3 6XHIS-CrUBC3∆ubc9 #1 Y0002 6XHIS-CrUBC9 6XHIS-CrUBC3 6XHIS-CrUBC3∆ubc9 #1

3047bp 5691bp 3069bp

KpnI ScaI

bp (x 1000)

20 10 7 5 4 3 2

KpnI Scal
ScUBC9 gene with the LEU2 marker, and a band of this size is produced exclusively in the potential disruption lines. However, digestion of genomic DNA with ScaI should produce bands of size 3047 bp and 5691 bp that hybridize with ScUBC9 cDNA probe in wild-type and post-homologous recombination genomic DNA, respectively. In wild-type (Y0002) genomic DNA and parental lines expressing 6XHIS-CrUBC9 or 6XHIS-CrUBC3, the correct band of 3047 bp is detected with the ScUBC9 cDNA probe. However, in potential disruption lines expressing either 6XHIS-CrUBC9 or 6XHIS-CrUBC3, both a band of 3047 bp and a larger band of presumably 5691 bp is detected.

The infrequency with which potential knockout lines were obtained, combined with these Southern results, suggest that neither CrUBC9 nor CrUBC3 can functionally substitute for ScUBC9 and that major chromosomal rearrangements occur to maintain the endogenous ScUBC9 gene when homologous recombination to disrupt the ScUBC9 gene occurs. The failure of CrUBC9 to functionally substitute for ScUBC9 is particularly surprising given its strong sequence similarity to ScUBC9 compared to other proteins that have successfully complemented the *ubc9* temperature-sensitive mutant. Table 3-3 shows a comparison of the percents identity and similarity of CrUBC9 and CrUBC3 to ScUBC9 compared to three other known SUMO E2 conjugases that have previously substituted functionally for the ScUBC9 protein. In fact, CrUBC9 is the most identical and similar to ScUBC9 in terms of amino acid sequence, so the observation that it cannot functionally substitute for ScUBC9 where other less similar/identical proteins can is unusual. This perhaps suggests that there are a few specific differences between CrUBC9 and other known SUMO E2 conjugases that prevent it from being able to function as the sole SUMO E2 conjugase in a heterologous system.
Table 3-3. Comparison of the percents identity and similarity of CrUBC9, CrUBC3, and other SUMO E2 conjugases to ScUBC9. SUMO E2 conjugase sequences were aligned with ScUBC9 using the NCBI BlastP for aligning two or more sequences (BLASTp (bl2seq)) and the predicted percent identity and percent similarity between ScUBC9 and each given protein sequence is given in the table. Proteins are sorted from most to least similar/identical.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Percent Identity to ScUBC9</th>
<th>Percent Similarity to ScUBC9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CrUBC9</td>
<td><em>C. reinhardtii</em></td>
<td>63%</td>
<td>81%</td>
</tr>
<tr>
<td>HUS5</td>
<td><em>S. pombe</em></td>
<td>62%</td>
<td>80%</td>
</tr>
<tr>
<td>SCE1</td>
<td><em>A. thaliana</em></td>
<td>60%</td>
<td>75%</td>
</tr>
<tr>
<td>hUBC9</td>
<td><em>H. sapiens</em></td>
<td>56%</td>
<td>75%</td>
</tr>
<tr>
<td>CrUBC3</td>
<td><em>C. reinhardtii</em></td>
<td>53%</td>
<td>71%</td>
</tr>
</tbody>
</table>
Identification of CrUBC3 homologs in other eukaryotic organisms.

Given the likely identification of a second, unique SUMO E2 conjugase in *Chlamydomonas*, we sought to determine if this phenomenon exists in other organisms. The amino acid sequence for CrUBC3 was used in a BLASTp search against the NCBI database to identify potential homologs. The top five homologs were hypothetical or predicted proteins from various other photosynthetic organisms and are shown in Table 3-4. When the identified homologs were aligned with CrUBC3 and CrUBC9, all but one showed more identity to CrUBC9 than CrUBC3, suggesting that these are not true CrUBC3 homologs but simply SUMO E2 conjugase homologs. The one exception to this was a predicted protein from *Volvox carteri*, a green alga closely related to *C. reinhardtii*. The *V. carteri* homolog (Accession number XP_002955932) was 69% identical to CrUBC3 but only 53% identical to CrUBC9. In addition, a similar BLASTp search against the NCBI database with CrUBC9 amino acid sequence identified a separate *V. carteri* protein (Accession number XP_002954400) which was 93% identical to CrUBC9. Together, this suggests that the presence of two distinct SUMO E2 conjugases is conserved in *Volvocales*, but perhaps not outside of this phylogenetic group. Interestingly, alignment of these two *V. carteri* protein sequences in the original alignment used to distinguish SUMO and ubiquitin conjugases (Figure 3-7) reveals that the CrUBC3 and CrUBC9 homologs in *V. carteri* appear to share the same pattern of residues for the SUMO-only consensus, ubiquitin-only consensus, and shared consensus. Specifically, the CrUBC3 homolog in *V. carteri* has 16 of the 17 SUMO E2 conjugase consensus residues that CrUBC3 has, and is also different from the consensus at the 8 residues at which CrUBC3 differs from the SUMO consensus. The *V. carteri* UBC3
Table 3-4. Potential homologs to CrUBC3. CrUBC3 amino acid sequence was queried against the NCBI database for potential homologs, and the top five hits are shown below. The E-value corresponds to the E-value reported for each hit when CrUBC3 was used as the query. The % identity between each protein and either CrUBC3 or CrUBC9 is shown.
<table>
<thead>
<tr>
<th>Organism Name</th>
<th>Accession Number</th>
<th>E-value</th>
<th>% Identity to CrUBC3</th>
<th>% Identity to CrUBC9</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Volvocar</em>teri f. nagariensi* (green alga)</td>
<td>XP_002955932</td>
<td>5e-72</td>
<td>69%</td>
<td>53%</td>
</tr>
<tr>
<td><em>Physcomitrella patens subsp. patens</em> (moss)</td>
<td>XP_001768256</td>
<td>1e-57</td>
<td>54%</td>
<td>68%</td>
</tr>
<tr>
<td><em>Picea sitchensis</em> (evergreen)</td>
<td>ABK23228</td>
<td>2e-57</td>
<td>54%</td>
<td>69%</td>
</tr>
<tr>
<td><em>Vitis vinifera</em> (grape vine)</td>
<td>XP_002284949</td>
<td>2e-57</td>
<td>54%</td>
<td>68%</td>
</tr>
<tr>
<td><em>Elaeis guineensis</em> (oil palm)</td>
<td>ACF06574</td>
<td>2e-57</td>
<td>54%</td>
<td>69%</td>
</tr>
</tbody>
</table>
Figure 3-7. Alignment of CrUBC9 and CrUBC3 homologs in *V. carteri* with known SUMO and ubiquitin conjugases. Protein sequences were aligned using ClustalW. Red highlighted residues are uniquely and 100% shared by SUMO-only E2 conjugase enzymes. Blue highlighted residues are uniquely and 100% shared by ubiquitin-only E2 conjugases. Green highlighted residues are shared by both SUMO and ubiquitin conjugases.
sequence appears to be slightly truncated at the N-terminus compared to other known SUMO E2 conjugase sequences, resulting in one of the 25 residues occurring in the alignment prior to the start of the *V. carteri* UBC3 amino acid sequence. Overall, however, there is a remarkable amount of agreement between CrUBC3 and its *V. carteri* homolog in terms of the SUMO E2 conjugase consensus residues that they share. Similarly, CrUBC9 and its *V. carteri* homolog share all 23 of the SUMO consensus residues initially identified as conserved in CrUBC9 and in addition the *V. carteri* CrUBC9 homolog lacks the same two SUMO consensus residues that CrUBC9 lacks in the alignment. Together, these data suggest not only that CrUBC3 and CrUBC9 homologs have been identified in the green alga *V. carteri*, but that the SUMO consensus residues that differ for CrUBC3 and CrUBC9 from the proposed consensus alignment are important in separating the two proteins into unique enzymes that presumably carry out non-overlapping functions within the cell.

**DISCUSSION**

The bioinformatic analysis of known SUMO and ubiquitin E2 conjugase enzymes presented here identified several key residues that were used to distinguish between a SUMO E2 conjugase and an ubiquitin E2 conjugase (Figure 3-1). These residues included 17 amino acids that were 100% conserved between both SUMO and ubiquitin conjugases. One of these 17 residues was the catalytic cysteine that forms a thioester with SUMO or ubiquitin (Geiss-Friedlander and Melchior, 2007). In addition, a His-Pro-Asn (HPN_ triad of amino acids was also strictly conserved among all SUMO and ubiquitin conjugases analyzed. The asparagine that makes up part of this triad has been shown to be critical for catalysis by E2 enzymes (Wu *et al.*, 2003). The remaining 13
residues are likely necessary to perform the chemistry associated with forming an isopeptide bond between the C-terminus of a SUMO or ubiquitin protein and the ε-amino group on a target lysine, as that is the shared activity between these two classes of E2 enzymes.

Perhaps unsurprisingly, more than twice as many residues were identified that were uniquely and 100% conserved among SUMO E2 conjugases compared to those residues that were uniquely and strictly conserved among ubiquitin conjugases. Given that dozens of E2 enzymes that conjugate ubiquitin are present in most species (Bachmair et al., 2001; Kraft et al., 2005) while only a single E2 for SUMO has been identified in most organisms, fewer strictly conserved residues among ubiquitin conjugases is likely to be expected.

The 25 residues identified as uniquely and 100% conserved among SUMO E2 conjugases include two residues in tandem (DG) that are part of a five amino-acid insertion that was identified as unique between UBC9 and other UBC enzymes when UBC9 was still thought of as a likely ubiquitin conjugase (Tong et al., 1997). CrUBC9 shares these same two residues as part of a five amino acid insertion as well, consistent with its activity as a SUMO E2 conjugase. The only other potential SUMO E2 conjugase identified in our bioinformatic analysis that had the same conserved five amino acid insertion at that position was CrUBC3, which was also the only other sequence to have all 17 of the shared consensus residues, none of the ubiquitin-only consensus residues, and more than half of the 25 SUMO-only consensus residues (Figure 3-2, Table 3-1). These findings suggest that CrUBC3 may be a second functional SUMO E2 conjugase in C. reinhardtii. It is important to note that neither CrUBC9 nor CrUBC3 conformed
100% to the SUMO-only consensus that was identified, as CrUBC9 shares just 23 of the 25 consensus residues, and CrUBC3 just 17 (Table 3-2). What could distinguish these UBC9 enzymes from those of other organisms is that neither has to function under all conditions to which a cell might be exposed. Given our results with regards to the stress phenotypes of mut5 (Chapter 2), CrUBC3 clearly does not function under stress conditions, and similarly CrUBC9 likely does not function to SUMOylate as part of the typical cell cycle of *C. reinhardtii*.

The fact that the ∆CrUBC9 mutant mut5 fails to SUMOylate in response to abiotic stress suggests that if CrUBC3 is a SUMO E2 conjugase it acts on targets distinct from CrUBC9 and likely is not involved in abiotic stress response. Consistent with this, a marked reduction in *CrUbc3* transcripts was observed upon shift to 42°C (Figure 3-3), compared to an increase in transcripts for *CrUbc9* in response to this same stress. This is consistent with the notion that CrUBC9 is involved in abiotic stress response while CrUBC3 is not.

In an attempt to confirm that CrUBC3 is a functional SUMO E2 conjugase, we sought to express the protein in yeast cells in an attempt to functionally substitute for ScUBC9. Attempts to complement the yeast temperature-sensitive mutant *ubc9* which fails to grow at 37°C were unsuccessful with both CrUBC9 and CrUBC3. This is interesting since multiple other SUMO E2 conjugase enzymes with less similarity to ScUBC9 than CrUBC9 have successfully substituted for ScUBC9 in this mutant (Yasugi and Howley, 1996; Ohsako and Takamatsu, 1999). This inability suggests that at 37°C, which is an elevated temperature for yeast growth, neither CrUBC9 nor CrUBC3 can functionally complement ScUBC9. Therefore, disruption of ScUBC9 was attempted
under conditions in which either CrUBC3 or CrUBC9 might have a higher probability of being functional. Results of these experiments at 30°C (Figure 3-4) initially suggested that CrUBC9 was able to allow the survival and growth of yeast cells lacking the native ScUBC9 gene. However, subsequent Southern blot analysis (Figure 3-6) revealed that the expression of neither CrUBC9 nor CrUBC3 allowed for the disruption of the endogenous ScUBC9 gene. The inability of these partial knockout lines (i.e., lines containing both a disrupted ScUBC9 gene and an apparently intact ScUBC9 gene) to grow at 37°C (Figure 3-5) is intriguing and could reflect several possibilities. First, the chromosomal rearrangements that have to take place to maintain an endogenous ScUBC9 gene somewhere in the genome while one copy of the gene is disrupted by homologous recombination could result in the elimination of a gene necessary for survival at 37°C. However, another possibility is that CrUBC9 and CrUBC3 are capable of forming a thioester with the SUMO protein of S. cerevisiae, but that these enzymes are incapable of efficiently conjugating this SUMO to target proteins. Because these proteins were expressed from the strong constitutive GPD promoter, if they acted in this capacity they could essentially act as a dominant negative to sequester endogenous SUMO within the cell and prevent its necessary conjugation to target proteins. In addition, we cannot rule out the possibility that CrUBC3 is not a functional SUMO conjugase, although the bioinformatic analysis presented here strongly suggests it encodes for a SUMO conjugase over a ubiquitin E2 conjugase.

The combination of bioinformatic analysis and failure to complement for the yeast ScUBC9 is suggestive that both CrUBC9 and CrUBC3 are possibly functional SUMO E2 conjugases, but neither is fully functional under all growth conditions, unlike
endogenous ScUBC9 which is functional at both 30°C and 37°C. The implication of two distinct, functional SUMO E2 conjugases in *C. reinhardtii* is unique, given that the rule of thumb has consistently been a single essential E2 conjugase for the SUMOylation pathway. One could envision a division of labor between CrUBC3 and CrUBC9 in which the former is responsible for constitutive SUMOylation that regulates normal cell growth and development while the latter mediates SUMOylation under stress conditions. This would explain the viability of *mut5* under standard laboratory conditions. This could be an analogous situation to the SUMO isoforms in human and *Arabidopsis* cells, in which one SUMO isoform (SUMO1 in the case of human cells) is conjugated to proteins under non-stress conditions, and other SUMO isoforms (SUMO2/3 in human cells and *AtSUMO1/2* in *A. thaliana*) modify proteins in response to abiotic stress (Saitoh and Hinchey, 2000; Saracco *et al.*, 2007).

This possible SUMO E2 conjugase adaptation in *C. reinhardtii* appears unique, as the only homolog to CrUBC3 that could be identified is in the green alga *Volvox carteri*. This suggests that the evolution of two functional SUMO E2 conjugase enzymes might be confined to the Volvocales. The evolutionary advantage that two distinct SUMO E2 conjugases might confer to this class of algae is unknown, although perhaps the growth of wild-type cells at 37°C compared to *mut5* provides a clue. *mut5* cells are apparently capable of normal cell growth and development at 20-25°C, and, by extrapolation, all of the SUMOylation reactions necessary for this growth and division under non-stress conditions. At 37°C, however, wild-type cells show not only cell survival but growth, whereas *mut5* cells show a rapid loss in cell viability (Figure 2-5). If only CrUBC9 was responsible for all SUMOylation in the cell, in response to continuous exposure to 37°C
the CrUBC9 protein could become saturated in terms of enzymatic activity acting on proteins modified in response to stress. However, if a second functional SUMO E2 conjugase devoted exclusively to SUMO modification under normal growth conditions was present, it could continue to SUMOylate in a manner that would allow cell growth and division to take place. Because CrUBC9 is so similar to UBC9 proteins from other organisms that are the sole functional SUMO E2 conjugase in that organism, it remains to be determined whether or not CrUBC9 is functional on both stress and non-stress protein substrates, as well as whether or not CrUBC3 is required for cell viability, although the failure of CrUBC9 to functionally substitute for ScUBC9 is suggestive that it has been specialized to act exclusively under stress conditions. The two residues in CrUBC9 and eight in CrUBC3 that differ from the SUMO-only consensus identified in the bioinformatic analysis above (Figure 3-2, Table 3-2) may provide clues as to specific locations in the amino acid sequences that code for CrUBC9 and CrUBC3 that modify it in such a way as to alter its function within the cell. This hypothesis is strengthened by the fact that the closest homologs to both of these proteins in V. carteri share the same differences with the SUMO consensus that their C. reinhardtii counterparts do (Figure 3-7).

**MATERIALS AND METHODS**

**C. reinhardtii strains and growth conditions**

Wild-type strain CC124 was obtained from the *Chlamydomonas* Genetics Center at Duke University (Durham, NC). Cultures were grown in Tris-acetate phosphate (TAP) media (Harris, 2009). For expression analysis under heat stress, 25ml aliquots of cells were shifted to pre-warmed flasks at 42°C for one hour.
Identification of SUMO or ubiquitin specific residues in E2 conjugase enzymes.

Known SUMO and ubiquitin conjugase enzymes were assembled in a large multiple-sequence alignment using CLUSTALW with default settings (http://www.ebi.ac.uk/Tools/msa/clustalw2/). SUMO E2 conjugase enzymes included: *Saccharomyces cerevisiae* UBC9 (NP_010219), *Arabidopsis thaliana* SCE1 (NP_191346), *Schizosaccharomyces pombe* Hus5 (NP_593204), *Danio rerio* UBC9-A (NP_571426), and *Mus musculus* UBC9 (NP_035795). Ubiquitin E2 conjugase enzymes included: *Saccharomyces cerevisiae* UBC4 (NP_009638), *Homo sapiens* UBCE2_D2 (NP_003330), *Danio rerio* UBCE2_D3 (NP_9562466), *Schizosaccharomyces pombe* Ubc4 (NP_595283), *Saccharomyces cerevisiae* UBC3 (NP_010339), and *Arabidopsis thaliana* UBCE2_4 (NP_568589). After assembling an alignment using all of the sequences above, residues 100% conserved across all ten sequences in the alignment were identified. Next, two additional alignments were assembled, one using only the SUMO E2 conjugase protein sequences, and a second using only ubiquitin conjugase protein sequences. Residues that were 100% conserved in the SUMO E2 conjugase alignment were compared to the larger multiple sequence alignment containing both SUMO and ubiquitin E2 enzymes to identify those residues that were 100% conserved in SUMO E2 enzymes and for which none of the ubiquitin E2 enzymes in the alignment had the same residue at that position. The reverse was also carried out in which residues 100% conserved in an ubiquitin E2 alignment were compared to residues in the larger alignment to identify ubiquitin residues conserved 100% in the alignment but not in SUMO.
Identification and analysis of putative E2 conjugases in the *C. reinhardtii* genome

For identification of additional E2 conjugases, the *S. cerevisiae* ScUBC9 protein sequence was queried against the *C. reinhardtii* genome using a BLASTp search (www.phytozome.net). Protein sequences returned as potential homologs to ScUBC9 were individually added to the SUMO/ubiquitin multiple sequence alignment above and the residues identified as part of either the shared, SUMO, or ubiquitin consensus residues were compared in that sequence to determine whether it more strongly resembled a SUMO E2 conjugase, ubiquitin conjugase or neither.

To compare the percent identity and similarity of *C. reinhardtii* SUMO E2 conjugases to ScUBC9, sequences were aligned using NCBI Blastp for aligning two more more sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi, program BLAST (bl2seq)) and identifying the reported percent identities and percent similarities after the alignment was generated.

To identify CrUBC3 homologs in other organisms, the CrUBC3 amino acid sequence was used in a BLASTp search against the NCBI database of non-redundant protein sequences using default parameters (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The top hits based on E-value were compared to both CrUBC3 and CrUBC9 sequences using a CLUSTALW alignment and the resulting percent identity between the putative homolog and either CrUBC3 or CrUBC9.

Quantitation of expression levels of CrUBC9 and CrUBC3

For quantitation of expression levels of *CrUbc9* and *CrUbc3* transcripts, total RNA was isolated from CC124 *C. reinhardtii* cells grown at 25°C and after a shift to 42°C for one hour using Trizol LS (Invitrogen, Grand Island, NY) according to the
manufacturer’s recommendations. Contaminating DNA was removed by treatment with DNaseI (ThermoScientific). Complementary DNA was synthesized from 2.4ug of total RNA with oligo-dT primers using the Plexor Two-Step qRT-PCR system (Promega, Madison, WI). Synthesized cDNA was diluted 1:2 in 1mM MOPS, 0.1mM EDTA and used as substrate for a qPCR reaction according the Plexor Two-Step qRT-PCR system recommendations, using 0.2mM of each primer (Biosearch Technologies, Novato, CA).

Primer pairs for qPCR were as follows: CrUBC3: (5’-FAM-isoC – GCTGGGGTACACG-TTTGGATG-3’ and 5’- GATACCAGGGCCGGAGAAGAC-3’); CrUBC9: (5’-CAL Fluor Orange 560-isoC - TGAGGCACACGGTACCGGAG-3’ and 5’- CTCACCATGGAGTTCAGCGAG-3’); G-Protein: (5’ - Quasar 670-isoC-GTTGGTG-GGTCAATGGGCAGAA-3’ and 5’-GACAAGACCATCAAGCTGTGGAAC-3’). G-Protein and CrUBC9 primers were multiplexed in a single reaction. The efficiency of amplification for each set of primers was calculated and used to quantify the relative transcript abundance at 25°C compared to 42°C (Pfaffl, 2001). Efficiencies for the primer pairs were as follows: CrUBC9: 83%, CrUBC3: 82%, G-Protein: 82%. The fact that the primer pair efficiencies were essentially the same for all three transcripts allowed the direct comparison of relative transcript level between the transcripts. In this case, the Ct values for CrUBC9 and CrUBC3 at 25°C were analyzed in the same manner as the ΔCt values above to determine the relative abundance of CrUBC3 when compared to CrUBC9 prior to any stress treatment. Final analysis therefore reflected transcript abundance relative to CrUBC9 transcript levels at 25°C and are the result of a technical triplicate of a biological triplicate (three separate cultures shifted to 42°C for one hour analyzed in triplicate by qRT-PCR).
Yeast methods

Yeast strain Y0002 was a generous gift of Stefan Jentsch. Wild-type yeast strain Y0002 (\textit{MAT}a his3-\text{\textDelta}200 leu2-3, 112 lys2-801 trp1-1 (am) ura3-52) was transformed by lithium acetate/heat shock using standard protocols (Gietz, 2002). Transformants obtained using the p423-GPD based plasmids were selected on complete minimal media lacking histidine (CM-HIS). Transformants using the LEU2 marker for homologous recombination were selected on complete minimal media lacking histidine and leucine (CM-HIS-LEU) (Sambrook, 1989).

For expression of CrUBC9 and CrUBC3 in \textit{S. cerevisiae}, cDNAs encoding both proteins were cloned into the plasmid p423-GPD which places the expression of a given cDNA under the control of the strong constitutive promoter, GPD and uses \textit{his} as a selectable marker for incorporation of the plasmid into yeast cells (Mumberg \textit{et al.}, 1995). CrUBC9 cDNA was amplified using the following primers: 5' – TAAA-GGATCCATGTCTGGCGTCGC – 3' and 5' – TTTTGTCGACTCAGGAGGTTG-GCGGG – 3' (BamHI and SalI sites underlined). CrUBC3 cDNA was first amplified from \textit{C. reinhardtii} RNA as a larger fragment including 5' and 3'UTR of the UBC3 transcript using the following primers: 5' – CTGCTTGCATATCAAAGG CCGCATACC – 3’. The product of this RT-PCR was used as template for PCR using the following primers: 5’ – TAAAGGATCC-ATGGCATCTCAGC – 3’ and 5’ – CCCCCGTCGACTCATTCCATCCTCC – 3’ (BamHI and SalI sites underlined). cDNAs were cut with BamHI and SalI and cloned into p423-GPD digested with BamHI and SalI. The resulting plasmids were named p423-GPD-CrUBC9 and p423-GPD-CrUBC3. A 6X-HIS tag was added to the plasmid...
by annealing the following primers together: 5’ – GATCATGCATCATCATCATCATTACACG – 3’ and 5’ - GATCCGT GATGATGATGATGATGCAT – 3’. Annealing was carried out by boiling equal volumes of 100mM concentrations of each primer for five minutes in the presence of 1X Pfu Polymerase Buffer (Agilent Technologies) and allowing the boiling reaction mix to slowly cool to room temperature in the same water bath in which it was boiled. The annealed primers have 5’ overhangs compatible with BamHI digested DNA, and were ligated into BamHI digested p423-GPD-CrUBC9 and p423-GPD-CrUBC3. The resulting plasmids were called p423-GPD-HIS-CrUBC9 and p423-GPD-HIS-CrUBC3.

To generate a LEU2 marker flanked by ScUBC9 sequence for homologous recombination, the ScUbc9 cDNA was amplified using the following primers: 5’ - CCCCCGGATCCATGAGTAGTTTGTGCTACAGC – 3’ and 5’ – CCCCCGTCCGACC-TATTTAGAGTACTGTTTAGC – 3’ (BamHI and SalI sites underlined) and cloned into BamHI-SalI digested pET28b resulting in the plasmid pET28b-ScUbc9. The ScUbc9 cDNA contains an endogenous DraI site that was cut 295bp into the 480bp cDNA. The LEU2 marker was amplified with the following primers: 5’ – TAAACCATGGCTGTGCGGATTCACACCAG – 3’ and 5’ – CCCCTTTAAAAAGATTTGTACTGAGAGTGCAC – 3’ using Phusion DNA Polymerase (Thermo Fisher Scientific, Pittsburgh, PA) to generate a blunt-ended PCR product which was cloned into DraI cut pET28b-ScUbc9. The resulting plasmid pScU-LEU2-bc9 was used as template for PCR using the ScUbc9 primers above (BamHI, SalI containing). The resulting PCR product was used for transformation of Y0002 cells previously transformed with either p423-GPD-HIS-CrUbc9 or p423-GPD-HIS-CrUbc3 in an attempt to knock out the endogenous ScUbc9.
Analysis of ScUbc9 knockouts

High throughput DNA isolation of wild-type yeast and potential transformants was carried out according to the method of Lõoke, et. al. (2011). One microliter of isolated DNA was used as template for PCR using primers designed to amplify a product in the event of a homologous recombination event between the LEU2 marker and endogenous ScUbc9. A set of primers was designed on either side of the insertion site to confirm disruption of the ScUbc9 gene. Each set contained a primer that annealed either upstream or downstream of the ScUbc9 gene and a second primer that annealed within the LEU2 marker. Therefore, a PCR product of the correct size would only be amplified if the LEU2 marker inserted into the yeast chromosomal DNA at the ScUbc9 locus. The first set of primers included a forward primer that annealed upstream of the ScUbc9 locus and a reverse primer that annealed within the LEU2 marker (5’ – GCTACCTGTACG-CCATCACTGTCC – 3’ and 5’ – AGTCATCGAATTGATTCTG-TGCGATAGC – 3’, respectively). The second set of primers was the reverse with a forward primer that annealed within the LEU2 marker and a reverse primer that annealed downstream of the ScUbc9 locus (5’ – AATTGATACTAATGGCTCAACGTGATA-AGG -3’, and 5’ – GGACAAATTGATGCAAATAAGGAGATTGGG – 3’, respectively).

For Southern Analysis, genomic DNA was isolated from yeast strains using standard protocols (Sambrook, 1989). Isolated DNA was digested overnight with KpnI and ScaI and DNA fragments were separated on a 0.8% 0.5X TBE gel. Southern analysis including transfer to positively charged nylon membrane, generation of DIG-labeled ScUBC9 probe, and hybridization and detection of probe was carried out according to established protocols for DIG-labeled probe Southern analysis (Roche, Indianapolis, IN).
with the modification that blots were hybridized to probe in Ultrahyb hybridization buffer (Ambion). DIG-labeled ScUBC9 probe was generated by PCR amplification of ScUBC9 cDNA with the following primers: 5’ – CCCCCGATCCATGAGTTTTGTGTCTACAGC – 3’ and 5’ - CCCCCGTCGACCTATTTAGAGTACTGTTTAGC – 3’ in the presence of DIG-dUTP nucleotide.

**Growth tests of ScUbc9 knockout lines**

For analysis of the growth of wild-type (Y0002) and knockout lines, cultures were normalized based on their OD<sub>600nm</sub> and spotted in a 1:4 dilution series on YPD, CM(-HIS), and CM(-HIS, -LEU) plates (Sambrook, 1989). Plates were incubated at either 20°C, 30°C, or 37°C to assess growth.
CHAPTER 4

Conclusions and Future Work
The role of the protein SUMO within the eukaryotic cell has rapidly broadened from its initial identification as a post-translational modification to regulate nuclear pore localization. We now know that this protein can target hundreds, if not thousands of proteins within a cell and that the consequences of SUMOylation on these target proteins can be highly varied. Although the name “ubiquitin-like” is appropriate for SUMO, both in terms of its structure and the enzymatic pathway leading to its conjugation to a target protein, in terms of function one could make the argument that it acts more like phosphorylation in terms of the diversity of consequences resulting from this modification. Although our understanding of this diversity, both in terms of the biochemical and functional consequences of SUMOylation, has greatly expanded in recent years, much remains to be understood about the role of this covalent modification in the cell.

*C. reinhardtii* presents a unique opportunity not available in any other organism examined to date to study the effects of a SUMO E2 conjugase mutant. This allowed us to focus specifically on the effect this *mut5* mutation had under abiotic stress. We demonstrated that CrUBC9 is essential for SUMOylation in response to a wide range of stresses, including abiotic stress treatments such as elevated temperature, as well carbon deprivation. The mere viability with no obvious growth defects of *mut5* is remarkable given the phenotypes observed in all other UBC9 mutants studied to date, and is strong suggestive evidence that a second functional SUMO E2 conjugase is likely present in *C. reinhardtii*. Nevertheless, it is clear from our experiments that under stress conditions, CrUBC9 is absolutely essential for SUMOylation and in many cases increases the tolerance of *C. reinhardtii* for a given stress condition. It would be interesting to
determine if overexpression of CrUBC9 could further increase the tolerance of *C. reinhardtii* to abiotic stress. However, the failure of CrUBC9 to complement the growth defect observed on high salt suggests that precise regulation of the SUMOylation state of proteins may be required for proper adaptation to stress.

There are several additional questions that remain regarding CrUBC9 and CrUBC3. The first is definitive identification of CrUBC3 as a functional SUMO conjugase. In addition, it remains to be seen whether or not there is a clear division of labor between the two, or if there might be some degree of functional redundancy. Based on the abiotic stress experiments described herein, it is quite clear that CrUBC3 does not act under stress conditions, however it remains to be seen whether or not CrUBC9 can SUMOylate target proteins under non-stress conditions. One can readily envision at least two plausible models for the functions of CrUBC9 and CrUBC3. In Model #1, CrUBC3 is the dedicated SUMO E2 conjugase for constitutive SUMOylation in the cell, while CrUBC9 only actively targets proteins for SUMOylation in response to stress. In Model #2, both CrUBC3 and CrUBC9 act upon proteins during normal growth and development, and under stress conditions, CrUBC9 switches to targeting stress-related proteins while CrUBC3 maintains constitutive SUMOylation so cells can continue to grow and divide. If Model #1 is correct, a CrUBC3 knockout mutant should be lethal, while if Model #2 is correct, knockout of CrUBC3 would not be expected to affect cell viability because CrUBC9 could functionally complement for CrUBC3 under these non-stress conditions. The failure of CrUBC9 to function in yeast cells as a SUMO E2 conjugase favors Model #1 in which both CrUBC3 and CrUBC9 have distinct, non-
overlapping functions. Although the fact that this was a negative results makes the
evidence only weakly suggestive of this fact.

What proteins are targeted by both CrUBC3 and CrUBC9 also remains to be
determined. In addition, what specific SUMO proteins are used for this SUMOylation is
unknown as well. It appears as though two distinct SUMO proteins are encoded in the
C. reinhardtii genome, CrSUMO96 and CrSUMO148 (Want et al., 2008). Whether or
not each SUMO E2 conjugase has a preferred SUMO, or if both can use either SUMO
protein with similar efficiencies is yet to be determined.

One major hurdle in answering many of these questions is the identification of the
other components of the SUMOylation pathway in C. reinhardtii. Given the likely
presence of at least two E2 enzymes in this green alga compared to other known
organisms, could there also potentially be multiple E1 activase enzymes as well?

In terms of intracellular localization, it appears as though CrUBC9 predominantly
localizes to the nucleus, although a small proportion of this protein localizes to the
cytoplasm. Whether or not the same pattern would be observed for CrUBC3 is unknown.
If CrUBC3 localized within the cell in a different pattern, this would be suggestive
evidence that the two proteins acted in completely separate ways within the cell (Model
#1 described above). However, given the fact that most SUMO proteins localize to the
nucleus, a nuclear localization of CrUBC3 would not rule out this possibility.

One of the most intriguing questions regarding the presence of CrUBC9 and
CrUBC3 in the C. reinhardtii genome is the question of “why?” Why is it that in all
other organisms a single E2 enzyme can carry out this function under both non-stress and
stress conditions, while in *C. reinhardtii* there are likely two? What sort of selective advantage could be acquired as a result of this adaptation that would have to be unique to the Volvocales?

The identification of a SUMO-only consensus that distinguishes a SUMO E2 conjugase from an ubiquitin E2 conjugase could potentially be used to begin to address this question. Knowing the residues that differ between CrUBC3 and CrUBC9 at those consensus sites that are 100% conserved among SUMO E2 conjugases, one could generate CrUBC3 and CrUBC9 “versions” of SUMO E2 conjugases for other organisms and express them together or separately to see what advantage or disadvantage it had on a cell.

While many questions remain regarding the role of CrUBC3 and CrUBC9 in *C. reinhardtii* the work described here advances our knowledge of both how and why cells SUMOylate proteins. The identification of two, likely separate SUMO E2 conjugases in a single organism that are distinct both in sequence and regulation is a novel discovery that differs from the dogma that a single E2 conjugase exists for the SUMOylation pathway. In addition, the role of CrUBC9 and, by extrapolation, SUMOylation in response to abiotic stress is established as absolutely critical for SUMO modification and for tolerance to a wide range of stress conditions in *C. reinhardtii*. 
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