Abstract

CDC48/p97 is a conserved essential homohexameric AAA-ATPase chaperone involved in membrane fusion, protein degradation, and other cellular processes. We have identified an *Arabidopsis* UBX domain-containing protein, PUX1, which functions to regulate the oligomeric structure of the AtCDC48 as well as its mammalian ortholog p97. Binding of PUX1 to the Nα domain of AtCDC48 is dependent through its UBX domain, however, disassembly of AtCDC48 hexamer requires the N-terminal domain of PUX1. These findings provide evidence that the assembly and disassembly of the hexameric CDC48/p97 complex is a dynamic process.

We have used reverse genetic and biochemical approaches to elucidate the function of AtCDC48 in plants. Our data show that AtCDC48 is essential for plant growth and development. AtCDC48 is localized throughout the cell including the nucleus, cytoplasm, ER, and the division midzone. Homozygous *Atcdc48* T-DNA insertion mutants are not viable. AtCDC48 is necessary for at least three stages of early development: fertilization, embryo development, and seedling growth. In order to avoid the lethality of *Atcdc48* mutants, we used an ethanol-inducible dominant negative system to study the functions of AtCDC48 during each developmental stage. Our data indicate that AtCDC48 has essential roles in cell division, elongation, and differentiation. Studies in this thesis support that AtCDC48 is an essential AAA-ATPase chaperone that functions in numerous diverse cellular activities in *Arabidopsis*, and the biochemical regulation of AtCDC48 activity by PUX1 is required for various cellular pathways critical for plant growth and development.
Dedication
This thesis is dedicated to my parents, ChanJin Park and BuSun Jung, and my family members whose love and support was very important in every aspect of life during my graduate years.

Acknowledgements
I would like to thank Sebastian Bednarek for providing me endless support and challenge that made me grow as a scientist from a humble graduate student. I also thank my colleagues, especially Dave Rancour who helped me in many ways scientifically during my graduate years. Lisa Koch, Colleen McMicheal, Steven Backues, and Catherine Konopka were excellent lab mates for helping me to adapt to the atmosphere in the lab with pleasant laughter. It was an honor for me to meet with my committee members, Rick Amasino, Jay Bangs, Betty Craig, and Tom Martin. Thanks for their great ideas and critical review of my experiments. At last, I thank Jin for supporting me during the rough times as well as good times of my life in US. Without all of you, I would not have my title as a Doctor of Philosophy.
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Chapter 1

Introduction

All the data presented in the thesis were generated by Sookhee Park except Figure 2 and Figure 3 in Chapter 1, which were generated by Carrie Dickey and David M. Rancour, respectively.

Thesis Objective

The CDC48/p97 family of AAA-ATPase chaperones is highly conserved in eukaryotes. This family is essential for viability in all the organisms (budding yeast, trypanosomes) tested thus far. Little is known of CDC48/p97 function in the plant kingdom. Early studies by Feiler and colleagues (Feiler et al., 1995) presented data indicating that AtCDC48 was highly expressed in the proliferating cells of the vegetative shoot, root, and flowers in rapidly growing plants. In addition, they provided localization data relying on cross reactivity of anti-mammalian p97 antibodies to Arabidopsis CDC48 to suggest that AtCDC48 primarily localized to the nucleus and the phragmoplast. The studies contained in this thesis describe the characterization of the Arabidopsis ortholog of CDC48/p97, AtCDC48 (AtCDC48A), at the biochemical and cellular levels. We demonstrate for the first time the drastic effects perturbation of AtCDC48 function has on overall plant development. In addition, data is provided that describes the novel biochemical regulation of AtCDC48 activity through association of a plant UBX domain containing protein, PUX1, and the subsequent disassembly of the functional homohexameric AAA-ATPase, AtCDC48, into non-active protomer subunits. Members
of the CDC48/p97 protein family have been implicated in diverse cellular processes such as membrane trafficking and fusion, organelle biogenesis, proteolysis and protein folding/degradation (Dalal and Hanson, 2001). The research will address the following primary questions: 1) How PUX1 regulates AtCDC48 activity? and 2) What are the main roles of AtCDC48 in plant growth and development?

**Background**

AAA proteins have been found in all prokaryotes and eukaryotes and play essential roles in cellular housekeeping, cell division and differentiation. All members of the CDC48/p97 family are Mg\(^{2+}\)-dependent AAA proteins, ATPase associated with a variety of cellular activity. Soluble and membrane-associated AAA ATPase proteins have been identified in the cytosol as well as in various membrane bound compartments. CDC48/p97 family proteins form homohexameric complexes, with a spherical or cylindrical shape and a central core. They are characterized by a 220-250 amino acid ATPase domain that contains conserved Walker A and B motifs as well as an AAA-specific region of homology (Neuwald et al., 1999). The conservation and widespread use of the AAA module suggests that AAA proteins may use common mechanisms to carry out a wide range of cellular functions. AAA protomers contain either one ATPase domain (type I) or two ATPase domains (type II). Several of the organelle-biogenesis AAA proteins are type II proteins including NSF and CDC48/p97 (Patel and Latterich, 1998a).

NSF (N-ethylmaleimide-sensitive factor), which is one of the best-characterized type II AAA ATPase, is a homohexameric chaperone required for vesicle docking/fusion
at all stages of the biosynthetic and endocytic secretory pathway (Rothman, 1994). It was first isolated from NEM (N-ethylnmaleimide) treated cytosolic fractions used to reconstitute an in vitro intra-Golgi transport assay (Block et al., 1988). The yeast homolog of NSF, Sec18p, whose temperature-sensitive mutant sec18-1 was identified initially as having a block in the secretory pathway, is required for multiple vesicle-transport steps (Novick and Schekman, 1979). Secretory vesicle targeting and fusion is mediated through the pairing of cognate, cytoplasmically oriented integral membrane proteins, SNAREs (SNAP-receptor), which reside on the two fusing membrane species (i.e. vesicle v-SNAREs pair with target membrane t-SNAREs) to yield SNARE complexes (Brunger, 2001; Jahn and Südhof, 1999). Sec18p/NSF functions as a molecular chaperone to disassemble, at the expense of ATP, these SNARE complexes to facilitate another round of secretory membrane targeting and fusion (Littleton et al., 2001; May et al., 2001).

SNAREs are not only involved in the heterotypic fusion of secretory vesicles with their appropriate acceptor compartment but also function in the homotypic fusion of like-like membranes such as vacuoles (Nichols et al., 1997).

CDC48 was originally identified as a mutant that displayed defects in the progression of cells through the cell division cycle (Moir et al., 1982) and p97 was initially isolated from Xenopus laevis oocytes (Peters et al., 1990). CDC48/p97 catalyzes certain homotypic membrane fusion events such as ER membrane fusion (Latterich et al., 1995), transitional ER assembly (Roy et al., 2000a), nuclear membrane fusion (Hetzer et al., 2001) and Golgi reassembly (Acharya et al., 1995; Rabouille et al., 1995b; Rabouille et al., 1995c). In addition, CDC48/p97 has been shown to participate in non membrane
fusion processes including for example ubiquitin-proteasome dependent protein degradation (Braun et al., 2002; Dai et al., 1998; Dai and Li, 2001; Ghislain et al., 1996; Jarosch et al., 2002a; Rabinovich et al., 2002) (Figure 1A).

Similar to NSF, CDC48/p97 has been shown to interact with two SNAREs, Ufe1p and syntaxin 5 (Patel et al., 1998) during ER membrane fusion and Golgi reassembly, respectively. However, the mechanism by which it promotes membrane fusion remains unknown. Although p97 and NSF are homologous, however, their interaction with SNAREs is mediated by distinct adapters; NSF requires SNAP while p97 requires the adapter p47 for interaction with syntaxin 5 (SYP31/32 in plants) (Kondo et al., 1997). p47 is involved in the control of homotypic membrane fusion of ER or Golgi membranes (Roy et al., 2000a; Yuan et al., 2001) and in post karyokinesis nuclear envelope formation (Hetzer et al., 2001). P97-p47 activity on Golgi membrane reformation after mitosis requires ubiquitin in a proteasome-independent manner and is dependent on another adapter protein, VCIP135 (valosin containing protein [VCP][p97]/p47 complex-interacting protein of 135 kDa), which resides on the membrane and can form a transient complex with p97-p47. VCIP 135 removes the ubiquitin and triggers membrane fusion in an undefined manner (Uchiyama et al., 2002; Wang et al., 2004) (Figure 1B). In addition, the adapter complex, Npl4/Ufd1 is required for p97 mediated formation of the chromatin-associated nuclear envelope network as well as formation of the closed nuclear envelope (Hetzer et al., 2001) (Figure 1C). Targeting of CDC48/p97 to other cellular pathways described above is accomplished via recruitment of additional adapter proteins.

The active form of CDC48/p97 is a ring-shaped complex comprised of six identical ~90 kDa subunits containing three domains; an N terminal domain followed by
two Walker-type ATPase domains (D1 and D2) that are joined by conserved linker regions (for review see (Lupas and Martin, 2002; Ogura and Wilkinson, 2001; Patel and Latterich, 1998b; Vale, 2000; Woodman, 2003)). CDC48/p97 protomers have the intrinsic ability to self-oligomerize, thereby creating the active hexameric ATPase (Davies et al., 2005; Peters et al., 1992; Rockel et al., 2002). Recent studies have revealed that the D2 domain of CDC48/p97 is responsible for the major enzyme activity of the chaperone (Dalal et al., 2004; Lamb et al., 2001). In contrast to the D1 domain, mutations in the D2 domain that inhibit ATP hydrolysis or binding displayed dominant negative properties in vivo. Ultrastructural studies of p97 have suggested that CDC48/p97 nucleotide hydrolysis and exchange in the D2 ATPase domain result in conformational changes that drive movement of the N-terminal domain. This provides the mechanical force necessary to act on substrate proteins most likely for protein assembly and disassembly (Beuron et al., 2003; Bruderer et al., 2004; Davies et al., 2005; Rouiller et al., 2002). The conformational changes in p97 that occur during nucleotide binding and hydrolysis have been studied (Beuron et al., 2003; Davies et al., 2005; DeLaBarre and Brunger, 2003; DeLaBarre and Brunger, 2005; Huyton et al., 2003; Pye et al., 2006; Rouiller et al., 2000; Zhang et al., 2000b). According to the p97 conformation studies, it can be assumed that Arabidopsis CDC48 also changes its conformation due to the nucleotide binding and hydrolysis.

The Arabidopsis genome encodes three CDC48 isoforms: AtCDC48A(AtCDC48), AtCDC48B and AtCDC48C. AtCDC48 is presumed to be the most abundant isoform because there exists greater than 100 expressed sequence tags (ESTs) and several full-length cDNAs in the Arabidopsis Information Resource (TAIR,
http://www.arabidopsis.org) and Salk Institute Genomic Analysis Laboratory (SIGnAL, http://signal.salk.edu/cgi-bin/tdnaexpress) databases compared with those identified to date for *AtCDC48B* (8 ESTs) and *AtCDC48C* (15 ESTs) (Rancour et al., 2002). These isoforms are predicted to share 91%(*AtCDC48B*) and 95%(*AtCDC48C*) amino acid identity with full length AtCDC48. Extensive characterization of AtCDC48 and the role of this protein family in plant growth and development have not yet been undertaken.

AtCDC48 has been shown to be re-localized from a diffuse cellular distribution to distinct localization at the division plane during plant cell cytokinesis. The division plane localization is similar to the SNARE proteins KNOLLE (Lauber et al., 1997) and the plant ortholog of syntaxin 5, SYP31 (Figure 2) (Feiler et al., 1995; Rancour et al., 2002). Initial biochemical studies have demonstrated that AtCDC48 associates with a membrane population that co-fractionates with both KNOLLE and SYP31. However, cytosolic AtCDC48 was found to interact with SYP31, whereas binding of AtCDC48 to KNOLLE was not detected (Lauber et al., 1997; Rancour et al., 2002). These results suggest that there are at least two distinct membrane fusion pathways that operate at the division plane to mediate plant cytokinesis, one involving AtCDC48 and one dependent on NSF.

In order to characterize the function of AtCDC48 including plant cytokinesis, two approaches were taken. First, to identify adapters that may be involved in AtCDC48/SYP31 interaction, preparative affinity chromatography was performed to identify soluble binding partners of AtSYP31 and AtCDC48. Second, reverse genetic studies were conducted to characterize the role of AtCDC48 and plant cell-specific components that interact with AtCDC48 during plant cell growth and development.
Two uncharacterized proteins binding specifically to GST-AtSYP31/AtCDC48 were identified as PUX1 (At3g27310), and PUX2 (At2g01650) (Figure 3A) (Rancour et al., 2004). Analysis of the deduced primary amino acid sequences for PUX1 and PUX2 revealed that these proteins are predicted to contain single UBX domains (Figure 3C), similar to the p97 adapter, p47. This domain has a characteristic ubiquitin fold with a \( \beta\alpha\beta\beta\alpha\beta \) secondary structure arrangement (Yuan et al., 2001).

In the case of p47, binding of the protein to the N-terminus of CDC48/p97 is mediated through two contiguous sites; a carboxyl-terminal UBX domain and middle ~100 amino acid region (amino acids 171-270) of undetermined structure (Uchiyama et al., 2003; Yuan et al., 2001). UBX domains have been identified in a number of functionally diverse eukaryotic proteins, typically at the carboxyl-terminus, including the putative yeast ortholog of p47, Shp1p (Kondo et al., 1997), and the yeast Cui1-3p protein family, which are required for sporulation (Decottignies et al., 2004). Similar to p47, the Cui1-3p proteins bind to CDC48 via UBX domains. Structural studies of the UBX domain of the human Fas-associated factor-1 (FAF1) (Schuberth et al., 2004) and p47 (Yuan et al., 2001) have shown that the UBX domain adopts a characteristic ubiquitin fold. In contrast to ubiquitin, the UBX domain lacks the carboxyl-terminal double glycine motif necessary for conjugation to target proteins (Buchberger et al., 2001).

The *Arabidopsis* genome contains 15 PUX (Plant UBX containing protein) genes (Rancour and Bednarek, unpublished data), which are postulated to be a family of AtCDC48 regulatory proteins. The characterization of PUX1 and AtCDC48 functions in plant growth and development will be presented in following chapters. A cDNA corresponding to PUX1 was generated and cloned from extracted *Arabidopsis* total RNA.
(Genbank accession no. AY572781). The sequence is predicted to encode a soluble protein of 251 amino acids with a molecular weight of 28,484 Da. **PUX1** is a unique gene in the *Arabidopsis* genome and putative **PUX1** orthologs have been identified in other plants including rice (*Oryza sativa; Os*). MPSS expression profile data from *Arabidopsis* (http://mpss.udel.edu/at/java.html) suggests that the **PUX1** gene is expressed ubiquitously *in planta*. Proteins containing regions of similarity and domain organization to full-length **PUX1** have also been identified using PSI-BLAST [Altschul, 1997 #839] in mouse (GenBank accession nos. NP_081153 and NP_937866; 26% identity and 43% similarity), human (GenBank accession no. NP_076988; 28% identity and 42% similarity), *Drosophila melanogaster* (GenBank accession no. NP_611356; 20% identity and 39% similarity) and *Caenorhabditis briggsae* (GenBank accession no. CAE64789; 15% identity and 31% similarity) suggesting that the function of **PUX1** may be conserved between plants and animals. In addition, putative **PUX1** orthologs were also identified in a limited number of fungi including *Eremothecium gossypii* (GenBank accession no. NP_984246; 22% identity and 38% similarity), *Aspergillus nidulans* (GenBank accession no. EAA60215; 17% identity and 31% similarity), and *Saccharomyces cerevisiae* (GenBank accession no. NP_013783; 17% identity and 31% similarity).

Because the predicted 28kDa **PUX1** protein was ~6kDa smaller in molecular mass than the 34kDa polypeptide isolated by GST-SYP31 affinity chromatography, the GST-SYP31/**PUX1** interaction results were verified by immunoblot analysis (Figure 3B). Immunoblot analysis of GST- and GST-SYP31 affinity purified T87 cytosolic proteins using affinity purified polyclonal anti-**PUX1** antibodies confirmed that the cytosolic 34 kDa polypeptide that bound to GST-SYP31 under conditions that support AtCDC48
interaction with SYP31 was indeed PUX1 (Figure 3B, lane 3). In addition to the 34 kDa protein, PUX1 immunoblot analysis detected a second cytosolic 38 kDa polypeptide that was isolated using SYP31 affinity chromatography (Figure 3B). Disruption of PUX1 resulted in the loss of the protein doublet (34 kDa and 38 kDa) detected with affinity-purified PUX1 antibodies demonstrating that both polypeptides are derived from the same gene product. The nature of the molecular mass difference between the two PUX1 isoforms is currently under investigation. Data in chapter 2 show that PUX1 binds and regulates AtCDC48 by inhibiting its ATPase activity and by promoting the disassembly of the active hexamer. Binding of PUX1 to hexameric AtCDC48 is mediated through the UBX-containing C-terminal domain. However, disassembly of the chaperone is dependent upon the N-terminal domain of PUX1. In addition, it is showed that the nucleotide status of AtCDC48 influences PUX1-mediated disassembly of the hexameric ATPase (Chapter 3). Phenotypic analysis of pux1 plants revealed that the loss of PUX1 accelerated the growth of various plant organs including roots and inflorescence shoots (Rancour et al., 2004). The growth of homozygous pux1 roots was ~5-fold faster than wild-type seedlings on nutrient agar medium. These results suggest that PUX1 functions as a regulator of AtCDC48 and that the activity of these two proteins is required for various cellular pathways important for plant growth and development.

For reverse genetic and biochemical approaches to elucidate the function of AtCDC48 in plants, three independent Arabidopsis mutant lines containing T-DNA insertions (obtained from Salk Institute Genomic Analysis Laboratory (SIGnAL, http://signal.salk.edu/cgi-bin/tdnaexpress) were obtained (Chapter 4). Data presented in chapter 4 shows that AtCDC48 is essential for plant growth and development.
Homozygous Atcdc48 T-DNA insertion mutants are not viable. AtCDC48 is necessary for at least three stages of early development: gametophyte development and/or fertilization, embryo development, and seedling growth. YFP-AtCDC48 was localized throughout the cell and the phenotype of an inducible dominant negative mutant of Atcdc48 suggests roles for AtCDC48 in cell division, expansion, and cell differentiation. In summary, results presented in this thesis provide strong evidence that AtCDC48 is a molecular chaperone that plays a critical role in plant growth and development by regulating membrane trafficking, and fusion, which is critical for overall plant morphogenesis and regulating signal transduction, which is critical for cell or tissue specific development.

References


Figure 1. Described biological roles for the CDC48 homolog, p97

A) p97 and its adapter Ufd1-Npl4 (UN) during ER-associated degradation. ER proteins destined for degradation are ubiquitinated at the cytosolic face of the membrane. P97 and Ufd1-Npl4 bind to the substrate via unfolded segments within the peptide and through interaction with the ubiquitin chain. ATP hydrolysis by p97 induces mobilization of the substrate into the cytosol for degradation by the proteasome. B) Phosphorylation (P) of p47 removes the p97-p47 complex from the membrane and at the end of mitosis, dephosphorylation allows rebinding. p97-p47 is then recruited to the substrate by direct interaction of p47. VCIP135, which is loosely attached to the Golgi and then interacts with p97-p47, triggers membrane fusion that leads to cisternal regrowth. C) Two types of fusion reactions are required to complete nuclear envelope assembly *in vitro*. p97/Ufd1/Npl4 (p97UN) mediates the closure of the nuclear membranes. p97/p47 mediates the fusion of vesicles with the outer membrane (ONM), which provide increased nuclear envelope surface area, allowing nuclear expansion. This figure was adapted and modified from Meyer, HH (Meyer, 2005a) and Burke, B (Burke, 2001)
Figure 2. Localization of AtCDC48 in interphase and dividing *Arabidopsis* cells

Dividing *Arabidopsis* cells were analyzed by wide-field indirect immunofluorescence microscopy (A-C) and confocal microscopy (D). (A-C) Cells were immunolabeled with anti-a-tubulin (green) and affinity-purified anti-KNOLLE (row A; red) or anti-AtCDC48 (rows B and C; red) antibodies and DAPI (blue). Electronically complied images (merged) were generated from the pseudocolored images. (D) Co-localization (yellow) of KNOLLE (red) and AtCDC48 (green) in dividing (middle) and non-dividing (top left and right) cells were examined by indirect confocal immunofluorescence microscopy. Solid arrows indicate the location of the cell plate. Solid arrowheads indicate the position of subcellular membrane compartments containing KNOLLE and AtCDC48 (see text for discussion). The open-headed arrow, (panel C) indicates nuclear localization of AtCDC48. Bar, 50 µm. This figure was adapted from Rancour et al. (Rancour et al., 2002).
Figure 3. Identification of *Arabidopsis* PUX1.

A) Coomassie Blue-stained analytical SDS-polyacrylamide gel of immobilized GST or GST-SYP31 affinity-purified cytosolic protein. MALDI-TOF mass spectrometric identification of individual proteins is indicated to the right of the respective protein bands. B) Immunoblot verification that PUX1 interacted with GST-SYP31. T87 cytosol (20 µg of S150 in lane 1) was subjected to affinity chromatography using immobilized GST (lane 2) and GST-SYP31 (lane 3), and eluted proteins were analyzed by immunoblotting with anti-AtCDC48 (upper panel) and anti-PUX1 (lower panel) antibodies. This figure was adapted from Rancour et al. (Rancour et al., 2004). C) Protein domain organization of UBX domain containing proteins. The protein domains presented include UBX, ubiquitin-like protein fold, and the UBA, ubiquitin-associated domain.
Chapter 2

Plant UBX domain-containing Protein 1, PUX1, regulates the Oligomeric Structure and Activity of Arabidopsis CDC48

Abstract

CDC48/p97 is a highly abundant hexameric AAA-ATPase that functions as a molecular chaperone in numerous diverse cellular activities. We have identified an Arabidopsis UBX domain-containing protein, PUX1, which functions to regulate the oligomeric structure of the Arabidopsis homolog of CDC48/p97, AtCDC48 as well as mammalian p97. PUX1 is a soluble protein that co-fractionates with non-hexameric AtCDC48 and physically interacts with AtCDC48 in vivo. Binding of PUX1 to hexameric AtCDC48 is mediated through the UBX-containing C-terminal domain. However, disassembly of the chaperone is dependent upon the N-terminal domain of PUX1. These findings provide evidence that the assembly and disassembly of the hexameric CDC48/p97 complex is a dynamic process.

Introduction

CDC48/p97 have been found to be associated with various other proteins involved in vesicle trafficking (Pleasure et al., 1993; Sugita and Sudhof, 2000) and DNA replication and repair, however, its function in these pathways has not been well
characterized (Joaquin Partridge et al., 2003; Yamada et al., 2000; Zhang et al., 2000a). The ATP-driven conformational changes in CDC48/p97 are utilized most likely for protein assembly and disassembly in the various pathways. The functional versatility of CDC48/p97 appears to be mediated by multiple adapter/regulatory proteins that dictate the substrate specificity of this molecular chaperone. In the fusion of mammalian ER and Golgi membranes, a CDC48/p97 complex containing the adapter protein, p47, interacts with the t-SNARE, syntaxin 5 (Rabouille et al., 1995a; Roy et al., 2000b). Dissociation of CDC48/p97-p47 from syntaxin 5 has recently been shown to require an additional factor, VCIP135 (Kondo et al., 1997; Uchiyama et al., 2002). Mammalian p97, p47 and another cofactor, the heterodimeric complex, Ufd1-Npl4, have also been found to regulate sequential steps during nuclear envelope assembly (Hetzer et al., 2001). In addition, CDC48/p97 functions with Ufd1-Npl4 and other members of the UFD pathway, Ufd2 (Koegl et al., 1999) and Ufd3 (Ghislain et al., 1996) in ubiquitin-proteasome protein processing and degradation including ERAD (Braun et al., 2002; Hitchcock et al., 2001; Jarosch et al., 2002b; Rabinovich et al., 2002; Ye et al., 2003). Interestingly, CDC48/p97, the CDC48/p97-p47 complex, and Ufd1-Npl4 bind mono- and/or polyubiquitin chains (Dai and Li, 2001; Meyer et al., 2000; Ye et al., 2003). It remains to be determined how their interaction with ubiquitin contributes to two seemingly distinct processes such as Golgi assembly and protein degradation. Binding of p47, Ufd1-Npl4, and another recently identified CDC48/p97 adapter, SVIP (Nagahama et al., 2003), which is required for the maintenance of ER integrity, is mutually exclusive. These proteins appear bind to the N-terminal domains of CDC48/p97 thereby coupling the outside edge of the active hexameric barrel to its targets. In the case of p47, binding of the protein to the N-terminus
of CDC48/p97 is mediated through two contiguous sites; a carboxyl-terminal UBX domain and middle ~100 amino acid region (amino acids 171-270) of undetermined structure (Uchiyama et al., 2003; Yuan et al., 2001). UBX domains have been identified in a number of functionally diverse eukaryotic proteins, typically at the carboxyl-terminus, including the putative yeast ortholog of p47, Shp1p (Kondo et al., 1997), and the yeast Cui1-3p protein family, which are required for sporulation (Decottignies et al., 2004). Similar to p47, the Cui1-3p proteins bind to CDC48 via UBX domains. Structural studies of the UBX domain of the human Fas-associated factor-1 (FAF1) (Schuberth et al., 2004) and p47 (Yuan et al., 2001) have shown that the UBX domain adopts a characteristic ubiquitin fold. In contrast to ubiquitin, the UBX domain lacks the carboxyl-terminal double glycine motif necessary for conjugation to target proteins (Buchberger et al., 2001). Our previous studies have suggested that the plant orthologs of CDC48/p97 and another structurally related member of the AAA ATPase, the N-ethylmaleimide-sensitive fusion protein, NSF, function in distinct membrane fusion pathways at the plane of cell division to mediate plant cytokinesis (Rancour et al., 2002). The Arabidopsis CDC48/p97 and syntaxin 5 orthologs, AtCDC48 and SYP31, respectively were found to co-localize at the division plane during cytokinesis and to interact in vitro and in vivo. To characterize further the function of AtCDC48 and SYP31 we have utilized affinity chromatography and MALDI-MS to identify plant proteins that interact with SYP31 and/or modulate the activity of AtCDC48. Here we show that one member of the Plant UBX domain containing protein family (PUX), PUX1, regulates AtCDC48 by inhibiting its ATPase activity and by promoting the disassembly of the active hexamer. Phenotypic analysis of pux1 plants revealed that the loss of PUX1 accelerated the growth of various
plant organs including roots and inflorescence shoots (Rancour et al., 2004). These results suggest that PUX1 functions as a regulator of AtCDC48 and that the activity of these two proteins is required for various cellular pathways important for plant growth and development.

Materials and Methods

General reagents and antibodies

Molecular biology enzymes were purchased from New England Biolabs (Beverly MA) or Amersham Biosciences (Piscataway, NJ). An E. coli expression plasmid for mouse p97 was provided by H. Meyer (ETH, Zurich, Switzerland). Monoclonal mouse anti-mammalian NSF antibodies (2E5) (Tagaya et al., 1993) and mammalian NSF E. coli expression plasmid were provided by T. Martin (University of Wisconsin-Madison). Donkey anti-rabbit, sheep anti-mouse and rabbit anti-chicken horseradish peroxidase conjugates were purchased from Amersham Biosciences (Piscataway, NJ) and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All other reagents unless specified were from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). Protein assays were performed using a Bradford assay kit (BioRad, Hercules, CA) and BSA as a standard.

Oligonucleotides used in this study

All oligonucleotides used in this study were synthesized by Integrated DNA Technologies (Coralville, IA) (Table 1). Capitalized sequences represent those
complementary to the *PUX1* (At3g27310) and *AtCDC48* locus. Lower case nucleotides correspond to sequences added to aid in cloning. Translational stop codon sequences are underlined.

**Cloning of PUX1 cDNA, and protein expression**

A *PUX1* cDNA was obtained by RT-PCR using primers SB214/SB215 from T87 suspension-cultured cell total RNA prepared as described (Kang et al., 2001). The amplified product was cloned into pGEM-T easy (Promega, Madison, WI) and sequenced using primers SB93 and SB94. An *E. coli* protein expression vector was constructed by subcloning an EcoRI *PUX1* fragment from pGEMT-easy/*PUX1*-cDNA into pGEX4T-3 (Amersham Biosciences). This strategy resulted in the addition of 5 amino acids (DSLVI), encoded by flanking nucleotide sequence derived from the pGEMT-easy vector, into the linker between the GST and the N-terminus of the PUX1. pGEX4T-3-PUX1 in *E. coli* strain BL21(DE3)pLys was used for GST-PUX1 protein expression. Cultures were grown at 37°C and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 hours. GST-PUX1 protein was affinity purified using glutathione-sepharose 4B as described (Rancour et al., 2002). *E. coli*-expressed GST-free PUX1 was generated from GST-PUX1 using a Thrombin Cleavage Capture Kit (Novagen, Madison, WI).

**Cloning and tobacco BY2 cell expression of the N-terminal his\textsubscript{6} and T7-epitope tagged AtCDC48 (H6T7-AtCDC48)**

*AtCDC48*, cDNA amplified by PCR with primer set of SB260 and SB149 from pBS-AtCDC48 (Dickey and Bednarek unpublished data). *AtCDC48* was inserted into
XmaI and KpnI sites of pPZP211, plant expression vector (Hajdukiewicz et al., 1994) that has double CaMV (Cauliflower Mosaic Virus) 35S promoter (Covey et al., 1981; Hull and Covey, 1983; Odell et al., 1985) in the multiple cloning sites of HindIII and XbaI. NOS terminator, derived from the nopaline synthase gene of Agrobacterium tumefaciens, is located in SacI and EcoRI sites of pPZP211. The N-terminal his<sub>6</sub> and T7–epitope (H6T7) tag was generated by primer extension using complementary primers SB405 and SB406, and ligated into the SmaI restriction site 5’ of 74 bp upstream of the endogenous translational start site of AtCDC48 in pPZP211. The tobacco BY2 cell was transformed with the H6T7-AtCDC48 using the Agrobacterium tumefaciens-mediated co-cultivation as described (An, 1985).

**Cloning and *E. coli* protein expression of H6T7-AtCDC48**

The cDNA encoding the H6T7-tagged *AtCDC48* in pPZP211 was PCR amplified using primers SB439/SB371, and TA-cloned into pGEM-T Easy (Promega, Madison, WI) followed by sequencing using primers SB40, 42, 43, 62, 149, 407 and 408. A Ndel/NotI restriction fragment containing the H6T7-*AtCDC48* cDNA was cloned into pET29A for protein expression in *E. coli*. H6T7-AtCDC48 protein was expressed in the ROSETTA (Novagen, Madison, WI) strain of *E. coli*. Protein induction was performed at 28°C using 100 µM IPTG for 2 hrs and the H6T7-AtCDC48A was purified as described (Meyer et al., 2000) with the exception that 2 mM β-mercaptoethanol was used instead of DTT.
Functional complementation of *S. cerevisiae* cdc48 mutants

Two *cdc48* conditional *S. cerevisiae* mutants, DBY2030 (*MATa* ade2-101 lys2-801 ura3-52 cdc48-1 cs), and MLY2006 (*MATa* ura3-52 cdc48-2 ts) provided by Dr. M. Latterich (McGill University) (Patel et al., 1998) were used to confirm that H6T7-tagged *AtCDC48* was functional. H6T7-AtCDC48 was cloned in the sense and antisense orientations into the NotI sites of pFL61 (Minet et al., 1992), a yeast expression vector which gives constitutive expression from the phosphoglycerate kinase promoter. The vector pFL61, pFL61 expressing the sense *AtCDC48* and pFL61 expressing the antisense *AtCDC48* were transformed into the conditional *cdc48* mutants. *Ura*+ transformants were selected on Dropout medium (0.67% Bacto-yeast nitrogen base, and 2% Dextrose, addition of 0.2% Adenine sulfate and 0.3% L-lysine-HCl added for DBY2030) without uracil at 28°C. Transformants were subsequently re-streaked onto YPD medium (1% yeast extract, 2% Peptone, and 2% Dextrose) for analysis of functional complementation at the non-permissive temperature of 16°C for DBY2030 and 37°C for MLY2006, respectively.

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed as described (Kang et al., 2001) using the fixed Tobacco BY2 cell protoplasts. For double-immunolabeling experiments the cells were incubated sequentially with affinity-purified primary antibodies of different species followed by a mixture of the corresponding secondary antibodies labeled with FITC and Cy3. Wide field microscopy and image processing were performed as described (Kang et al., 2001). Separate images were collected from each fluorescence emission channel and images were processed using Photoshop 6.0 (Adobe Systems, San
Jose, CA). The labeling pattern observed in fixed protoplasts versus cells that were fixed prior to enzymatic removal of the cell wall resulted in no observed difference in the distribution of AtCDC48 or other marker proteins relative to the images shown in Figure 2B.

**Cloning and *E. coli* protein expression PUX1 truncation mutants**

GST-PUX1 truncation-mutant constructs were generated by PCR amplification of cDNA fragments from pGEX4T-3-*PUX1*. Fragments were cloned into a modified pGEX4T-3 vector with a Tev protease cleavage site (pGEX4T-3-TEV) (Rancour and Bednarek unpublished data) using either BamH I/Sal I or BamH I/EcoR I. The mutants were generated by PCR amplification using the following oligonucleotide sets: (a) N-terminus (amino acids 1-100; primers SB227 and SB483), (b) UBX (aa101-181; primers SB502 and SB484), (c) C-terminus (aa181-251; primers SB503 and SB228), (d) N-terminus+UBX (aa1-181; primers SB227 and SB484), and (e) UBX+C-terminus (aa101-251; primers SB502 and SB228). Proteins were expressed in *ROSETTA E. coli* at 37°C with 100 μM IPTG induction for 3 hrs and affinity purified using glutathione-sepharose 4B as described above. GST-free forms were generated by TEV protease treatment of glutathione-sepharose bound GST fusion proteins in TBS pH7.4 overnight at 22°C at a mass ratio of 1 mg recombinant His<sub>6</sub>-TEV to 20 mg GST-protein. Recombinant His<sub>6</sub>-TEV was removed using Ni-NTA agarose (Qiagen Inc., Valencia, CA) and the GST-free proteins were quantified and stored at –80°C. The purity of all *E. coli*-expressed proteins was assessed by SDS-PAGE and Coomassie staining.
PUX1 binding studies with AtCDC48, mouse p97 and mammalian NSF

Mammalian His6-p97 and His6-NSF-myc were prepared as described (Meyer et al., 2000; Söllner et al., 1993). PUX1 binding assays contained 10 μg/ml of purified AAA-ATPase in 200 μl binding buffer (20 mM HEPES pH 7.4, 150 mM KCl, 1 mM MgCl₂, 2 mM β-mercaptoethanol, 0.1% (v/v) TX-100). T87 S150 containing AtCDC48 was prepared as described (Rancour et al., 2002) and the concentration of AtCDC48 was quantified by immunoblotting and scanning densitometry using E. coli expressed H6T7-AtCDC48 as a standard. AtCDC48 constituted approximately 0.4 % (w/w) of a T87 S150 protein extract. Therefore, 275 μg S150 protein containing 1 μg (11.2 pmol) of AtCDC48 was utilized. Purified E. coli-expressed GST-PUX1 constructs were added in molar ratios to AtCDC48 of 0.5, 1, 2 and 3 moles and ratios of 1 and 2 for p97 and NSF, respectively. Reactions were incubated on ice for 30 min followed by affinity isolation using glutathione-sepharose 4B resin. Bound complexes were washed 3 times with binding buffer and processed for SDS-PAGE. AtCDC48 samples were processed for immunoblotting with anti-AtCDC48 antibodies. NSF and p97 protein samples were analyzed by SDS-PAGE followed by staining with Coomassie R-250.

Characterization of the oligomeric structure of AtCDC48/PUX1 complexes

E. coli-expressed H6T7-AtCDC48, His6-p97, and GST-free PUX1 were used to examine the effect of GST-free PUX1 on the oligomeric status of these AAA-ATPases. To test the effect of full-length and PUX1-domain fragments on the oligomeric status of AtCDC48 and p97, PUX1 derived proteins were mixed with the AAA-ATPase at molar ratios of 1:3, 1:1, and 3:1 PUX1:AAA-ATPase and incubated for 30 min on ice prior to
sucrose gradient fractionation. 30 µg of total protein was fractionated on a 5 ml 20-40% (w/w) sucrose gradient (20 mM HEPES pH 7.4, 150 mM KCl, 1 mM MgCl₂, 2 mM β-mercaptoethanol). Gradients were centrifuged at 128Kxg in a SW50.1 rotor (Beckman Coulter, Fullerton, CA) for 18 hrs at 4°C. The protein sedimentation standards used included ovalbumin (3.6 S, 43.5 kDa), bovine serum albumin (4.4 S, 66 kDa), and apoferritin (17.7S, 480 kDa). Standards were run on gradients parallel to samples. Fractions were collected and analyzed as described (Rancour et al., 2002).

Results

Generation of N-terminal-tagged AtCDC48

To characterize the biochemical role of AtCDC48, an epitope-tagged, *E.coli* expressed AtCDC48 was generated. The N-terminal his₆ and T7–epitope (H6T7) tagged AtCDC48 was generated in order to facilitate the detection and purification of AtCDC48 via immunoblotting and affinity chromatography (Figure 1A). *E.coli*-expressed H6T7-tagged AtCDC48 was further characterized by glycerol/sucrose gradient velocity sedimentation followed by immunoblot analysis as shown in Figure 1B. The fractionation profile of *in vivo* AtCDC48 was highly reproducible between separate gradients and a typical immunoblot of gradient fractions (Rancour et al., 2002). The majority of cytosolic AtCDC48 was found to sediment at ~17S. The peak of *E. coli*-expressed H6T7-tagged AtCDC48 sedimented at 17S consistent with an *in vivo* hexameric complex of AtCDC48. A minor fraction of AtCDC48 was also observed to sediment near ~4.4S (fraction 6) which is likely to correspond to monomeric AtCDC48. These results suggest that *E.coli*-


expressed H6T7-AtCDC48 is predominantly associated with homohexameric and high-ordered protein oligomers.

*E. coli* expressed oligomeric H6T7-AtCDC48 demonstrated ATP hydrolytic activity with a $V_{\text{max}}$ of 4.33 µM P$_i$ min$^{-1}$ µg$^{-1}$ and a $K_m$ of 128.7 µM in the presence of 5mM magnesium (see chapter 3); values indicative of an enzyme with much higher *in vitro* activity than reported for either purified Xenopus p97 (Peters et al., 1992) or *E. coli* expressed mammalian p97 (VCP) (Song et al., 2003). ATPase assays were carried out at 25°C for various time periods and the released inorganic phosphate was quantified by a malachite green assay as described (Cogan et al., 1999; Rowlands et al., 2004).

Feiler and colleagues (Feiler et al., 1995) have also been demonstrated that AtCDC48 could functionally replace yeast CDC48 *in vivo*. To determine the N-terminal tag attached AtCDC48 *in vivo* function, we tested the ability of the H6T7-AtCDC48 to rescue yeast *cdc48* mutants. H6T7-AtCDC48 fully rescued the growth of the yeast *cdc48-1* temperature and cold sensitive mutant at 37°C and 16°C (Figure 2A).

An additional test of function was to determine if H6T7-AtCDC48 exhibited an *in vivo* sub-cellular localization similar to AtCDC48, which has been shown to be re-localized from a diffuse cellular distribution to distinct localization at the division plane during plant cell cytokinesis. (Chapter 1, Figure 3B and C) (Rancour et al., 2002). Previously Feiler and colleagues (Feiler et al., 1995) also provided evidence that mammalian p97 antibodies weakly immunolabeled the phragmoplast midzone of dividing *Arabidopsis* cells. We therefore examined the localization of H6T7-AtCDC48 in dividing tobacco cells using affinity-purified AtCDC48-specific antibodies and anti-T7 antibodies (Figure 2B). H6T7-AtCDC48 was targeted to the division plane in transgenic tobacco
cells during cytokinesis (Figure 2B-C). Cells in cytokinesis (Figure 2B) were identified using DAPI, to detect binucleated cells, and anti-α-tubulin antibodies were used to visualize phragmoplast microtubules. These results taken together strongly support that the N-terminal epitope tagged AtCDC48 is functional.

**PUX1 interacts with the AtCDC48/p97 class of AAA-ATPases**

Subcellular fractionation and immunoblot analysis of *Arabidopsis* T87 cell extracts suggested that PUX1 was a soluble cytosolic protein. We wanted to know if PUX1 interacts with soluble AtCDC48. As shown in Figure 3B, GST-PUX1 precipitated cytosolic and purified AtCDC48. Full length GST-PUX1 was able to bind to equivalent amounts of either cytosolic AtCDC48 (S150) or H6T7-AtCDC48 (Figure 3B, middle panel) and to inhibit the ATPase activity of H6T7-AtCDC48. At molar ratios of 1:1 and 3:1 (PUX1: H6T7-AtCDC48), the ATPase activity of H6T7-AtCDC48 was 60% and 30% of untreated samples, respectively (data not shown).

To determine which domains of PUX1 interact with AtCDC48, a series of truncation mutants were generated and their capacity to bind AtCDC48 was tested. As shown in Figure 3B and Figure 7B (lane 3), PUX1/AtCDC48 binding was not dependent on the N terminus of PUX1 (N-term; aa 1-99, schematic in figure 3A). However, the PUX1 UBX-C truncation mutant (UBX-C; aa 100-251; schematic in figure 3A) interacted with H6T7-AtCDC48 with the same efficiency as full length PUX1 (Figure 3B, lower panel). Interestingly, the efficiency of binding of the UBX-C PUX1 mutant with cytosolic AtCDC48 was reduced relative to full length PUX1 (compare Figure 3B, lanes 25 and 30). *Arabidopsis* PUX1 was found also to bind to the mammalian ortholog
of AtCDC48, p97, but not to another related type II hexameric AAA-ATPase, NSF (Figure 3C). These results indicate that the activity of PUX1 is conserved but limited to the CDC48/p97 sub-family of type II AAA-ATPases.

**PUX1 facilitates disassembly of CDC48/p97 hexamers**

PUX1 physically interacts with AtCDC48 in vitro. Results from PUX1 immunoprecipitation experiments indicated that PUX1 with AtCDC48, physically interact in vivo. Glycerol gradient fractionation and immunoblot analysis of *Arabidopsis* cytosol demonstrated co-migration of PUX1 with non-hexameric AtCDC48. Thus, it was tested whether PUX1 modulated the oligomeric structure of AtCDC48. Purified GST-free PUX1 and H6T7-AtCDC48 fractionated by velocity sedimentation analysis as predicted for a monomer (~30 kDa), and hexamer (≥ 17.7 S), respectively (Figure 4B). A minor peak of non-hexameric *E. coli* expressed H6T7-AtCDC48A (≤8S) was also detected (Figures 4B). However, several significant changes occurred to the fractionation profiles of PUX1 and H6T7-AtCDC48 when they were mixed together at a molar ratio of 3:1 and fractionated by velocity sedimentation centrifugation (Figure 4C). First, the migration of PUX1 into the gradient increased in the presence of H6T7-AtCDC48 (Figure 4C, upper panel). Conversely, the sedimentation of H6T7-AtCDC48 was retarded in the presence of PUX1 such that it fractionated predominantly at ~8 S (Figure 4C, lower panel, migration peak at fractions 8 and 9) with little hexamer remaining (fraction 15). Cytosolic AtCDC48 present in an *Arabidopsis* S150 preparation also fractionated predominantly at ~8 S in presence of *E.coli* expressed PUX1 (Figure 5A). These results suggested that PUX1 facilitates the disassembly of hexameric AtCDC48, forming PUX1/AtCDC48
heterodimers. Similar to AtCDC48, we found that PUX1 mediated the disassembly of hexameric mouse p97 (Figure 5B). The efficiency of H6T7-AtCDC48 disassembly was proportional to the molar ratio of PUX1:H6T7-AtCDC48 used in the assay. PUX1-mediated disassembly of AtCDC48 was independent of exogenous nucleotide substrate (ATP), product (ADP), or a non-hydrolyzable analog (AMP-PNP) (Figure 6).

**PUX1 requires both the UBX-C terminus and the N-terminus for activity.**

As described above, the primary AtCDC48 interaction determinants of PUX1 were contained within the PUX1 UBX-C terminal fragment (Figure 3B). To test whether UBX-C was sufficient to facilitate the disassembly of the AtCDC48 hexamer, GST-free UBX-C PUX1 was incubated with H6T7-AtCDC48 at a molar ratio of 3:1, fractionated by velocity sedimentation centrifugation and analyzed by immunoblotting with anti-AtCDC48 antibodies (Figure 7A). In contrast to full length PUX1 (Figure 4C), the UBX-C and N-terminal fragments of PUX1 alone did not promote the disassembly of the AtCDC48 complex (Figure 7A). However, when the UBX-C and N-terminal domains were added in trans, significant, albeit not equivalent to full-length PUX1, levels of non-hexameric AtCDC48A were observed (Figure 7A, bottom panel).

Oligomerization of the UBX-C and N-terminal domains prior to their interaction with AtCDC48 may facilitate the in trans disassembly of hexameric AtCDC48. Alternatively, binding of the PUX1 UBX-C domain to AtCDC48 could permit subsequently the binding of the PUX1 N-terminus thereby promoting the disassembly of the core hexamer. The latter model would predict that interaction between the PUX1 N-terminus and AtCDC48 would be dependent on the presence of PUX1 UBX-C. To test
these models, we examined if a GST-N-terminal-PUX1 fusion protein interacted in vitro with the UBX-C domain in the presence or absence of H6T7-AtCDC48 (Figure 7B). As shown above (Figure 3B), GST-UBX-C interacts with H6T7-AtCDC48, however, the GST-tagged N-terminus of PUX1 alone did not interact directly with H6T7-AtCDC48 (Figure 7B, lane 3). Likewise, limited interaction between the UBX-C region and N-terminus of PUX1 was observed (Figure 7B, lane 1). However, binding of the PUX1 N-terminus with H6T7-AtCDC48 was enhanced by the presence of the PUX1 UBX-C domain (Figure 7B, lane 2).

Discussion

The functional oligomeric structure of CDC48/p97 AAA ATPases has been demonstrated by biochemical and structural studies to be a hexamer (Beuron et al., 2003; DeLaBarre and Brunger, 2003; Dreveny et al., 2004; Huyton et al., 2003; Lamb et al., 2001; Peters et al., 1992; Peters et al., 1990; Rabinovich et al., 2002; Rancour et al., 2002; Rockel et al., 2002; Rouiller et al., 2000; Rouiller et al., 2002; Zhang et al., 2000b). However, small but reproducible quantities of non-hexameric-associated CDC48/p97 have been observed in various organisms (Egerton and Samelson, 1994; Rancour et al., 2002; Roggy and Bangs, 1999). The hexameric structure of CDC48/p97 is likely to be an intrinsic property of the ATPases as urea-dissociated p97 subunits can self-reassemble (Wang et al., 2003a). Furthermore, recent evidence showing that epitope-tagged wild-type and ATPase-defective mutant subunits can be incorporated into pre-formed CDC48/p97 hexamers suggests that the oligomeric structure of the chaperone is dynamic
in vivo (Dalal et al., 2004; Lamb et al., 2001). The molecular mechanism by which subunit exchange occurs is unknown. To our knowledge, no reports have been made regarding the rate and mechanism of turnover for the CDC48/p97 oligomeric complexes. This work is the first evidence that suggests the quaternary structure of the CDC48/p97 family of AAA-ATPases is itself subjected to control and that this control has biological significance. In vitro assays have demonstrated that PUX1 promotes the disassembly of hexameric AtCDC48. Consistent with this, PUX1 co-fractionates and physically associates with soluble non-hexameric AtCDC48 from plant cell extracts.

Database analysis has shown that PUX1 orthologs exist throughout the plant kingdom and that functional homologs may also be expressed in animals and fungi. Indeed, it was shown that PUX1 interacted and promoted the disassembly of the hexameric mouse p97 complex (Figure 3C and 5B). The putative animal homologs of PUX1 can be distinguished from other UBX-containing proteins by the presence of a PUX1-like module that shares amino acid similarity to full-length PUX1 including conserved N- and C- terminal regions flanking a centrally located UBX domain. Interestingly, putative PUX1 orthologs have been identified in only a limited number of unicellular organisms.

A reoccurring feature, with regard to the binding to its various effector proteins, is CDC48/p97 ATPase interaction with ubiquitin and ubiquitin-related protein fold domains (Dai and Li, 2001; Dalal et al., 2004; Decottignies et al., 2004; Dreveny et al., 2004; Flierman et al., 2003; Jarosch et al., 2002b; Meyer et al., 2002; Rape et al., 2001; Uchiyama et al., 2002; Ye et al., 2001; Yuan et al., 2001). Targeting of the ATPase activity of CDC48/p97 to its various cellular substrates is dependent on specific protein
cofactors/adapters (Meyer et al., 2000). These adapters either recruit CDC48/p97 to mono- or poly-ubiquitinated substrates (Meyer et al., 2002) or the adapters themselves contain an ubiquitin-related protein domain (Decottignies et al., 2004; Dreveny et al., 2004; Kondo et al., 1997; Uchiyama et al., 2002; Yuan et al., 2001; Yuan et al., 2004). Several recent studies (Decottignies et al., 2004; Dreveny et al., 2004), including data presented here indicate that UBX domains may serve as a general interaction domain for CDC48/p97 (Decottignies et al., 2004; Dreveny et al., 2004). The Arabidopsis genome is predicted to encode 15 PUX proteins and our current working hypothesis is that this protein family may function as specific regulators of CDC48/p97.

Based upon crystallographic analysis, Dreveny and colleagues (2004) proposed that a FP-containing loop between the p47 UBX domain S3 and S4 β-strands (Buchberger et al., 2001) is required for interaction with the N-terminal domain of CDC48/p97. In contrast to p47, however, the FP-loop is not conserved in the PUX1 UBX domain. Further work is therefore necessary to define the structural feature(s) contained within the PUX1 UBX-C terminal fragment that are sufficient for its interaction with AtCDC48 and mouse p97. Similar to p47 (Uchiyama et al., 2002), it is shown that PUX1 contains a second binding site for AtCDC48 in the N-terminal (aa 1-100) fragment. However the binding of the PUX1 N-terminus to AtCDC48 is facilitated in trans by the UBX-C-terminal (aa 101-251) domain. Perhaps binding of the UBX-C domain to AtCDC48 causes a conformational change that allows the PUX1 N-terminus to bind. Alternatively, AtCDC48 may stabilize weak intramolecular interactions between the PUX1 N-terminus and UBX-C domain. Nevertheless the end result of PUX1 N-terminus binding is the disassembly and inactivation of the AtCDC48 oligomer.
PUX1 and PUX2 were identified *in vitro* binding assay under conditions that supported the ATP-dependent interaction of AtCDC48 with the *Arabidopsis* ortholog of mammalian syntaxin 5, SYP31 (Rancour et al., 2002). PUX1 and the putative *Arabidopsis* homologs of mammalian p47, PUX3 and PUX4 (which share 34 and 36% identity and 53 and 52% amino acid similarity, respectively, with the human protein), bind AtCDC48 *in vitro* but do not interact with SYP31. Rather, PUX2 functions as the *Arabidopsis* adapter for interaction between AtCDC48 and SYP31 (D. Rancour and S. Bednarek, in preparation). The role if any for PUX1 in AtCDC48A/SYP31 interaction remains to be determined. With the exception of AtCDC48 no other soluble proteins, including the AAA ATPase NSF, were found to interact with PUX1 suggesting that its function is limited to AtCDC48.

Our hypothesis is that PUX1-mediated disassembly regulates the overall cellular activity of AtCDC48. Previous studies have shown that the chaperone activity of CDC48/p97 is required for a variety of biochemical processes including and membrane fusion, proteolysis. Not surprisingly, mutations in CDC48/p97, that affect its activity and/or localization, cause defects in cell cycle progression and cell growth (Frohlich et al., 1991; Lamb et al., 2001; Madeo et al., 1998). Consistent with these observations, the loss of a negative regulator of AtCDC48 oligomerization and ATPase activity *in vitro* leads to enhanced root and shoot growth in *pux1* mutants (Rancour et al., 2004). The relative abundance of AtCDC48 (~1% total T87 protein of which ~85% is soluble) (Rancour et al., 2002)] versus PUX1 (~0.029% of soluble T87 protein) and small amount of AtCDC48-associated PUX1 suggests that PUX1-mediated regulation of AtCDC48 activity maybe highly potent during various stages of plant growth and development.
References


Table 1: Oligonucleotides used in study.

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<th>Name</th>
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Figure 1. Purification and partial characterization of *E. coli* expressed H6T7-AtCDC48

A) Total protein homogenate and Ni\(^{2+}\)-NTA-agarose purified protein from *E. coli* expressing H6T7-AtCDC48. Protein was analyzed by SDS-PAGE followed by coomassie staining.  B) Purified H6T7-AtCDC48 was subjected to sizing by 10 to 40% glycerol velocity sedimentation to examine the native oligomeric structure. Sedimentations standards are indicated.
Figure 2. Characterization of H6T7-AtCDC48: Yeast mutant complementation and tobacco cell expression with immunolocalization

A) Temperature sensitive (Ts) and cold sensitive (Cs) growth of conditional *S. cerevisiae cdc48* mutants H6T7-AtCDC48 cDNA cloned into Ts and Cs yeast *cdc48* conditional mutants A) Control; YPD media at permissive temperature of 28°C, A; Selective media at permissive temperature of 28°C B; Selective media at restricted temperature - Ts; 36°C, Cs; 16°C. Untransformed mutants and mutants transformed with the following constructs were analyzed. (Ts); 1, H6T7-AtCDC48, 2, H6T7 tagged anti-sense AtCDC48, 3, empty vector (PFL61), 4, Temperature sensitive *cdc48* mutant. (Cs); 1, H6T7-AtCDC48, 2, H6T7 tagged anti-sense AtCDC48, 3, Temperature sensitive *cdc48* mutant. B) Immunolocalization of CaMV (*Cauliflower Mosaic Virus*) 35S promoter driven *Arabidopsis* AtCDC48 in tobacco BY2 suspension cell line. 35S::H6T7-AtCDC48 transformed BY2 cells. Blue; nucleus, green; tubulin, red; H6T7-AtCDC48. Cell lines were labeled with either α-AtCDC48 or α-T7 antibody. Arrowheads indicate the division plane of dividing cells.
A

Control
YPD media at 28°C

A
selective media
28°C

B
selective media
36°C

Ts
YPD media at 28°C
selective media
28°C

selective media
16°C

Cs
YPD media at 28°C
selective media
28°C

selective media
16°C

B

BY2 cell (transformed with 35S::H6T7-AtCDC48)

A
Without α- AtCDC48

B
α- AtCDC48

C
α- T7
Figure 3. PUX1 interacts specifically with the CDC48/p97 class of AAA-ATPases

A) Protein domain organization of *Arabidopsis* PUX1. B) Interaction of PUX1 with AtCDC48 requires the UBX and C-terminus of PUX1. AtCDC48 immunoblot data for *in vitro* AtCDC48 binding assays performed with GST control (upper panel), full length GST-PUX1 (middle panel) and the PUX1 truncation mutant fusion protein, GST-UBX-C (lower panel). The source of AtCDC48 in the binding reactions (11.2 pmol per reaction) was from either a T87 cytosolic protein fraction (S150; lanes 1-5, 11-15, and lanes 21-25) or purified E. coli expressed AtCDC48 (His6-T7-AtCDC48; lanes 6-10, 16-20, and 26-30). GST and GST-PUX1 fusion proteins were added at molar ratios of 0.5 (lanes 2, 7, 12, 17, 22, and 27), 1 (lanes 3, 8, 13, 18, 23, and 28), 2 (lanes 4, 8, 14, 19, 24, and 29), and 3 moles (lanes 5, 10, 15, 20, 25, and 30) per mole of AtCDC48. AtCDC48 loading controls are given in lanes 1, 6, 11, 16, 21, and 26. C) PUX1 interacts with mammalian p97 but not NSF. Coomassie stained *in vitro* binding assay results of either mammalian p97 (upper panel) or mammalian NSF (lower panel). Interactions with GST (lanes 2, 3, 7 and 8) or GST-PUX1 (lanes 4, 5, 9, and 10) were tested. 11.2 pmol of p97 or NSF were incubated in the presence of GST fusion proteins added at molar ratios of 1 (lanes 2, 4, 7, and 9) and 2 moles (lanes 3, 5, 8, and 10) per mole of AAA-ATPase. Loading controls are presented in lanes 1 and 6 for p97 and NSF, respectively.
Figure 4. PUX1 promotes the disassembly of AtCDC48 hexamers

(A) PUX1 fractionates as a monomer. *E. coli* expressed PUX1 protein was fractionated by sucrose gradient [10-25% (w/w)] velocity sedimentation. (B) Active *E. coli* expressed H6T7-AtCDC48 fractionates as a hexamer. *E. coli* expressed His6-T7-AtCDC48 protein was fractionated by sucrose gradient [20-40% (w/w)] sedimentation. (C) Full length PUX1 promotes the disassembly of hexameric H6T7-AtCDC48 *in vitro*. *E. coli* expressed PUX1 and H6T7-AtCDC48 were mixed, incubated 30 min on ice and fractionated by sucrose gradient [20-40% (w/w)] sedimentation. Fractions were analyzed by immunoblotting using anti-PUX1 or anti-AtCDC48 antibodies as indicated. The migration of sedimentation marker proteins ovalbumin (3.6 S, 43.5 kDa), bovine serum albumin (4.4 S, 66 kDa) and/or apoferritin (17.7S, 480 kDa) is indicated.
Figure 5. PUX1 promotes the disassembly of cytosolic AtCDC48 hexamers and *E. coli* expressed mouse p97

A) Full length PUX1 promotes the disassembly of soluble hexameric AtCDC48. *E. coli*-expressed PUX1 and the *Arabidopsis* soluble fraction were mixed, incubated 30 min on ice and fractionated by sucrose gradient [20-40% (w/w)] sedimentation. B) PUX1 promotes the disassembly of *E. coli* expressed mouse p97. Fractions were analyzed by immunoblotting using α-PUX1 or α-AtCDC48 antibodies as indicated. The migration of sedimentation marker proteins ovalbumin (3.6 S, 43.5 kDa), bovine serum albumin (4.4 S, 66 kDa) and/or apoferritin (17.7S, 480 kDa) is indicated.
Figure 6. PUX1 promotes the disassembly of hexameric AtCDC48 regardless of exogenous nucleotides.

H6T7 AtCDC48 was incubated 30 min on ice with the indicated exogenous nucleotide (1mM ATP, ADP or AMP-PNP) in the absence (-) or presence (+) of *E.coli* expressed GST free PUX1 and fractionated by sucrose gradient (20-40%(w/v)) sedimentation. Fractions were analyzed by immunoblotting using anit-AtCDC48 antibodies.
**Figure 7. PUX1 requires both the UBX-C terminus and the N-terminus for activity**

A) Both the N-terminal and UBX-C terminal domains of PUX1 are required for AtCDC48 disassembly activity and can function in trans. E. coli-expressed PUX1 fragments (as indicated to left of blot panels) and His6-T7-AtCDC48A were mixed, incubated 30 min on ice and fractionated by sucrose gradient [20-40% (w/w)] sedimentation. Fractions were analyzed by immunoblotting for AtCDC48. The migration of sedimentation markers is indicated.

B) Binding of the PUX1 N-terminus to AtCDC48 is dependent on the UBX-C. *In vitro* binding assays were performed using GST-PUX1-N-terminus as bait. Incubations in the presence of the PUX1 UBX-C terminal fragment (lanes 1 and 2) or His6-T7-AtCDC48A (lanes 2 and 3) were assessed. Bound protein was analyzed by SDS-PAGE and immunoblotting for either PUX1 UBX-C (using anti-PUX1 antibodies; upper panel) or AtCDC48 (lower panel).
Chapter 3

Protein domain-domain interactions and requirements for the negative regulation of Arabidopsis CDC48/p97 by the plant UBX-domain containing protein, PUX1

Abstract

CDC48/p97 is an essential AAA-ATPase chaperone that functions in numerous diverse cellular activities through its interaction with specific adapter proteins. The UBX-containing protein, PUX1, functions to regulate the hexameric structure and ATPase activity of AtCDC48. To characterize the biochemical mechanism of PUX1 action on AtCDC48, we have defined domains of both PUX1 and AtCDC48 that are critical for interaction and oligomer disassembly. Binding of PUX1 to AtCDC48 was mediated through a region containing both the UBX-domain and immediate C-terminal flanking amino acids (UBX-C). Like other UBX domains, the primary binding site for the UBX-C of PUX1 is the Nₐ domain of AtCDC48. Alternative plant PUX protein UBX domains also bind AtCDC48 through the N-terminus but were found not to be able to substitute for the action imparted by the UBX-C of PUX1 in hexamer disassembly, suggesting unique features for the UBX-C of PUX1. We propose that the PUX1 UBX domain modulates a second binding site on AtCDC48 required for the N-terminal domain of PUX1 to interact with and promote dissociation of the AtCDC48 hexamer. Utilizing Atcdc48 ATP hydrolysis and binding mutants, we demonstrate that PUX1 binding was not affected but hexamer disassembly was significantly influenced by the ATP status of
AtCDC48. ATPase activity in both the D1 and D2 domains was critical for PUX1-mediated AtCDC48 hexamer disassembly. These results together provide new mechanistic insight into how AtCDC48 the hexameric status and ATPase activity is modulated.

**Introduction**

CDC48 and its mammalian ortholog, p97/VCP, are highly abundant and conserved members of the AAA family (ATPases associated with diverse cellular activities) of molecular chaperones (for review see (Lupas and Martin, 2002; Ogura and Wilkinson, 2001; Patel and Latterich, 1998b; Vale, 2000; Woodman, 2003)). The active form of CDC48/p97 is a ring-shaped complex comprised of six identical ~90 kDa subunits containing three domains; an N terminal domain followed by two Walker-type ATPase domains (D1 and D2) that are joined by conserved linker regions.

CDC48/p97 protomers have the intrinsic ability to self-oligomerize, thereby creating the active hexameric ATPase (Davies et al., 2005; Peters et al., 1992; Rockel et al., 2002). Recent studies have revealed that the D2 domain of CDC48/p97 is responsible for the major enzyme activity of the chaperone (Dalal et al., 2004; Lamb et al., 2001). In contrast to the D1 domain, mutations in the D2 domain that inhibit ATP hydrolysis or binding displayed dominant negative properties *in vivo*. Ultrastructural studies of p97 have suggested that CDC48/p97 nucleotide hydrolysis and exchange in the D2 ATPase domain result in conformational changes that drive movement of the N-terminal domain. This provides the mechanical force necessary to act on substrate proteins most likely for
protein assembly and disassembly (Beuron et al., 2003; Bruderer et al., 2004; Davies et al., 2005; Rouiller et al., 2002).

Targeting of CDC48/p97 activity to cellular pathways is accomplished via its recruitment by adapter/cofactor proteins. For example, p97 requires the cofactor p47 to modulate ER/Golgi membrane assembly (Kondo et al., 1997; Roy et al., 2000a; Yuan et al., 2001), nuclear envelope reformation (Hetzer et al., 2001), and spindle pole dynamics (Cao et al., 2003). Alternatively, the cofactor heterodimer complex Ufd1-Npl4 is required for p97-mediated formation of the chromatin-associated nuclear envelope network, consolidation of the nuclear envelope (Hetzer et al., 2001), and ERAD (Alzayady et al., 2005; Schuberth and Buchberger, 2005)-21. The interaction of studied adapter/cofactor proteins with CDC48/p97 occurs via the ~200 amino acid N-terminal domain of CDC48/p97 (Beuron et al., 2003; Bruderer et al., 2004; Meyer, 2005b; Rouiller et al., 2000). The N-terminal domain of CDC48/p97 is also responsible for multiubiquitin chain binding necessary for the in vitro degradation of target proteins (Dai et al., 1998; Song et al., 2003). However, binding of p47 and Ufd1-Npl4 to p97 is mutually exclusive (Meyer et al., 2000). Recent studies have also shown that proteins interact via the C-terminus of CDC48/p97 (Allen et al., 2006; DeLaBarre et al., 2006; Rientes et al., 2005) (Rancour et al., 2006 manuscript in prep).

The CDC48/p97 adapter p47 contains two independent CDC48/p97 binding sites, the ubiquitin regulatory X (UBX) domain and Binding Site 1 (BS1) (Bruderer et al., 2004; Uchiyama et al., 2002). The two binding sites interact independently with CDC48/p97. The UBX domain is comprised of ~80 amino acids and shares common secondary-structure organization with ubiquitin, characterized by a β-β-α-β-β-α-β-β-
Grasp fold. In contrast to ubiquitin, however, UBX domains lack the C-terminal glycine motif necessary for covalent coupling to target proteins and are typically located towards the C-terminus of a variety of eukaryotic proteins that interact with CDC48/p97 (Buchberger et al., 2001; Yuan et al., 2001). *S. cerevisiae* encodes seven UBX-containing proteins that have been shown to interact with Cdc48p. Deletion of the UBX domains abolishes their binding to Cdc48p indicating that the UBX domain is a general Cdc48p binding module (Schuberth et al., 2004). In addition to the UBX domain, p47 contains a second CDC48/p97 interaction domain known as BS1, which is located between the C-terminal UBX domain and a conserved SEP domain of undefined function. The hydrophobic residues of BS1 in p47 are conserved in Ufd1 (located between residues 215 and 241) and are believed to be involved in the exclusive nature of interaction of CDC48/p97 with either p47 or Ufd1-Npl4 (Bruderer et al., 2004).

The *Arabidopsis* genome encodes a family of 15 UBX containing proteins (Rancour and Bednarek, unpublished data). Recently we have shown that one of these proteins, PUX1, can regulate the oligomeric status and activity of the *Arabidopsis* CDC48/p97 ortholog, AtCDC48, as well as mammalian p97 but not the related AAA ATPase NSF (Rancour et al., 2004). *In vitro*, PUX1 association with the CDC48/p97 complex inactivated the ATPase and promoted the disassembly of the hexamer. Loss-of-function *pux1* mutants displayed accelerated growth relative to wild-type plants suggesting that PUX1 functions as a negative regulator of AtCDC48 function *in vivo*.

Here we have defined further the interaction regions between PUX1 and CDC48/p97 and the requirement of the D1 and D2 ATPase domains for PUX1-mediated
hexamer disassembly. Our results have demonstrated that the central UBX domain of PUX1 interacts through the N-terminus of AtCDC48. In addition we showed that the nucleotide status of AtCDC48 influences PUX1-mediated disassembly of the hexameric ATPase.

Materials and Methods

Oligonucleotides used in this study

All oligonucleotides used in this study were synthesized by Integrated DNA Technologies (Coralville, IA) (Table 1). Capitalized sequences represent those complementary to the PUX1, PUX5 or AtCDC48. Lower case nucleotides correspond to sequences added to aid in cloning. Restriction enzyme and point mutant sequences are underlined.

Expression and purification of GST-free and GST-tagged PUX fusion proteins

GST-tagged full-length PUX1, UBX-C (amino acids (aa) 101-251) and the N-terminal domain (aa 1-100) of PUX1 were prepared as described (Rancour et al., 2004). All other PUX1 truncation mutant constructs used in this study were generated by polymerase chain reaction (PCR) using primers listed in Table 1 and subcloned into the bacterial expression vector pGEX4T-3-TEV (Rancour et al., 2004). The PUX5 (At4g15410) cDNA clone C104907 (Yamada et al., 2003) was obtained from Arabidopsis Biological Resource Center (Ohio State University) and verified by DNA sequencing. The PUX5 cDNA was used as a template for PCR amplification with primers
SB814/815 (Table 1) of the region encoding the UBX domain (aa 335-421). The amplified product was cloned into pGEMT easy (Promega) verified by DNA sequencing and subcloned as an EcoR1 fragment into the bacterial expression plasmid pGEX4T-2-TEV (Rancour et al., 2004). Protein expression was performed in the ROSETTA (Novagen) E. coli strain. Cultures were grown at 37°C to mid-log phase and induced with 250 µm (PUX5 constructs) or 100 µm (PUX1 constructs) IPTG for 3 hours. Cells were disrupted by sonication in cold TBS pH 7.4, 1 mM DTT, 2.5 mM PMSF, 5 µg/ml pepstatin A, 1 µg/ml chymostatin, 1 mM p-aminobenzamidine, 1 mM e-aminocaproic acid, 5 µg/ml aprotinin and 1 µg/ml leupeptin and affinity purified using glutathione Sepharose 4B as described (Rancour et al., 2002). GST-free proteins were prepared by cleavage with His6-tagged TEV protease. All E. coli expressed proteins were quantified by Bradford using BSA as a standard, and their purity determined by SDS-PAGE and Coomassie Blue staining.

**Cloning and E. coli protein expression of H6T7-tagged AtCDC48 and Atcdc48 mutant proteins**

Full-length H6T7-AtCDC48 containing an N-terminal tag consisting of 6 histidine residues followed the 12 amino acid T7 epitope tag (H6T7) was generated as described (Rancour et al., 2004) and used for the production of H6T7-tagged Atcdc48 mutant proteins. Truncation mutant constructs used in this study were amplified by PCR from the H6T7-AtCDC48 cDNA using primers listed in Table 1. Single and double ATP binding and hydrolysis mutant of H6T7-Atcdc48 D1 (K254A and E308Q) and D2 (K527A and E581Q) were also engineered. Site directed mutagenesis by the Stratagene
Quik-Change protocol (see table 1 for oligonucleotide sequence details) was used to alter residues that have previously been shown to be required for CDC48/p97 ATPase activity (Song et al., 2003; Wang et al., 2003a; Wang et al., 2003b; Ye et al., 2003). For the binding mutants the lysine residue in the D1 and D2 Walker A motif was replaced with alanine and for the hydrolysis mutants the glutamate amino acid of the Walker B DExx motif was changed to glutamine (Figure 2A constructs 6-11). Mutant sequences were verified by DNA sequencing and subcloned into the bacterial expression vector pET29A (Novagen). Wild-type and mutant H6T7-AtCDC48 proteins were expressed in ROSETTA (Novagen) strain of E. coli at 28°C using 100 μM IPTG for 2 hours and purified using Ni-NTA (Qiagen Inc., Valencia, CA) as described (33).

**In vitro binding studies**

PUX1 binding assays contained 1 μg of purified E. coli expressed H6T7-AtCDC48 or H6T7-Atcdc48 mutant proteins in 100 μl of reaction buffer (20 mM HEPES/KOH pH7.4, 150 mM KCl, 1mM MgCl₂, 2 mM β-mercaptoethanol, 0.1 % (v/v) Triton X-100). Full-length and GST-pux1 truncation mutant proteins were added at a molar ratio of 3:1 relative to H6T7-AtCDC48 or H6T7-Atcdc48 mutant proteins. Reactions were incubated in reaction buffer for 30 minutes on ice followed by affinity isolation using glutathione-Sepharose 4B resin (Amersham Biosciences). Isolated complexes were washed five times with binding buffer and subjected to SDS-PAGE followed by either staining with Coomassie Brilliant Blue (R-250) or immunoblotting with anti-T7 antibodies (Novagen).
ATPase enzyme activities of Atcdc48 mutant proteins

Kinetic analysis was performed as previously described (Rancour et al., 2004) with purified *E. coli* expressed wild-type and mutant H6T7-Atcdc48. Fixed time point assays were conducted for 3 or 15 minutes in ATPase reaction buffer (20 mM HEPES/NaOH pH7.4, 150 mM NaCl, 5mM MgCl$_2$, 1mM DTT) with the specified ATP concentrations (0.05 – 0.2 mM) at 22°C. Colorimetric detection of phosphate product release was performed in a 96-well microtiter plate format and absorbance measurements were measured using a microplate reader (Bio-Tek, Winooski, VT) equipped with a 660-nm filter. Data analysis and curve fitting were performed using GraphPad Prism version 4 (GraphPad Software, Inc, San Diego, CA).

Analysis of oligomeric structure of full-length and mutant Atcdc48/pux1 complexes

To assess the oligomeric status of full-length and mutant H6T7-Atcdc48 and the effect of PUX1-derived proteins on their structure, PUX1-derived proteins were mixed with wild-type and mutant H6T7-Atcdc48 proteins at a molar ratio of 3:1 and incubated on ice for 30 minute prior to sucrose gradient fractionation. Approximately 20 μg of total protein was fractionated on a 5 ml 20~40 % (w/w) sucrose gradient (20 mM HEPES/KOH pH7.4, 150 mM KCl, 1mM MgCl$_2$, 2 mM β-mercaptoethanol). Protein sedimentation standards were analyzed on parallel gradients. Gradients were fractionated as described (Rancour et al., 2004) and the fractions were subjected to SDS-PAGE followed by immunoblotting with anti-T7 antibodies. Refractive indices of gradient fractions were used to allow comparison of different gradients.
Results

The PUX1 UBX domain interacts with AtCDC48

Recent studies have suggested that the UBX motif may serve as a general CDC48/p97 interaction domain (Bruderer et al., 2004; Decottignies et al., 2004; Dreveny et al., 2004; Schuberth and Buchberger, 2005; Schuberth et al., 2004). Consistent with this we have shown that a fragment of the PUX1 protein containing a UBX domain plus an additional 70 C-terminal amino acids (UBX-C aa 101-252; see figure 1A) binds to AtCDC48, together with the N-terminal domain (aa 1-100) of PUX1. This fragment can mediate AtCDC48 complex disassembly in trans (Rancour et al., 2004). Compared to the majority of UBX-containing proteins, the predicted PUX1 UBX domain however has several atypical features. First, the PUX1 UBX domain is positioned near the middle of the protein (Figure 1A). Second, the PUX1 UBX-domain lacks several critical features including the conserved set of amino acids “FP (phenylalanine-proline)” in the proposed protein loop between the S3 and S4 β-strands. These residues have been postulated to be required for the interaction of the p47 UBX domain with the N-terminus of CDC48/p97 (Bruderer et al., 2004; Dreveny et al., 2004)

To test if the isolated PUX1 UBX domain is sufficient for binding to AtCDC48, we examined the interaction of a GST-tagged fusion protein containing only the UBX domain lacking the C-terminal 70 amino acids with AtCDC48 in vitro. Based upon the SMART (http://smart.embl-heidelberg.de) protein motif search algorithm, the PUX1 UBX domain resides between amino acids 101 and 181. Unfortunately a GST-fusion protein containing this 81 amino acid region was insoluble when expressed in E. coli
Addition of the 13 and 30 amino acids that flank the N- and C-terminus of the “SMART-defined” UBX domain, respectively, were required to maintain the solubility of a GST-tagged UBX fusion protein (GST-UBX aa 88-211). As shown in figure 1B, GST-UBX (aa 88-211) bound to AtCDC48 with approximately the same efficiency as full-length PUX1 protein. In contrast, binding to AtCDC48 of a soluble truncated GST-UBX fusion protein, GST-UBX (aa 88-191), lacking the 19 C-terminal amino acid segment (aa 192-211) was nearly abolished (Figure 1B).

Similar to the PUX1 UBX-C-terminus truncation mutant (101-252) (Rancour et al., 2004), the GST-free UBX (aa 88-211) region greatly promoted the binding (Figure 1C lane 5) of the PUX1 N-terminal domain (1-100) to AtCDC48 and subsequent dissociation of the AtCDC48 hexamer in trans (data not shown). In the absence of GST-free UBX (aa 88-211) we did not detect any interaction between AtCDC48 and the PUX1 N-terminal domain (Figure 1C lane 3).

We next wanted to examine if the UBX domain from another AtCDC48-interacting PUX protein could also mediate the interaction of the N-terminus of PUX1 with AtCDC48 or if this is a unique property of the PUX1 UBX domain. For these experiments the UBX domains of PUX5 (At4g15410) and PUX4 (At4g04210) were chosen to be analyzed due to their high sequence similarity to mammalian p47. PUX5, PUX4, and PUX3 (At4g22150) represent the closest Arabidopsis homologues to mammalian p47 (Rancour and Bednarek, unpublished data). As shown in Figure 1D (lane 2) a GST-fusion protein containing the PUX5 UBX domain (aa 332-421) (Figure 1A) bound to AtCDC48. In contrast however to PUX1 UBX (aa 88-211), GST-free PUX5 UBX (aa 332-421), which binds to AtCDC48 with similar efficiency as GST-PUX5 UBX
(data not shown), did not promote binding of the PUX1 N-terminal domain (Figure 1D lane 5). Similar results were obtained with the UBX domain from PUX4 (data not shown). These data suggest a functional difference exists between UBX domains for CDC48/p97 complex disassembly.

**Domain interactions between AtCDC48 and PUX1**

Previous studies have shown that the UBX domain of p47 and several other UBX-domain containing proteins binds to the N-terminal domain of CDC48/p97 protomers (Bruderer et al., 2004; Decottignies et al., 2004; Dreveny et al., 2004; Romisch, 2006; Schuberth and Buchberger, 2005; Schuberth et al., 2004). Given the difference we observed in PUX1 and PUX5 UBX domain-mediated hexamer disassembly, we wanted to define the region(s) of AtCDC48 required for PUX1 interaction with the hexameric ATPase. For this purpose, we generated several soluble ATPase active (see below) AtCDC48 truncation mutants (Figure 2A and B, 2-5) and tested their ability to bind GST-PUX1 in vitro (Figure 3A). Binding of PUX1 to the N-terminal deletion mutant (aa 192-809) was not detectable (Figure 3A) even though the mutant was competent to assemble into an ATPase active oligomeric complex (Figure 3B). In addition, velocity sedimentation analysis demonstrated that PUX1 was unable mediate disassembly of the N-terminal deletion mutant complex, AtCDC48 (aa 192-809) (Figure 3B). However, PUX1 did bind to the AtCDC48 truncation mutant containing only the N-terminal 213 amino acids (Figure 3A, lane 4). Therefore, the N-terminus of AtCDC48 is the main interacting domain of PUX1.
To define further which sub domain(s) of the N-terminus of AtCDC48 are required for interaction with PUX1, GST-tagged protein fusion containing the N terminal AtCDC48 sub domains, N_a (aa 1-114) and N_b (aa 115-213) were generated based on previous studies (DeLaBarre and Brunger, 2003). \textit{In vitro} binding studies demonstrated that the N_a (aa 1-114) region of AtCDC48 contains critical binding site(s) for PUX1 (Figure 3A). These data support a model whereby PUX1 associates primarily through the N-terminal domain of AtCDC48 to mediate complex disassembly.

**Role of ATP binding and hydrolysis in PUX1-mediated AtCDC48 interaction and dissociation**

To determine if the ability of PUX1 to bind and/or disassemble oligomeric AtCDC48 is dependent upon the ATPase activity of the individual and/or combined contribution of the D1 and D2 domains of the chaperone, we generated a series of AtCDC48 truncation and point mutants (Figure 2) that putatively affect the function of the D1 and D2 ATPase domains.

\textit{E. coli} expressed Atcdc48 mutant proteins (Figure 2A constructs 6-11) were soluble and their purity was confirmed by SDS-PAGE and Coomassie Blue staining (Figure 2B lanes 6-11). In contrast, the Atcdc48 truncation mutant (aa 214-809) lacking both the N-terminal domain as well as the 22 amino acid linker region, L1, showed no detectable ATPase activity and did not properly assemble into a hexameric complex (data not shown) and was therefore not further characterized.

Previously we have shown that \textit{E. coli} expressed wild-type AtCDC48 has robust ATP hydrolytic activity. The enzyme exhibited a $K_m$ of 40.5 $\mu$M and a $V_{max}$ of 12.5 $\mu$M.
Pi min$^{-1}$ µM$^{-1}$ (Rancour et al., 2004). Several of the other mutant proteins also exhibited detectable ATPase activity including the truncation mutants, Atcdc48 (aa 192-809) and Atcdc48 (aa 1-475) and the ATP hydrolysis and binding point mutants, Atcdc48 (E308Q), and Atcdc48 (K254A), respectively (Table 2). However, these mutant proteins displayed distinct properties compared to the wild-type protein. In contrast to 214-809 the N-terminal deletion mutant Atcdc48 (aa 192-809), which contained the L1 segment was found to assemble into hexameric complexes (Figure 3B). Atcdc48 (192-809) mutant exhibited a $V_{max}$ of 17.2 µM Pi min µM$^{-1}$, which is 1.4 times the wild-type $V_{max}$ of 12.5 µM Pi min$^{-1}$ µM$^{-1}$ (Table 2). In addition, this truncation mutant exhibited cooperativity in its ATPase cycle manifest in a Hill coefficient of 2 (Figure 4).

As shown in Table 2, D2 ATP hydrolysis (E581Q), D2 ATP binding (K527A) mutants, and both D1/D2 ATP hydrolysis (E308/581Q) and binding (K254/527A) double mutants did not exhibit any detectable ATPase activity. However, both D1 ATP hydrolysis (E308Q) and ATP binding (K254A) mutants exhibited reduced but detectable ATPase activity levels relative to the wild-type protein. The D1 hydrolysis mutant had higher ATPase activity, 8.75 µM Pi min$^{-1}$ µM$^{-1}$, than the D1 ATP binding mutant, 3.5 µM Pi min µM$^{-1}$.

To determine whether ATP binding and/or hydrolysis influence binding of PUX1 to AtCDC48, wild-type and mutant H6T7-Atcdc48 were incubated with GST-PUX1 in the presence and absence of ATP, and affinity purified using immobilized glutathione. As shown in figure 5A, PUX1 interaction with AtCDC48 was independent of the nucleotide status of the hexameric complex. In addition, binding of the PUX1 N-terminal domain in the presence of the PUX1 UBX-C truncation mutant to the hexameric
chaperone was not dependent upon H6T7-Atcdc48 ATP hydrolysis or binding (Figure 5B). Surprisingly, unlike wild-type AtCDC48, interaction of the PUX1 N-terminal domain with Atcdc48 double hydrolysis or binding mutants in the absence of PUX1 UBX-C was extremely low but detectable (Figure 5B, lanes 3 and 8, respectively). Therefore, binding of PUX1 to AtCDC48 is not dependent on the ATP status nor the capacity of AtCDC48 to bind or hydrolyze ATP.

Though binding of PUX1 to AtCDC48 is not dependent on ATP, we wanted to test whether the PUX1-mediated disassembly of AtCDC48 hexamers was influenced by the ATP status of AtCDC48. We first verified by velocity sedimentation analysis that all single and double mutants assembled into oligomeric complexes with sedimentation characteristics indistinguishable from wild-type E. coli expressed H6T7-AtCDC48 (Figure 6) (Rancour et al., 2004). We then tested whether PUX1 could promote the disassembly of these mutant forms of AtCDC48. As shown in figure 6A and B, PUX1 facilitated dissociation of the single D1 or D2 ATP hydrolysis (E308Q and E581Q) and ATP binding (K254A and K527A) mutant oligomeric H6T7-Atcdc48 complexes. PUX1-mediated disassembly of hexameric D1/D2 double ATP hydrolysis and binding mutants (Figure 6A and 6B), however, was significantly inhibited.

Discussion

Plant UBX-containing protein 1, PUX1, was identified through protein interaction studies and found to be a regulator of AtCDC48 function in vitro and in vivo (Rancour et al., 2004). This work provided direct evidence that the hexameric structure of
CDC48/p97 family member is under dynamic protein-mediated regulation. In this study, we focused in greater detail on the structural and nucleotide requirements for PUX1 interaction with AtCDC48 and their role in the AtCDC48 disassembly process. This work has identified several distinct features of AtCDC48 that regulate hexamer formation and influence its ATPase activity. These are likely to be common to the regulation of other CDC48/p97 family members.

Previous work has demonstrated the capacity of CDC48/p97 orthologs to bind ubiquitin and ubiquitin-like protein folds of the β-GRASP fold family (Buchberger et al., 2001; Yuan et al., 2001). This protein-protein interaction feature is used to facilitate the recruitment of CDC48/p97 to a variety of biological processes via either adapters that contain these protein folds or by direct binding to ubiquitin. Our previous work with PUX1 established the requirement of a domain region that included the PUX1 UBX-C terminus region (aa 101-252) in binding AtCDC48. Further deletion analysis of PUX1 (Figure 1A) narrowed the domain requirements of PUX1 for binding to AtCDC48 to PUX1 amino acids 88-211, which included the UBX domain and 30 amino acids of C-terminal flanking sequence. Inclusion of these 30 additional C-terminal flanking amino acids had significant consequences for binding but it is unclear whether this is due to direct binding of the flanking amino acids to AtCDC48 or their requirement for truncated protein stability. These issues remain to be tested.

CDC48/p97 interaction with ubiquitin and ubiquitin-like protein folds of the β-GRASP fold family have been shown to be mediated through the N-terminal domain of CDC48/97. Similarly, we show the primary binding interaction of PUX1 with AtCDC48 occurs through the N-terminus of AtCDC48 (Figure 3A). Truncation mutants of
AtCDC48 lacking the N-terminal domain (aa 192-809) lose their ability to bind PUX1. The N-terminal domain of CDC48/p97 has been further divided into two subdomains, referred to Na and Nb, based on structural analysis of CDC48/p97 (DeLaBarre and Brunger, 2003). Analogous truncation mutants of AtCDC48 were made and protein-binding studies indicated that the Na region from AtCDC48 (aa 1-114) was necessary and sufficient for binding of PUX1 UBX-containing regions (Figure 3A). These data, taken together, suggest that PUX1 has evolved to take advantage of the conserved binding site for β-GRASP folds to bind and mediate hexamer disassembly. Binding of the PUX1 UBX domain to AtCDC48 is mutually exclusive with the UBX domain of PUX4, which displays amino acid sequence similarity to mammalian p47 (data not shown).

In addition to the Na and Nb regions, our data also indicated that the linker 1 between N-terminus and D1 domain of AtCDC48 was critical for hexamer stability and ATPase activity of AtCDC48. Without linker 1, the N-terminus deletion mutant of Atcde48 did not form an oligomer and thus lost its ATPase activity.

It has been reported that the conformation of the N-terminus of CDC48/p97 changes upon a nucleotide binding and hydrolysis (Beuron et al., 2003; Davies et al., 2005; Rouiller et al., 2002; Zhang et al., 2000b). In addition to the binding of interacting proteins, the N-terminal domain of AtCDC48 regulates the ATPase activity of AtCDC48. An Atcdc48 lacking its N-terminal domain (retaining aa 192-809) showed cooperative ATP binding and higher ATPase activity than wild type (Figure 4). Similarly, the N-terminal deletion of the Thermoplasma VCP-like ATPase (VAT) showed up to 24 fold enhanced ATP hydrolysis and 250 fold enhanced protein unfolding activity than wild type VAT indicating that N terminus either regulates and/or inhibits the ATPase activity
of VAT (Gerega et al., 2005). Our hypothesis is that the N-terminus of AtCDC48 is important for negatively regulating ATP hydrolysis in the absence of protein substrates.

In addition to the structural requirements necessary for PUX1 binding, we also showed that PUX1 interaction with AtCDC48 was independent of ATP hydrolysis and binding in the D1 or D2 ATPase domains (Figure 5A). Full-length *E. coli* expressed AtCDC48 forms functional hexamer and assembly of oligomer is not affected by the exclusion of exogenous nucleotide nor the presence of exogenous ATP, ADP, and AMP-PNP (Rancour et al., 2004). Likewise the Walker A/Walker B mutant Atcdc48 proteins generated in this study assembled into hexamers providing additional evidence that hexamerization of AtCDC48 is independent of the nucleotide hydrolysis and/or binding capacity of the ATPase domains (Figure 6).

Previously, we showed that PUX1-mediated AtCDC48 hexamer disassembly is partially inhibited in the presence of the ATP analog, AMP-PNP (Rancour et al., 2004). In this manuscript we have investigated the individual and combined contributions of the ATPase domains for PUX1-mediated AtCDC48 disassembly. Single Walker A/Walker B ATP hydrolysis and binding Atcdc48 mutant proteins were found to be sensitive to PUX1 mediated disassembly (Figure 6). However, the double Walker A and B mutants exhibited significant inhibition in their ability to go through the disassembly process than either of the single ATPase domain mutants. We hypothesize that inhibition of both D1 and D2 ATPase domains, alters the conformational flexibility of AtCDC48 preventing the necessary conformational changes required for hexamer disassembly. These conformational changes are apparent due to the ability of the PUX1 N-terminus to bind in the absence of the UBX-C (Figure 5B, lanes 3 and 8) but the mechanistic downstream
steps for disassembly do not occur nor have they been resolved. Unfortunately, attempts at mapping the binding site of the PUX1 N-terminus have not been successful to date.

The overall structure of the CDC48/p97 hexamer is drastically influenced by the ability of the two ATPase domains to bind and subsequently hydrolyze ATP (Davies et al., 2005; Rouiller et al., 2002). These dynamic structural changes are postulated to be the primary mechanism through which the CDC48/p97 family of ATPases function as molecular chaperones. This plasticity in structure appears to be taken advantage of by PUX1 resulting in the demise of AtCDC48 through disassembly of the active hexamer.

**References**


Table 1: Oligonucleotides used in this study

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<th>NAME</th>
<th>SEQUENCE (5'-3')</th>
<th>PURPOSE</th>
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<td>CATATTCATTGATCAGATCGACTCTATTGCACCG</td>
<td>5' Site-directed mutagenesis for Atcdc48 E308Q</td>
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<td>5' H6T7-AtCDC48; NdeI</td>
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<td>5' 13 amino acid extension from UBx of PUX1; BamHI</td>
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<tr>
<td>SB525</td>
<td>cGCATCCGCCAGTTTTTCCG</td>
<td>5' 27 amino acid extension from UBx of PUX1; BamHI</td>
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<td>3' 10 amino acid extension from UBx of PUX1; EcoRI</td>
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Table 2. Steady-State Enzyme Kinetics

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</tr>
<tr>
<td>192-809</td>
<td>100</td>
<td>17.20</td>
<td>1.4</td>
</tr>
<tr>
<td>214-809</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1-461</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1-475</td>
<td>16*</td>
<td>4.1*</td>
<td>0.3</td>
</tr>
<tr>
<td>E308Q</td>
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<td>8.75</td>
<td>0.69</td>
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<tr>
<td>E581Q</td>
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<td>E308/581Q</td>
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* The mutant exhibited minimal activity that did not be fit with Michaelis-Menten kinetics. Best estimates of the value is reported.
- no detectable activity
Figure 1. The UBX (88-211) domain of PUX1 is required for interaction with AtCDC48

A) Protein domain organization of Arabidopsis PUX1 and PUX5 (At4g15410). B) E. coli expressed GST-UBX truncation fusion proteins of PUX1 (lanes 2 and 3) or full length GST fusion of PUX1 (lane 4) were incubated with H6T7-AtCDC48 protein at a 3 to 1 molar ratio for 30 minutes at 4°C, isolated with gluthathione-Sephrose 4B resin and analyzed by immunoblotting with anti-T7 antibody. Purified PUX1-UBX truncations or full length PUX1 were incubated with AtCDC48. C) PUX1-N terminus binds AtCDC48 in the presence of the PUX1 UBX (88-211). GST or GST-fusion protein of PUX1 was incubated with H6T7-AtCDC48. Protein complexes isolated with gluthathione-Sephrose 4B, washed and analyzed by SDS-PAGE and coomassie blue staining. D) PUX5 UBX binds AtCDC48 but does not promote PUX1 N-terminus binding. GST or GST-fusion protein of PUX1 was incubated with H6T7-AtCDC48. Protein complexes isolated with gluthathione-Sephrose 4B, washed and analyzed by SDS-PAGE and coomassie blue staining. I;
Figure 2. H6T7-AtCDC48 truncation and point mutants used in this study

A) Schematic representation of AtCDC48 wild type and mutant proteins. The site-specific mutations in the ATPase domains are depicted by \( \Delta \) (D1) \( \nabla \) (D2) for ATP hydrolysis and \( \blacktriangle \) (D1) \( \blacktriangledown \) (D2) for ATP binding mutants.  

B) Purified wild-type and mutant H6T7-Atcdc48 proteins. One microgram of H6T7-tagged fusion proteins were resolved by SDS-PAGE and stained with Coomassie Blue. The molecular mass markers are shown on the left (M) and lane numbers shown above correspond to order of wild type (1) mutant (2)-(11) shown in figure 2A.
Figure 3. The $N_a$ subdomain of the AtCDC48 N-terminus (amino acids; 1-114) is the primary binding site for PUX1

A) *E. coli* expressed GST fused PUX1 and H6T7-Atcdc48 truncation protein binding assays. Atcdc48 truncation mutant proteins were incubated with either GST fused PUX1 (lanes 2, 4, 6, and 8) or GST alone (lanes 1, 3, 5, and 7) for 30 minutes at 4°C. Protein complexes were isolated with gluthathione-Sepharose 4B. Bound protein was washed extensively and analyzed by SDS-PAGE followed by immunoblotting for H6T7 Atcdc48 using anti-T7 antibody. B) *E. coli* expressed GST-free PUX1 and H6T7 tagged Atcdc48 (192-809) were incubated together at a 3 to 1 molar ratio on ice for 30 min in the absence of exogenous nucleotide and fractionated by sucrose gradient (20-40%) velocity sedimentation. Gradient fractions were analyzed by SDS-PAGE followed by immunoblotting. The migration of sedimentation marker proteins bovine serum albumin (4.4 S, 66 kDa) and apoferritin (17.7 S, 480 kDa) is indicated. Protein peak migrations were identical between gradients as determined by the refractive indices of the gradient fractions.
A

Atcdc48a mutants (amino acids) (192-809) (1-213) (1-114) (115-213)
GST-PUX1 - + - + - + - +
GST + - + - + - - +

B

Sucrose
20%  1  5  10  15  20  40% (w/w) 

Atcdc48 (192-809) 
Atcdc48 (192-809) + PUX1

α-T7

↑ 4.4S
↑ 17.7S
Figure 4. ATPase activity of AtCDC48 and Atcdc48 mutant (192-809)

The substrate concentration dependence of ATPase activity for full length and 192-809. 10-min fixed point ATPase assay was used. Values represent the mean ± S.D. of 3 independent measurements.
The diagram shows the relationship between ATP concentration (mM) and released phosphate (μM/μM of protein) for two different protein samples: 192-809 (△) and Full length AtCDC48 (□). The graph illustrates an increase in released phosphate as ATP concentration increases for both samples, with the 192-809 sample showing a slightly higher or steeper increase compared to the Full length AtCDC48 sample.
Figure 5. Binding of PUX1 and AtCDC48 is ATP independent

A) *E. coli* expressed GST-PUX1 and H6T7-Atcdc48 ATPase mutant protein binding assays. Purified GST (lanes 1, 4, 7, 10, 13, 16, 19, and 22) or GST-PUX1 was incubated with Atcdc48 mutant proteins in the presence (lanes 2, 5, 8, 11, 14, 17, 20, and 23) or absence (lanes 3, 6, 9, 12, 15, 18, 21 and 24) of 1mM ATP for 30 minutes at 4ºC. B) *In vitro* binding assays were performed using GST fused N-terminus of PUX1 with either Atcdc48 (E308Q/E581Q) (lane 1-5) and Atcdc48 (K254A/K527A) (lane 6-10). Protein complexes were isolated, washed, and bound protein was analyzed by SDS-PAGE followed by immunoblotting for Atcdc48 using anti-T7 antibody. I; Input
Figure 6. ATP hydrolysis and binding in both D1 and D2 ATPase domain is required for PUX1-mediated AtCDC48 disassembly

A) Atcdc48 double ATP hydrolysis mutant is resistant to PUX1 mediated disassembly.
B) Atcdc48 double ATP binding mutant is resistant to PUX1 mediated disassembly.

Sucrose gradient sedimentation analysis of Atcdc48 site-directed mutant proteins in the absence or presence of PUX1. *E. coli* expressed GST-free PUX1 (26.8 µg) and H6T7 tagged Atcdc48 mutant proteins (20 µg) were mixed in a 3 to 1 ratio, incubated 30 min on ice in the absence of exogenous nucleotide and fractionated by sucrose gradient (20-40% (w/w)) velocity sedimentation. The migration of sedimentation marker proteins bovine serum albumin (4.4 S, 66 kDa) and apoferritin (17.7 S, 480 kDa) is indicated. A, Atcdc48 ATP binding mutants and PUX1 complex.
**A**

Sucrose

Atcdc48(E308Q)

Atcdc48(E308Q) + PUX1

Atcdc48(E581Q)

Atcdc48(E581Q) + PUX1

Atcdc48(E308Q/E581Q)

Atcdc48(E308Q/E581Q) + PUX1

---

**B**

Sucrose

Atcdc48(K254A)

Atcdc48(K254A) + PUX1

Atcdc48(K527A)

Atcdc48(K527A) + PUX1

Atcdc48(K254A/K527A)

Atcdc48(K254A/K527A) + PUX1

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Chapter 4

Characterization of Atcdc48 mutants and the localization study of AtCDC48 in Arabidopsis thaliana

Abstract

CDC48/p97 is an essential conserved AAA-ATPase chaperone that functions in numerous diverse cellular activities. CDC48/p97 is recruited to perform its specific functions through its interaction with adapter proteins. Our working hypothesis is that the Arabidopsis CDC48 homolog (AtCDC48) has a necessary role in plant cytokinesis and cell expansion. We have used reverse genetic and biochemical approaches to elucidate the function of AtCDC48 in plants. Our data shows that AtCDC48 is essential for plant growth and development. AtCDC48 is localized throughout the cell including the nucleus, cytoplasm, ER, and the division midzone. The homozygous Atcdc48 T-DNA insertion mutants are not viable. AtCDC48 is necessary for at least three stages of early development: fertilization, embryo development, and seedling growth. In order to avoid lethality of Atcdc48 mutants, we used an ethanol-inducible dominant negative system to study the functions of AtCDC48 during each developmental stage. Our data indicate that AtCDC48 is required during growth and development of the shoot apical meristem and the primary root.
Members of the AAA (ATPase Associated with different cellular Activities) ATPase protein family are characterized by either one (type I) or two (type II) 220-250 amino acid ATPase domains containing conserved Walker A and B motifs (Neuwald et al., 1999). The conservation and widespread use of the AAA domain suggests that AAA ATPase proteins may use common mechanisms that utilize their ATPase activity to carry out a wide range of cellular functions.

Targeting of the AAA ATPase family member CDC48/p97 to cellular pathways is accomplished via recruitment of adapter proteins or cofactors. For example the mammalian/animal homolog of CDC48, p97, requires the cofactor p47 to mediate ER and Golgi membrane assembly (Kondo et al., 1997; Roy et al., 2000a; Yuan et al., 2001), nuclear envelope reformation (Hetzer et al., 2001), and spindle pole dynamics (Cao et al., 2003). Alternatively, the cofactor complex Ufd1-Npl4 is required for p97-mediated formation of the chromatin-associated nuclear envelope network, consolidation of the nuclear envelope (Hetzer et al., 2001), and ERAD (Alzayady et al., 2005; Schuberth and Buchberger, 2005) (Romisch, 2006).

In *S.cerevisiae*, it has been shown that Cdc48p is essential for cell cycle progression and has a role in spindle pole body duplication and separation (Frohlich et al., 1991). Conditional mutants of *cdc48* arrest in the medial nuclear division stage of the cell division cycle with a large bud and microtubules indicating arrest occurs at a critical checkpoint during G2 (Frohlich et al., 1991). It has also been reported that Cdc48p has the role in sister chromatid separation in fission yeast *Schizosaccharomyces prombe* (Ikai
Yanagida, 2006) and Cdc48 and its adapters, Ufd1-Npl4, has been shown to regulate spindle disassembly at the end of mitosis in *Xenopus* (Cao et al., 2003; Cao and Zheng, 2004; Cheeseman and Desai, 2004). Eyes closed (Eyc) is the *Drosophila* p47 homolog. Loss of Eyc function causes a lethal failure of nuclear envelope assembly in early zygotic divisions in *Drosophila* (Sang and Ready, 2002). These results taken together illustrate the significant importance of the CDC48/p97 in various aspects of cell cycle control and organismal development.

The *Arabidopsis* genome encodes three *CDC48* isoforms: *AtCDC48A*, *AtCDC48B* and *AtCDC48C*. *AtCDC48A* (hence forth referred to as *AtCDC48*) is presumed to be the most abundant isoform because there exists greater than 250 expressed sequence tags (ESTs) and several full-length cDNAs in the *Arabidopsis* Information Resource (TAIR, http://www.arabidopsis.org) and Salk Institute Genomic Analysis Laboratory (SIGnAL, http://signal.salk.edu/cgi-bin/tdnaexpress) databases compared with those identified to date for *AtCDC48B* (19 ESTs) and *AtCDC48C* (15 ESTs) (Rancour et al., 2002). These isoforms are predicted to share 91% (*AtCDC48B*) and 95% (*AtCDC48C*) amino acid identity with full length AtCDC48. Feiler and colleagues (Feiler et al., 1995) demonstrated that AtCDC48 is highly expressed in the proliferating cells of the vegetative shoot, root, and flowers in rapidly growing plants. In addition, immunolocalization studies showed that AtCDC48 was primarily localized to the nucleus and the phragmoplast during cytokinesis (Feiler et al., 1995; Rancour et al., 2002). However, extensive characterization of AtCDC48 and the role of this protein family in plant growth and development have not yet been undertaken.
Our hypothesis is that AtCDC48 plays a critical role in plant growth and development. The functional characterization and subcellular localization of AtCDC48 is presented in this chapter.

Materials and Methods

Oligonucleotides Used in This Study

Oligonucleotide sequences shown in Table 1 were synthesized by integrated DNA Technologies (Coralville, IA). Capitalized sequences represent those complementary to the AtCDC48 locus. Underlined lowercase letters in the oligonucleotide sequences indicate added restriction enzyme sites used for cloning.

Plant Transformation Vector Construction

A H6T7 epitope-tag was generated as described in chapter 2. The cDNA encoding the H6T7-tagged AtCDC48 in pPZP211 was PCR amplified using primers SB439/SB371, and TA-cloned into pGEM-T Easy (Promega, Madison, WI). The AtCDC48 native promoter (754 bp upstream of the AtCDC48 start codon) was amplified by PCR with SB504 and SB656 including 5’ SalI and 3’ NdelI sites and subcloned into the H6T7-AtCDC48 fragment. The entire construct was cut with SalI and KpnI restriction enzymes and inserted into pPZP211 (Hajdukiewicz et al., 1994). To generate the AtCDC48 promoter-EYFP-AtCDC48 fusion vector, EYFP was amplified with SB695 and SB683 from pCAM-35S-EYFP-C1 (Preuss et al., 2004) to replace the existing H6T7 tag of AtCDC48 in pGEMT-easy. This cassette was then subcloned into the SalI and KpnI sites
of pPZP211. Images of plants expressing EYFP fused AtCDC48 were taken using a confocal laser-scanning microscope (Nikon Eclipse TE 2000-U, Japan) equipped with an argon laser. Images were captured and processed using Image J 1.32 (Wayne Rasband, National Institutes of Health, USA) and Adobe Photoshop/Illustrator (Adobe Systems, San Jose, CA, USA) imaging software on Macintosh computers (Apple Computer, Cupertino, CA, USA).

**Generation of ethanol inducible dominant negative mutant**

The ethanol inducible transcription factor and promoter from the *alc* cassettes of pSRN1 and pACN1 (pSRNACN_bin) (Caddick et al., 1998) were amplified with SB723 and SB737 including NotI sites. The amplified fragment was subcloned into the Bsp120I site of the modified pPZP211 (Bsp120I, compatible with NotI, was engineered into the HindIII site of pPZP211) (Scott Michaels, Indiana University) 5’ to H6T7-AtCDC48. The double ATP hydrolysis and binding dominant negative of *Atcdc48* mutants were generated as described in Chapter 3. The H6T7-AtCDC48 and mutant expression constructs were cloned into the modified pPZP211 as described above. Vertically grown ethanol inducible wild-type and dominant negative *Atcdc48* seedlings (about 60 seedlings per plates) were treated with 1 ml of 0.5%, 1%, and 2% ethanol at the bottom of plates 4 days after germination. Ethanol treated plates were placed horizontal for 20 minutes and re-positioned to vertical at 22°C. Root length was measured at 1, 2, and 4 days after ethanol treatment. Averages and standard errors were calculated for groups of 24 seedlings. Pictures of primary leaves and roots of seedling were obtained with a dissecting microscope (Leica MZ6) 2hrs, 6hrs, 1day, 2days, and 4days after ethanol
treatment. The bright-field micrographs (Carl Zeiss, Thornwood, NY, USA) of roots 4 days after ethanol treatment were obtained. Total protein extracts from ethanol inducible lines from each time point were prepared. Four seedlings were homogenized in 100 µL of 2X SDS-PAGE sample buffer (Laemmli, 1970) and incubated at 65°C for 15 min. The samples were cleared of insoluble debris by centrifugation at 16,000×g for 5 min at room temperature, and 15 µL of the supernatant was resolved on a 12.5% (w/v) SDS-polyacrylamide minigel and analyzed by immunoblotting with anti-T7 and anti-AtCDC48 antibodies.

Isolation of Atcdc48 T-DNA Insertion Mutants

Three independent Arabidopsis mutant lines containing T-DNA insertions, salk_064573, salk_064893, and salk_116074 (Alonso et al., 2003), obtained from the Salk Institute Genomic Analysis Laboratory (SIGnAL, http://signal.salk.edu/cgi-bin/tdnaexpress). The mutant lines were screened for Atcdc48::T-DNA using oligonucleotide primers SB372, SB400, SB401, SB513, and SB514. DNA sequencing of the PCR-amplified products determined the T-DNA insertion positions within AtCDC48. The genotype of segregating plants was confirmed by PCR using allele-specific primer pairs. For analysis of developing embryo of Atcdc48 mutants, siliques were cleared in Hoyers solution (7.5 g gum Arabic, 100 g chloral hydrate, and 5 mL glycerol in 30 mL water; (Liu and Meinke, 1998) and observed by bright-field light microscopy. For imaging by confocal laser scanning microscopy, roots were briefly stained with 0.1 mg/ml propidium iodide for 30 seconds to visualize cell walls.
Plant Material and Growth Conditions

Surface-sterilized seeds were plated on solidified half-strength Murashige and Skoog (Murashige and Skoog, 1962) medium plates [base recipe: 2.15 g/liter MS salts (Invitrogen Life Technologies), 2.6 mM 2-(N-morpholino)-ethanesulfonic acid, 1% (w/v) Phytagar (Invitrogen Life Technologies), pH to 5.7 with KOH] and cold treated (4°C) in the dark for 3-4 days. Plated seeds were germinated and grown vertically under constant fluorescent lighting at 22°C. Wild-type *Arabidopsis thaliana* Colombia 2 (Col 2) and *Atcdc48* T-DNA insertion lines were grown as above and transplanted into soil (Superfine Germination Mix, Con-rad Fafard, Agawam, MA) treated with Adept® insecticide (Uniroyal Chemical Company Inc., Middlebury, CT) and grown under fluorescent light illumination cycles of 16 hours light and 8 hours dark at 22°C. Wild-type and *AtCDC48/Atcdc48* plants were transformed using the *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent, 1998). Transgenic plants transformed with the YFP translational fusion of AtCDC48 (YFP-AtCDC48) under the native promoter and the ethanol inducible AtCDC48 and Atcdc48 ATP binding/hydrolysis mutant constructs were selected on solidified MS media containing 100 µg/mL Kanamycin (An, 1985). A tobacco suspension-cultured cell line was also transformed with YFP-AtCDC48 as described in Chapter 2.

*In vitro* pollen germination

Pollen viability and germination was monitored as described (Li et al., 1999). Flowers just about to open were harvested and allowed to dehydrate at room temperature for 90 min on the bench. Pollen grains were then transferred to pollen germination media
[18% (w/v) sucrose, 0.6% (w/v) phytagar, 0.01% (w/v) boric acid, 1mM MgSO₄, 2.5 mM CaCl₂, 2.5 mM Ca(NO₃)₂, pH 7.0] by streaking the dried flower on the surface of the pollen germination medium. Pollens grains were germinated at 28°C for 6 to 24 hours and were imaged with a Zeiss Axioskop (Carl Zeiss, Thornwood, NY, USA) in combination with phase contrast filter sets. Images were processed using IPLab Spectrum (Signal Analytics, Vienna, VA, USA). Images of pollen expressing YFP-AtCDC48 were obtained from a confocal laser scanning microscope (Nikon Eclipse TE 2000-U, Japan) equipped with an argon laser and processed using Image J 1.32 and Adobe Photoshop/illustrator imaging software.

**Cross-section of Arabidopsis roots**

The ethanol inducible wild-type and mutant seedlings before and 2 days after 2% ethanol treatment were fixed overnight at 4°C with 4% (v/v) gluteraldehyde in 0.05 M KPO₄, pH7.0, followed by rinsing with KPO₄ buffer and dehydration through a graded ethanol series (30% ~ 100%). Tissue was then embedded in LR White (EMS, Hatfield, PA) with 25% increments. Thin sections (3 to 5 µm) were cut with a Reichert-Jung Ultracut model E microtome (Vienna, Austria). Sections were stained with toluidine blue O as described previously (Kang et al., 2001) and bright-field images were captured as described above.

**Analysis of inducible AtCDC48 mRNA Expression**

Total RNA was isolated from ethanol-induced wild-type and mutant seedlings using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). 2 µg of
total RNA was treated with RQ DNAs e (Promega, Madison, WI) to eliminate genomic DNA. After heat inactivating RQ DNAs e, 1 µg of total RNA was used as input template for reverse transcription by Moloney Murine Leukemia Virus RT (Promega) using oligo (dT) to produce the first-strand cDNA in a 20 µL reaction mixture. A 1 µL aliquot of a 20-fold dilution of the cDNA was subsequently PCR-amplified with the oligonucleotide primer pairs for AtCDC48 (SB439 and SB36) and ubiquitin (SB747 and SB748) as a control.

Results

T-DNA insertion mutants of *AtCDC48*

Genomic *AtCDC48* is 3.3 kb in length and composed of 8 exons. AtCDC48 is ubiquitously expressed throughout all plant tissue (Feiler et al., 1995). PCR-based identification of T-DNA insertions in *AtCDC48* was performed. The T-DNAs in *Atcdc48-1, Atcdc48-2*, and *Atcdc48-3* were inserted in the 1st intron, 3rd exon, and 3rd intron, respectively, of *AtCDC48*, which are upstream of the sequences encoding the two ATPase domains (Figure 1). The T-DNA insertion site in each of the alleles was verified by DNA sequence analysis. All three Atcdc48 lines were heterozygotes for the T-DNA insertion and 10% of the progeny from self-fertilized heterozygous *Atcdc48* plants displayed germination defects and seedling lethality. It was found that the mutant phenotypes observed were specific to *AtCDC48*, using PCR-based genotypic analysis of embryos (data not shown) and complementation experiments using the native promoter driven YFP-AtCDC48 (see below).
Phenotypic analysis of *Atcdc48::T-DNA insertion mutants*

Seedlings from wild-type and heterozygous parents were grown on solid MS media. The mutants from all three *Atcdc48* alleles displayed identical phenotypes, and growth of mutant *Atcdc48* seedlings arrest 1 day after germination (Figure 2A). PCR-based genotypic analysis confirmed that the arrested embryos and seedlings were homozygous for the T-DNA insertion in *AtCDC48* (data know shown). The homozygous *Atcdc48* seedling root cells were severely disorganized (Figure 2B). The entire mutant root as shown in the right panel of figure 2B was less than 0.5 mm in length and the root tip was narrower than wild-type. The mutant root had abnormal cell files, no elongation zone, no meristematic region, and no organized root cap based on visual inspection. These results indicate that *AtCDC48* is critical for plant development, including seedling growth.

Because only 2% of the progeny we recovered from heterozygous *AtCDC48/Atcdc48* plants were non-viable homozygous, we tested to determine at what stage in seed development homozygous *Atcdc48* mutants were affected, immature siliques from self-fertilized heterozygous plants were split open and examined under a dissecting microscope. The mutant seeds, approximately 10%, were pale green relative to wild-type seeds in the same silique from heterozygous *Atcdc48* (*AtCDC48/Atcdc48*) plants (Figure 2C). To determine the growth and development defects of *Atcdc48* mutants, wild-type and heterozygous siliques were cleared with Hoyers solution. Mutant embryo growth was arrested around the heart stage of development (Figure 2D and E). However, no defects in cell division and elongation were observed in embryo.
As indicated above, only total 12% of the progeny, including seeds of self-fertilized heterozygous plants were homozygotes. The segregation ratio of wild-type versus heterozygotes was variable ranging 0.6 to 1 suggesting that transmission of the mutant allele through the gametes was impaired. To confirm this, male and female transmission efficiencies of the two defective Atcdc48 alleles were determined by performing reciprocal crosses between heterozygous mutants and wild-type plants (Figure 2F). Both Atcdc48 alleles showed significantly reduced male transmission efficiency, however, weak female transmission efficiency was also detected. Thus, the observed segregation ratio was effected in both gametophytes. The great reduction in transmission efficiency via male germ line in AtCDC48/Atcdc48 plants suggests a critical role for AtCDC48 in pollen function and development.

**Analysis of pollen development in Atcdc48 mutants**

To determine which stage of pollen development was defective in cdc48 mutants, pollen grains from wild-type and heterozygous plants were examined by differential interference contrast (DIC) and epifluorescence microscopy after staining with 4,6-diamidino-2-phenylindole (DAPI) to monitor nuclear DNA content. No apparent defects in microsporogenesis, including tetrad formation, and microspore release were observed. After microspore release, all the pollen grains contained two generative and a vegetative nucleus (Figure 3b, and d). Both pollen grains from wild-type and AtCDC48/Atcde48 showed approximately 70% of germination frequency. The pollen tube growth of heterozygous mutant progeny in germination media did not differ significantly compared to the wild-type (Figure 3a, c).
Phenotypic analysis of transgenic \textit{AtCDC48} dominant-negative mutant plants

To further study the role of \textit{AtCDC48} in plant growth and development, transgenic plant expressing conditional dominant-negative mutant proteins were generated. It has been demonstrated that expression of ATP binding or hydrolysis mutant of CDC48/p97 protein effectively turns off CDC48/p97 function \textit{in vivo} (Dalal et al., 2004; Lamb et al., 2001). Therefore, to further test the role of \textit{AtCDC48} function in plants, an ethanol-inducible dominant negative protein expression system was used to circumvent the gametophyte and seedling lethal phenotypes observe with the \textit{AtCDC48} insertion alleles. Ethanol-inducible \textit{AtCDC48} and \textit{Atcdc48} ATP hydrolysis (E308Q/E581Q) and binding (K254A/K572A) mutants were generated. Mutant and wild-type \textit{AtCDC48} constructs containing an N-terminal H6T7-epitope tag was used for ease of detection. It was determined that the tight regulation of ethanol inducible transgenic protein expression could be easily controlled with low concentrations of ethanol, 0.5 – 2%, without damaging wild-type seedling growth. This regulated promoter displayed no basal activity in the absence of ethanol and was strongly up-regulated in the presence of non-toxic levels of ethanol in \textit{Arabidopsis} (Figure 4 and 5C). Results shown in figure 4 and 5 were treated with 2% ethanol, however, the same phenotype was observed in lower ethanol applications (0.5 and 1%). Transgenic plants showed clear visible phenotypes, which correlated with the expression of the mutant Atcdc48 protein. Root hairs of mutant plants were affected in number and position as shown in figure 4B. In addition the dominant negative mutant cotyledons became chlorotic and arrested growth (Figure 4C). Root growth was also arrested after ethanol treatment (Figure 5A) and there was lack of an elongation zone in the dominant negative mutant. The \textit{Atcdc48} mutant seedlings
showed higher levels of protein as compare to the wild-type seedlings (Figure 5B). The mutant protein was detected 6 hours after 2% ethanol treatment and mutant phenotypes were visible within 1 day after treatment. RT-PCR analysis has shown that mRNA expression was detectable 2 hours after ethanol treatment and accumulated in ethanol treated seedlings, which was consistent with the protein level seen above (Figure 5C). Atcdc48 mutants were subjected to microscopic analysis before and after induction with ethanol to observe the root cross-section. Wild-type root hairs were initiated where the vascular tissue was fully matured, near the developmental zone (Figure 5A). In contrast, root hairs of Atcdc48 dominant negative mutants were apparent immediately above the root cap (Figure 6B-a) and some root hairs initiated from non-root hair cells (atrichoblasts) (Figure 6B-b and c). Cell wall stubs and cytokinesis-defective cells were also observed in pericycle and cortex cells (Figure 6B-d).

**Characterization and localization of YFP-fused AtCDC48 in plants**

To further examine the role of AtCDC48, Arabidopsis plants expressing the YFP-AtCDC48 fusion protein under the native promoter were generated. We tested the in vivo functionality of this construct by verifying that it rescued Atcdc48 homozygous plants. Complementation was confirmed by PCR analysis corroborated by seedling, silique, and flower appearance (Figure 7). However, siliques were not full of seeds (Figure 7B) because anther and filament development were delayed resulting in the style growing taller without enough pollen on the stigma for fertilization of all the ovules (Figure 7D and F). In contrast, the wild-type flower had full siliques (Figure 7A) and the anthers already started to release pollen before the papillae were fully matured (Figure 7C).
Furthermore, the pollen sac was almost empty when the stigmatic papillae were taller than the filament of the stamen (Figure 7E) indicating that the transgene containing a long N-terminal extension of AtCDC48, only partially complements the mutant phenotype during pollen development, maturation and fertilization.

YFP-fused AtCDC48 was localized throughout the pollen sac, pollen, and ovules in the ovary. In pollen, the two sperm nuclei did not have any YFP signal but the vegetative nucleus displayed strong YFP-AtCDC48 expression. After germination of the pollen tube, YFP signal of the vegetative nucleus and surrounding the nucleus was usually seen behind the growing tip of the pollen tube (Figure 8A). YFP-AtCDC48 was expressed throughout transgenic seedlings, however, it was strongest in meristematic cells (Figure 8B and C). Punctuated dots (puncta) were also observed near the plasma cell membrane (Figure 8D). Distinct nuclear staining was consistently observed in post-karyokinesis and interphase cells of the root (Figure 8E and F). There were also small vesicle-like structures near segregated chromosomes before karyokinesis (Figure 8G). Following nuclear membrane reformation after cytokinesis, evenly distributed puncta containing YFP-AtCDC48 were observed around the nuclear membrane. Later after cytokinesis, No YFP puncta were observed, however, there was a diffuse, continuous YFP signal around the nucleus (Figure 8E and G). In interphase nuclei, the YFP puncta was also found within the nucleus (Figure 8F). YFP-AtCDC48 was found to be associated with phragmoplast midzone during cytokinesis (Figure 8E, G, H, and I arrow head). In the transgenic tobacco lines, YFP-AtCDC48 was also associated with the spindle during mitosis (Figure 8H, arrow).
AAA proteins are conserved across all kingdoms and play essential roles in cellular housekeeping, cell division and differentiation. AAA-ATPases have been identified in the cytosol as well as membrane-enclosed compartments. CDC48/p97 protein family members function in membrane trafficking, organelle biogenesis and protein degradation pathways (Acharya et al., 1995; Peters et al., 1992; Rabouille et al., 1995b). AtCDC48 is expressed throughout the cell cycle. Previous studies from our lab implicate a role for AtCDC48 during plant cytokinesis because AtCDC48 was re-localized to the division zone during cytokinesis (Rancour et al., 2002).

Atcdc48 loss of function plants are non-viable indicating a critical role in plant growth and developments. The T-DNA insertion mutants of AtCDC48 showed abnormal mutant phenotypes in various stages of pollen, embryo, and seedling growth and development (Figure 2). A small number of homozygous Atcdc48 plants (12% of total progeny) can be isolated because the pollen transmission defects are not complete. The development of homozygous embryos was delayed in respect to wild-type arresting at the heart stage of embryogenesis. Some homozygous mutant embryos were able to bypass this arrest and germinate, however, these seedlings died shortly after germination. One possible hypothesis to explain these result is that either AtCDC48B or AtCDC48C may weakly compensate for the loss of AtCDC48A (AtCDC48) in Atcdc48A homozygous mutants during seed development, since AtCDC48B and C share 91% and 95% amino acid sequence identity, respectively, with full-length AtCDC48A (Rancour et al., 2002).
However homozygous Atcdc48 (Atcdc48A) seedlings never survived past germination indicating that AtCDC48 is absolutely required for seedling development.

Disorganized mutant root cells in homozygous Atcdc48 mutants indicated that many aspects of root development are effected including cell proliferation, and cell expansion (Figure 2E). Dyes used for staining penetrated the mutant root faster than wild-type roots and accumulated in cells that should have become part of the vasculature indicating there is a possible defect in cell wall and plasma membrane dynamics. In addition, the cells of the mature vascular tissue showed severe deformation. CDC48 has been shown to be associated with plasma membrane in soybean (Shi et al., 1995). These results indicate that AtCDC48 also has a role in plasma membrane and cell wall integrity, in the processes of cell expansion and cytokinesis during seedling root growth.

One of the postulated function of CDC48/p97 ATPases is to interact with cis-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complexes, priming them for fusion by making SNAREs available to engage in trans with cognate SNAREs of target membranes promoting another round of fusion (Mayer et al., 1996; Rabouille et al., 1995b). One possible hypothesis is that without functional AtCDC48, SNAREs may not be recycled for another round of certain membrane and vesicular fusion at the site of new plasma membrane and cell wall deposition in certain cell types.

In order to define pollen defects in Atcdc48 mutants, we have utilized microscopic analysis of pollen and found no significant difference compared to wild-type during pollen morphogenesis and germination, indicating there could be possible defects during pollen tube growth through the pistil or ovule fertilization in the ovary. Further
examination needs to be done to confirm this hypothesis. The phenotypes observed from the T-DNA insertion mutants were complemented by native promoter-driven YFP-AtCDC48. In the seedling stage, there were no differences in growth and morphology in both wild-type and YFP-AtCDC48::Atcdc48/Atcdc48 plants, while homozygous Atcdc48 seedlings were lethal. However, transgenic plants did not have full siliques (Figure 7B), indicating that the transgene containing a long N-terminal extension of AtCDC48, only partially complements the mutant phenotype during fertilization. The interaction of many adapter/cofactor proteins with CDC48/p97 occurs via the ~200 amino acid N-terminal domain of CDC49/p97, and the N-terminus is the site of conformation change depending on the ATP status of the molecule (Beuron et al., 2003; Bruderer et al., 2004; Davies et al., 2005; Meyer, 2005b; Rouiller et al., 2000; Rouiller et al., 2002). Therefore, one possible hypothesis is that an essential N-terminal interaction with an adaptor during fertilization was partially inhibited.

The presence of YFP-AtCDC48 signal in regions of cell proliferation and expansion are consistent with our hypothesis that AtCDC48 plays a role in cell division and expansion. The subcellular localization of YFP-AtCDC48 to the developing nuclear envelope following karyokinesis suggests a conserved mechanism of nuclear envelope formation between animals and plants. Variable inner and exo-nuclear staining implies that the function of AtCDC48 differs according to cell type and stage of the cell cycle. In yeast, Cdc48p has a role in spindle pole body duplication or separation (Frohlich et al., 1991). In Xenopus, when paired with its adapters Ufd1-Npl4, p97 has been shown to regulate spindle disassembly at the end of mitosis (Cao et al., 2003; Cao and Zheng, 2004; Cheeseman and Desai, 2004). The mitotic spindle localization of YFP-AtCDC48 in
plant and cultured cells (tobacco) is further evidence that the *Arabidopsis* homolog of CDC48/p97, AtCDC48, may have a similar role in spindle disassembly.

An alternative approach to gene knock out, utilizing an inducible dominant negative gene expression method was undertaken to test the *in vivo* roles of AtCDC48 in plant growth and development. An ethanol inducible dominant negative system was an ideal system to control the transgenic gene expression while avoiding lethality enabling us to study the primary effect of the Atcdc48 mutants directly. As expected, the dominant negative mutants showed a mutant phenotype upon ethanol treatment. The accumulation of mutant protein correlated with the arrest of leaf cell growth and expansion as well as early leaf senescence. AtCDC48 is also essential to cell division and elongation in roots (Figure 4, 5 and 6). The mutant root phenotype of excessive ectopic root hairs provides evidence that AtCDC48 has a critical role in controlling cell signal transduction essential to trichoblast/atrichoblast cell differentiation. These *in vivo* dominant negative studies provide the first functional hints that AtCDC48 may have a role in plant cytokinesis. In a number of samples, we observed incomplete cell walls and cell wall stubs along the endodermis/cortex interface resulting in an inappropriate number of cell layers within the root. The endodermis and cortex arise from a specific longitudinal division of a progenitor cell to give rise to two distinct cell type fates. From our data, it appears that AtCDC48 has a role in the completion of that longitudinal cell division. It would be very interesting for future work to determine the cell fate identity of the resulting mutant cell types. These data clearly demonstrate the great importance that AtCDC48 has in many facets of plant growth and development. In addition to its roles in plant growth and development, our dominant negative expression work has provided clues into how
AtCDC48 expression is regulated endogenously. Our data indicate that AtCDC48 may auto-regulates its own protein expression level. Although we observed that ethanol induced mRNA levels in plants expressing wild-type or mutant were comparable, induced mutant Atcdc48 protein levels were much higher than induced wild-type protein levels. Higher mutant protein expression levels correlated with severe mutant phenotypes. We hypothesized that Atcdc48 mutant protein degradation is not occurring in dominant negative mutants because the mutant protein cannot be disassembled by PUX1. As reported in chapter 3, PUX1 was not able to dissociate the double ATP binding/hydrolysis mutant \textit{in vitro}. Therefore, mutant protein accumulated over time after ethanol treatment in contrast to the wild-type, even though RNA levels were the same between the wild-type and mutant plants. This suggests that AtCDC48 controls its own protein expression level possibly through its association with PUX1, hexamer dissociation, and protein degradation. In support of this proposal, over-expression of PUX1 in plant resulted in a compensatory up-regulation of AtCDC48 protein (Rancour and Bednarek, unpublished data). Cdc48p was conjugated with polyubiquitin in yeast which implies that Cdc48p may be a substrate of the proteasome (Mayor et al., 2005). Taken together, these data support a model whereby a certain level or range of AtCDC48 activity is required for cell function and that AtCDC48 protein activity levels are self maintained in order to keep this homeostasis. It is still unclear as to how this activity level is sensed.

We hypothesized that the dominant negative Atcdc48 mutant protein subunits incorporated into the hexameric complex along with the endogenous AtCDC48 \textit{in vivo}. The results showed that inducible ATP binding mutants had a more severe phenotype
than ATP hydrolysis mutants during seedling growth and development. A significant conformational change, including a rearrangement of the N terminal domains occurs upon nucleotide binding to the CDC48/p97 hexamer (Rouiller et al., 2000). It has been hypothesized that the critical conformational change in the mammalian homolog of AtCDC48, p97, is a large rearrangement upon nucleotide binding rather than the smaller changes associated with nucleotide hydrolysis. The location and number of p47 molecules bound to p97 varies significantly in the nucleotide-free and ADP-bound states, suggesting that p47 requires ATP to form a tight complex with p97 (Dalal and Hanson, 2001). High affinity binding of substrates in the ATP-bound state may be a common feature of AAA proteins (Vale, 2000) and we hypothesized that the high affinity of AtCDC48 to its adaptors is also closely associated with ATP binding. Therefore, AtCDC48 interaction with adaptors, which is critical for seedling growth and development is inhibited in Atcdc48 ATP binding (K254A/K527A) mutants. In comparison, ATP hydrolysis mutants showed minor phenotypes and survived after transfer to soil (free of ethanol), indicating adaptor interaction with AtCDC48/Atcdc48(E308Q/E581Q) hetero-hexamer in conditional mutants is less affected than ATP binding mutants. However, further analysis of the inducible mutant phenotype after seedling stage needs to be investigated.

In conclusion, the essential role of AtCDC48 with respect to cell division, elongation, and differentiation shown in this chapter support our hypothesis that AtCDC48 is an essential AAA-ATPase chaperone that functions in numerous diverse cellular activities in Arabidopsis.


Figure 1. Schematic representation of *Arabidopsis* CDC48 T-DNA mutants gene disruptions

The exon/intron structure of AtCDc48 is shown to scale, with black boxes representing exons. The position and directions of T-DNA inserts with left border sequences are indicated. Kan, T-DNA neomycin phosphotransferase selectable gene marker.
Table 1: Oligonucleotides used in study.

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<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Purpose</th>
</tr>
</thead>
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<td>SB371</td>
<td>tcaagtctcgggaAGTAATCCAAAGTAGAG</td>
<td>3’ H6T7-AtCDC48A cloning; Not I</td>
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<tr>
<td>SB372</td>
<td>caaaccagctggacgctgcaact</td>
<td>Salk T-DNA left border</td>
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<td>SB400</td>
<td>ATTTGGGAGAAGTAGTTGG</td>
<td>5’Atcdc48 T-DNA insertion screening</td>
</tr>
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<td>GTCAGAACCACATAAGAATCC</td>
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</tr>
<tr>
<td>SB439</td>
<td>ggaattccatatgCATCATCATCATCATCAC</td>
<td>5’ H6T7-AtCDC48 cloning; Nde I</td>
</tr>
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<td>agatgTACGATGGAGATTCGGAGGTTGAAG</td>
<td>5’ AtCDC48 UTR; Sal I</td>
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<tr>
<td>SB513</td>
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<td>3’ AtCDC48 UTR; Nde I</td>
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<tr>
<td>SB683</td>
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<td>3’ YFP; Sma I</td>
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Figure 2. Phenotypes of *Atcdc48* seeds, seedlings and embryos

A) Portion of an immature silique from a heterozygous *Atcdc48* plant approximately 10 days after ovule fertilization. 10% of the seeds in a green silique are pale green (arrowhead). B-C) Embryonic phenotype of *Atcdc48* mutants. Mutant embryo growth is arrested at around primarily heart stage of development. D) Five-day-old wild-type and homozygous mutant (arrowhead) seedlings. E) Root phenotype of five-day-old homozygous mutant seedling (F) Reciprocal cross results between heterozygous Atcdc48 and WT Col-2.
Figure 3. *In vitro* pollen tube germination assay and cytochemical analysis of heterozygous mutant pollen development

Pollen grains from wild-type (A) and heterozygous *AtCDC48/Atcdc48* (B) were assayed. (b, d) Pollen grains were examined by epifluorescence microscopy after 4, 6-diamidino-2-phenylindole (DAPI) staining at onset of desiccation. SN, sperm nuclei (arrowhead); VN, vegetative nucleus (arrow).
Figure 4. Phenotypic analysis of conditional \textit{Atcdc48} dominant negative mutant

A) Schematic diagram of ethanol inducible system. B) Five-day-old seedlings treated with 2% ethanol. Images are taken three days after ethanol treatment. Ethanol inducible \textit{AtCDC48} (WT) shows no phenotypic defects. Seedlings expressing the mutant forms of \textit{Atcdc48} show root growth defects and lack a root elongation zone and cotyledons are chlorotic. Seedlings were grown on vertical sucrose-free half-strength MS solid medium at 22°C under continuous light.
Figure 5. Conditional dominant negative *Atcdc48* mutant plants displayed inhibition of root growth

A) Seedling root growth was monitored 4 days after 2% ethanol treatment. The data represent a minimum sample size of 25 plants for each construct. The standard deviation is presented for each group. B) Expressed H6T7-Atcdc48 mutant proteins were monitored by SDS-PAGE followed by an immunoblot probed with anti-T7 antibody. 1, 4, and 7: before ethanol treatment; 2, 5, and 8: 6 hours after ethanol treatment; 3, 6, and 9: 1 day after ethanol treatment. C) RT-PCR analysis of H6T7-AtCDC48 expression. cDNA was synthesized from total RNA of each time point. 1, 5, and 9: before ethanol treatment; 2, 6, and 10: 2 hours after ethanol treatment; 3, 7, and 11: 6 hours after ethanol treatment; and 4, 8, and 12: 1 day after ethanol treatment. AtCDC48-specific primers were used to amplify a 1kb fragment of AtCDC48 containing the H6T7 tag.
Figure 6. Phenotypic analysis of conditional Atcdc48 dominant negative mutant root cross-sections

The ethanol inducible wild-type (A) and mutant seedlings (B) of 2-days after 2% ethanol treatment were post-fixed overnight at 4°C. Tissue was then embedded in LR and thin sections (3 - 5 μm) were cut using a microtome. Sections were stained with toluidine blue O and bright-field microscopy images were taken. (A-a); About 4 μm above the quiescent center (QC), (A-c and d); About 20 μm and 60 μm above QC, (A-e and f); About 150 μm above QC; (B-a) About 4 μm above the quiescent center, (B-b and c); About 10 μm and 20 μm above the QC. (B-d); Cell wall stubs and cytokinesis-defective cells (arrow). Abnormal root hairs; arrowhead.
Figure 7. Seedling lethal phenotype of homozygous \textit{Atcdc48} rescued by YFP-\textit{AtCDC48} under the native promoter

A) Siliques from wild-type, B) Siliques from “rescued”, C and E) Flowers from wild-type, D and F) Flowers from “rescued”.
Figure 8. Localization of YFP-AtCDC48 in 3-day-old transgenic homozygous

Atcdc48 seedlings

A, D, and E) Root division zone, B and C) Root elongation zone, F, G and H) Leaf apical meristem, I) YFP-AtCDC48 was expressed strongly in pollen vegetative nucleus. YFP-AtCDC48 proteins shown in yellow; Expanding cell plates in dividing root cells labeled with arrowheads and spindles labeled with arrow. Nucleus staining differs between cell types in the root.
Chapter 5

Summary, Conclusions and Future Directions

CDC48/p97 is a highly abundant hexameric AAA-ATPase that functions as a molecular chaperone in numerous diverse cellular activities. We have identified an *Arabidopsis* UBX domain-containing protein, PUX1, which functions to regulate the oligomeric structure of the *Arabidopsis* homolog of CDC48/p97, AtCDC48 as well as mammalian p97. PUX1 is a soluble protein that co-fractionates with non-hexameric AtCDC48 and physically interacts with AtCDC48 *in vivo*. Binding of PUX1 to hexameric AtCDC48 is mediated through the UBX-containing C-terminal domain. However, disassembly of the chaperone is dependent upon the N-terminal domain of PUX1. These findings provide evidence that the assembly and disassembly of the hexameric CDC48/p97 complex is a dynamic process. PUX1 functions to regulate both the hexameric structure as well as ATPase activity of AtCDC48. The primary binding site for the UBX-C of PUX1 is the Na domain of AtCDC48. UBX domains from other *Arabidopsis* PUX proteins (PUX4 and PUX5) tested bind the N-terminus of AtCDC48 but are not to be able to substitute for the UBX-C of PUX1 in hexamer disassembly, suggesting unique features for the UBX-C of PUX1. Utilizing Atcdc48 ATP hydrolysis and binding mutants, it was found that PUX1 binding was not affected by the ATP status of AtCDC48 but hexamer disassembly was significantly influenced. ATPase activity in both the D1 and D2 domains was critical for PUX1 mediated AtCDC48 hexamer disassembly. These results together provide new mechanistic insight into how AtCDC48 hexameric status and ATPase activity is modulated.
I have used reverse genetic and biochemical approaches to elucidate the function of AtCDC48 in plants. Our data show that AtCDC48 is essential for plant growth and development. AtCDC48 is present throughout the cell cycle and localizes to the nucleus, cytoplasm, ER, and the division midzone. Homozygous Atcdc48 T-DNA insertion mutants are not viable. AtCDC48 is necessary for at least three stages of early development: fertilization, embryo development, and seedling growth. In order to avoid the lethality of Atcdc48 mutants, an ethanol-inducible dominant negative system was used to study the functions of AtCDC48 during each developmental stage. Data in this thesis indicate that AtCDC48 has essential roles in cell division, elongation, which are essential for plant growth and development. AtCDC48 controls its own protein expression level possibly by ubiquitin associated protein degradation pathways and PUX1-association with the AtCDC48 hexamer is the initial step in this degradation process.

For future study, it will be important to elucidate the molecular nature of the pollen defect in Atcdc48. Studies have been initiated to identify the stage of pollen development at which Atcdc48 pollen fails. One approach to identify the stage Atcdc48 mutant pollen become defective is to take advantage of the pollen mutant quartet (Preuss et al., 1994). In quartet, the four products of male meiosis II fail to dissociate and continue their development to maturity resulting in a tetrad of pollen grains. Based on germ allele segregation, if mutant Atcdc48 is deleterious to pollen development, then we would observe tetrad pollen with two viable pollens and two defective. Therefore, a heterozygous Atcdc48 mutant was a crossed to homozygous quartet mutant. The progeny were allowed to self-fertilize and the F2 will be used for the analysis of pollen defects. If
the pollen from a *AtCDC48/Atcdc48/quartet* plant shows defects in an early stage of development (i.e. meiosis), then only two products of pollen remain attached. If the defect occurs late in pollen development (i.e. mitosis), then two pollens will exhibit aberrant nuclei content in the vegetative or sperm nuclei. At last if the defect occurs during pollen tube growth, then mutant pollen tube growth arrests compare to wild-type pollen tube. Pollen of wild-type and *AtCDC48/Atcdc48::qrt/qrt* lines will be analyzed for cytoplasmic density by the staining method of Alexander staining, for chromosome staining by DAPI, and pollen tube growth will be also monitored using bright field microscopy. Therefore, we could elucidate the critical function of *AtCDC48* during pollen development.

*AtCDC48* activity is recruited to biochemical pathways through the association of adapter proteins. Given the broad range of phenotypes observed with various *Atcdc48* mutant plants, we postulate that identifying *AtCDC48* interacting proteins, will allow us to begin dissecting these important biological processes at the molecular level. To identify additional proteins associated with *AtCDC48*, immobilized *E.coli*-expressed H6T7-*AtCDC48* will be incubated with both soluble and detergent extracted membrane protein fractions of T87 *Arabidopsis* suspension cultured cells, and bound proteins will be eluted and analyzed by SDS-PAGE. Alternatively H6T7-*AtCDC48* will be purified from transgenic plants by affinity chromatography. Polypeptides that co-purify specifically with H6T7-*AtCDC48* will be subjected to trypsin digestion followed by MALDI-TOF-MS for identification. We strongly believe that identification of additional *AtCDC48* associating factors will allow for the targeted molecular characterization of
many processes critical for plant growth and development in addition to providing clues into how AtCDC48 functions to regulate them.

AtCDC48 has been shown to be re-localized from a punctate cellular distribution to distinct localization at the division plane during plant cell cytokinesis in a manner similar to KNOLLE (a cell plate-specific SNARE) and SYP31 (another SNARE, the plant ortholog of syntaxin 5) (Feiler et al., 1995; Rancour et al., 2002). Initial biochemical studies demonstrated that AtCDC48 associates with a membrane population that co-fractionates with both KNOLLE and SYP31. However, cytosolic AtCDC48 was found to interact specifically with SYP31, whereas binding of AtCDC48 to KNOLLE was not detected. These results suggest that there are at least two distinct membrane fusion pathways that operate at the division plane to mediate plant cytokinesis, one involving AtCDC48 and one independent of AtCDC48. The null mutant of SYP31 showed no phenotype during plant growth and development, suggesting functional redundancy. A likely candidate is SYP32, which shares 41 % amino acids identity with SYP31. SYP32 T-DNA and RNAi mutant lines are currently being analyzed. RNAi of SYP32 seedling caused a growth retardation and cell expansion phenotype in seedling leaves. The homozygous SYP31 and SYP32 RNAi lines had been crossed and further study will follow.

By completing the proposed experiments, it would be possible to determine what kinds of role AtCDC48 has during plant growth and development and to identify new components that are involved in the function of AtCDC48 in *Arabidopsis*. 
References

