PROTEINS THAT INTERACT WITH UBIQUITIN-CONJUGATING ENZYME 2, UBC-2, IN THE NEMATODE *CAENORHABDITIS ELEGANS*

by

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ABSTRACT

A major route for protein degradation occurs via the ubiquitin-proteasome pathway. Proteins are targeted for destruction by the proteasome through covalent ubiquitin attachment. This tagging system involves the concerted action of an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3), with the latter two being involved in achieving target specificity. UBC-2, an essential E2 in the nematode *C. elegans*, is a functional homolog of *Saccharomyces cerevisiae* UBC4, a member of a branch of ubiquitin-conjugating enzymes required for the degradation of short-lived and abnormal proteins. In order to identify proteins that interact with UBC-2, a yeast two-hybrid analysis was employed. Putative UBC-2 interacting proteins identified include: a ubiquitin-like budding yeast DSK2 ortholog referred to as DSK-2, two RING finger proteins, a protein containing a FYVE domain, a calcium ATPase called MCA-1, and ubiquitin. Since DSK-2 represented 60% of the putative interactors isolated from the screen, this project focused on the interaction of UBC-2 and DSK-2, and demonstrated a novel interaction between an E2 and an ubiquitin-domain protein (UDP) *in vitro*. The ability of DSK-2 to bind polyubiquitin and itself was also demonstrated. RNA interference (RNAi) was employed in an attempt to determine the biological function of UBC-2 interacting proteins *in vivo*. RNAi with *mca-1* produced a subtle body shortening phenotype, although genes encoding UBC-2 interacting proteins appear to be non-essential .

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Å	angstrom
ABC	ATP-binding cassette
AD	Alzheimer's disease
APC	anaphase promoting complex
3-AT	3-aminotriazole
ATP	adenosine triphosphate
β-TrCP	β-transducing repeat-containing protein
BLAST	basic local alignment search tool
BSA	bovine serum albumin
bp	base pairs
CDC	cell division cycle
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
dsRNA	double stranded RNA
DSK	dominant suppressor of *kar1*
DTT	dithiothreitol
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal-growth factor
ERAD	endoplasmic reticulum-associated degradation
GST	glutathione s-tranferase
HD	Huntington disease
HECT	homologous to E6-AP carboxyl-terminus
IPTG	isopropyl-β-D-thiogalactopyranoside

IκB	inhibitor of NFκB
LB	Luria broth
LiAc	lithium acetate
LSB	loading sample buffer
NFκB	nuclear factor κB
NG	nutrient growth
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
PLIC	protein linking integrin associated protein with cytoskeleton
Rad	radiation sensitivity abnormal
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
SCF	Skp1-Cdc53-F-box protein
SDS	sodium dodecyl sulphate
SPB	spindle pole body
TGF-β	transforming growth factor-β
Tris	tris(hydroxymethyl)aminomethane
UBA	ubiquitin associated
UBC	ubiquitin-conjugating enzyme
U-box	UFD-2 homology domain
UBL	ubiquitin-like protein
Ubp	ubiquitin protease
UDP	ubiquitin domain protein
UFD	ubiquitin fusion degradation
X-gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

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I. INTRODUCTION

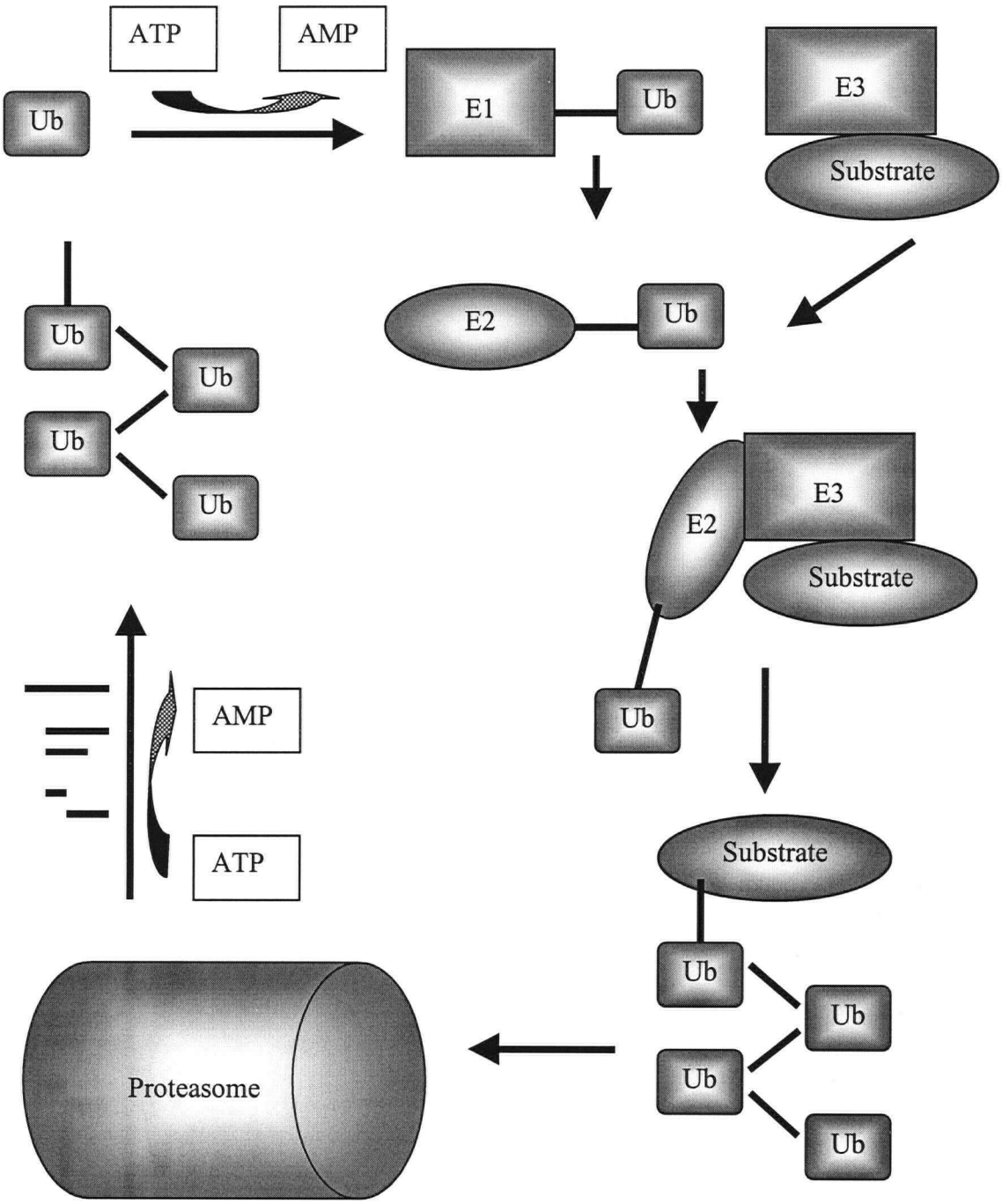
1. UBC-2 and a summary of the ubiquitin pathway

The ubiquitin-proteasome pathway is a major route for selective protein degradation in the cell (Figure 1). Ubiquitin is activated through ATP hydrolysis and an ubiquitin activating enzyme (E1). Ubiquitin is attached via the carboxy-terminal glycyl residue to an active site cysteinyl residue on the E1, resulting in the formation of a thiolester bond. Activated ubiquitin is subsequently transferred to an ubiquitin-conjugating enzyme (E2). Ubiquitin is attached through the carboxy-terminal glycyl residue to an active site cysteinyl residue on the E2 through a thiolester bond. The ubiquitin protein ligase (E3) provides substrate specificity for this protein degradation pathway. The E3 either transfers activated ubiquitin to substrates directly or provides a scaffold to facilitate substrate protein ubiquitylation by placing the E2 and the substrate in close proximity. The carboxy-terminal glycyl residue of activated ubiquitin is attached to an internal lysyl residue of the substrate protein. Subsequently, the E2 transfers additional activated ubiquitin moieties onto the initial ubiquitin residue to form a multi-ubiquitin chain on the substrate protein. This chain is recognized by the proteasome as a signal for degradation. The ubiquitin chains are hydrolyzed by isopeptidases to facilitate recycling of ubiquitin in the cell.

UBC-2, an ubiquitin-conjugating enzyme in *Caenorhabditis elegans*, is a member of the highly conserved and functionally overlapping "UBC4 branch" of E2s. In budding yeast, *Saccharomyces cerevisiae*, simultaneous mutation of all UBC4 branch members, UBC1, UBC4, and UBC5, produces lethality (Seufert *et al.*, 1990). Budding yeast UBC4 members are implicated in the degradation of short-lived proteins such as mitotic cyclins and misfolded proteins such as those produced during the stress response (Seufert and Jentsch, 1990). The amino acid sequence of UBC-2 is 85% identical to UBC4 and UBC5 in *S. cerevisiae* and 95% identical to UbcD1 in *Drosophila melanogaster* (Zhen *et al.*, 1993). The importance of members of the UBC4 branch *in vivo* is underscored by the high degree of conservation between UBC4 branch members, the fact that UBC-2 is essential for larval development in *C. elegans* and that UbcD1 is required for proper telomere behaviour in *Drosophila* (Cenci *et al.*, 1997; Zhen *et al.*, 1996). Furthermore,

Figure 1. The ubiquitin pathway of protein degradation

Ubiquitin (Ub) is activated by an ubiquitin activating enzyme (E1) and ATP hydrolysis. Activated ubiquitin is subsequently transferred to the ubiquitin-conjugating enzyme (E2). The ubiquitin protein ligase (E3) is thought to provide a scaffold for the E2 and the substrate to bring them into close proximity and facilitate substrate protein ubiquitylation. The carboxy-terminal glycyl residue of activated ubiquitin is attached to an internal lysyl residue of the substrate protein. The E2 transfers subsequent activated ubiquitins onto the initial ubiquitin molecule on the substrate protein to form a multi-ubiquitin chain which is recognized by the proteasome as a signal for degradation to peptides (▬) in an ATP dependant manner. The ubiquitin chains are hydrolyzed by isopeptidases, allowing recycling of ubiquitin in the cell.



UBC-2 is constitutively expressed and *ubc-2* mutants exhibit pleiotropic defects which affect embryonic, somatic and germline development in *C. elegans* (Stevens, Ph.D. thesis, 1999; Zhen *et al.*, 1996).

2.Components of the ubiquitin pathway

2.1. Ubiquitin

Ubiquitin is a small 76 amino acid protein which exhibits high sequence homology in a wide variety of organisms studied to date. A comparison of amino acid sequences of ubiquitin in budding yeast versus in mammals reveals differences at only three residues (Özkaynak *et al.*, 1984). This high degree of conservation in eukaryotes underscores the essential function of ubiquitin in the cell.

In vivo, ubiquitin exists in an unbound state or in covalent linkage to a diversity of proteins (Özkaynak *et al.*, 1984). Conjugation of ubiquitin to proteins serves as a signal for degradation via the proteasome in an ATP-dependent manner (Ciechanover *et al.*, 1978; Hershko *et al.*, 1980). The crystal structure of ubiquitin, determined to 1.8 Å, revealed that the protein is compact and globular with a carboxy-terminal extension from the hydrophobic core. This extension facilitates conjugation of the carboxy-terminal glycine carboxyl group to the ε-amino group of a lysyl residue on target proteins via an isopeptide bond (Vijay-Kumar *et al.*, 1987).

Ubiquitin itself is the target of ubiquitylation during multiubiquitin chain formation. Interestingly, ubiquitin chains linked between the carboxyl-terminal glycine of one ubiquitin and lysyl 63 of the next ubiquitin moiety appears to be implicated in DNA repair and protection from stress conditions (Arnason and Ellison, 1994; Spence *et al.*, 1995). In *S. cerevisiae*, ubiquitin-conjugating enzymes UBC4 and UBC5, present at elevated levels in the cell under stress, are involved in lysyl-63-linked polyubiquitin formation (Arnason and Ellison, 1994; Seufert and Jentsch, 1990). However, isopeptide bond formation, mediated by UBC4 and UBC5, between the carboxyl-terminal glycine of one ubiquitin moiety and lysyl 48 residue of the next ubiquitin in a growing chain appears to be the most common linkage used as a signal for proteasomal targeting under normal conditions (Finley *et al.*, 1994). Interestingly, the S5a/Rpn10 subunit of the proteasome 19S particle interacts with lysyl-48-linked polyubiquitin chains of at least

four ubiquitin moieties in length (Devereaux *et al.*, 1994). This indicates that ubiquitin chains target substrates for degradation potentially through direct physical interaction with the proteasome, although other factors are plausibly involved.

2.2. Ubiquitin-activating enzyme

Ubiquitin activation is accomplished through an ubiquitin-activating enzyme (E1). The E1 attaches AMP to the carboxy-terminal glycyl residue of ubiquitin subsequent to catalyzing hydrolysis of ATP to AMP and PPi. The AMP-ubiquitin intermediate is subsequently attacked by the E1 sulfhydryl group of the active site cysteinyl residue, resulting in the formation of a thiolester bond (Ciechanover *et al.*, 1981). In *S. cerevisiae*, the UBA1 essential gene encodes a 114 kDa ubiquitin-conjugating enzyme which contains a nucleotide-binding site (McGrath *et al.*, 1991). Genes encoding E1 enzymes with active site cysteinyl residues have been cloned and functionally analyzed in wheat (UBA1, UBA2, UBA3) (Hatfield *et al.*, 1990; Hatfield and Vierstra, 1992), rabbit (Sun *et al.*, 1997), and human (Handley *et al.*, 1991). The E1 enzyme is approximately 100 kDa in size, exhibits a GXGXXG nucleotide-binding motif (X is any residue), and contains an active site cysteinyl residue in the center of the primary amino acid sequence (Hatfield and Vierstra, 1992). *In vivo*, the 105 kDa E1 was found to dimerize in reticulocyte lysates (Ciechanover *et al.*, 1982).

2.3. Ubiquitin-conjugating enzyme

Activated ubiquitin is transferred from the E1 to an ubiquitin-conjugating enzyme (E2). The E2 forms a thiolester bond with the carboxy-terminal glycyl residue of an activated ubiquityl moiety through the active site cysteinyl residue. The E2 transfers ubiquitin to either a substrate protein lysyl residue or to an ubiquitin ligase active site cysteinyl residue. Genes encoding 20 E2s in *C. elegans*, 13 E2s in *S. cerevisiae*, 17 E2s in human and E2s in other eukaryotic organisms have been identified (Jones *et al.*, 2002; reviewed in Peters *et al.*, 1998; reviewed in Pickart, 2001). All E2s exhibit a highly conserved (35-40% identity) 150 amino acid ubiquitin-conjugating (UBC) domain which includes the FHPNIXXXGXICLDL consensus sequence (Haas and Siepmann, 1997; reviewed in Peters *et al.*, 1998). This sequence contains the active site cysteinyl residue

necessary for thioester bond formation with ubiquitin and an HPN tripeptide essential for appropriate folding of the protein. Mutation of the active site cysteinyl residue of E2s results in the elimination of ubiquitin-conjugating activity (Sommer and Jentsch, 1993; Sung *et al.*, 1991). Deletion analysis has suggested that the amino-terminal region of the UBC domain might be involved in binding the E1 (Sullivan and Vierstra, 1997). However, a direct interaction between the E1 and an E2 has not been demonstrated.

Further E2 classification is based on the presence or absence of the amino-terminal and/or carboxy-terminal extensions beyond the UBC domain. The extensions are often required for enzyme function and are believed to be important for subcellular localization and/or interaction with substrate or ubiquitin ligase proteins. Class I E2s consist of the UBC domain alone and are approximately 16 kDa in size. The lack of sequence beyond the UBC domains in Class I E2s is consistent with their dependence on an ubiquitin ligase to transfer ubiquitin to a substrate protein (Girod and Vierstra, 1993). Examples of Class I E2s include UBC-2 from *C. elegans* (Zhen *et al.*, 1993), UBC4 and UBC5 from *S. cerevisiae* (Seufert and Jentsch, 1990), and UbcD1 from *Drosophila* (Treier *et al.*, 1992). UBC4 and UBC5 are involved in the degradation of short-lived and abnormal proteins (Seufert and Jentsch, 1990). Interestingly, UBC-2 or UbcD1 can functionally substitute for yeast UBC4 activity in *ubc4 ubc5* double mutant cells (Treier *et al.*, 1992; Zhen *et al.*, 1993).

Class II E2s contain the UBC domain in addition to a carboxyl-terminal extension. UBC-1, a homolog of UBC2(RAD6), is an example of a Class I E2 in *C. elegans* (Leggett *et al.*, 1995). Examples of Class II E2s in *S. cerevisiae* include UBC2(RAD6), UBC3(CDC34), and UBC6. The acidic carboxy-terminal tail of UBC2(RAD6) is necessary for polyubiquitylation of histones H2A and H2B and is essential for sporulation (Sung *et al.*, 1988). The carboxyl-terminal extension of UBC3(CDC34) was determined to be essential for progression of the cell cycle. It is thought that the carboxyl-terminal tail may mediate interaction with UBC3 substrates or specific E3s which determine the substrate selection of UBC3(CDC34) (Kolman *et al.*, 1992; Silver *et al.*, 1992). A hydrophobic region of the carboxy-terminal tail of UBC6 appears to be responsible for localization of this E2 at the endoplasmic reticulum membrane (Sommer and Jentsch, 1993).

Class III E2s consist of the UBC domain with an amino-terminal extension. Members of this class have been identified in *Drosophila*, mouse and human. An ability of Class III E2s to partially complement *ubc4* mutants indicates the possibility that they have overlapping functions with UBC4-mediated proteolysis (Matuschewski *et al.*, 1996).

Class IV E2s contain both amino-terminal and carboxy-terminal extensions beyond the UBC domain. The amino-terminal extension of UbcM1 in mouse exhibits transmembrane domains, indicating a possible subcellular targeting function for this portion of the protein (S. Jentsch, pers. comm. in Stevens, Ph.D. thesis, 1999). The carboxy-terminal extension of a 230 kDa E2 in rabbit exhibits a cysteinyl residue, in addition to the active site cysteinyl residue of the UBC domain, possibly indicating a dual ubiquitin ligase function and ubiquitin conjugating activity of this E2 (Berleth and Pickart, 1996).

2.4. Ubiquitin ligase

The ubiquitin ligase (E3) is a component involved in the final transfer of ubiquitin to a target substrate. The E3 is thought to promote ubiquitylation by direct transfer of ubiquitin to the target substrate or by acting as a scaffold to enable facile transfer of ubiquitin from the E2 to the substrate. In the former case, the E3 forms a thiolester bond with ubiquitin at an active site cysteinyl residue after transfer from the E2.

The variety of E3s that have been identified and characterized is thought to reflect the specificity of E3s for a wide range of substrates that are targeted for multiubiquitylation and subsequent degradation by the proteasome (reviewed in Jackson *et al.*, 2000). E3s are classified on the basis of their structure, composition, and substrate specificity. These E3 classes include: HECT domain proteins, the SCF complex, the APC complex, RING finger proteins, and U-box containing proteins.

2.5. Multiubiquitin assembly factor

The multiubiquitin assembly factor (E4), known as UFD2 in *S. cerevisiae*, is a recent addition to current knowledge of component proteins involved in the ubiquitin pathway of selective protein degradation (Koegl *et al.*, 1999). The E4 is not involved in

the process of transferring ubiquitin to the substrate but appears to be necessary for efficient assembly of multiubiquitin chains of sufficient length for recognition by the proteasome. UFD2 enhances the formation of multiubiquitin chains catalyzed by UBC4 *in vitro*. It has been proposed that the E4 might promote multiubiquitylation through a proven ability to bind ubiquitin conjugated to substrate proteins and a possible function in directing proteolysis by dictating a preference for specific lysyl residues of ubiquitin in multiubiquitin chain formation. Homologues of yeast UFD2 have been identified in human, *C. elegans*, *Dictyostelium*, and *S. pombe* (Koegl *et al.*, 1999; Pukatzki *et al.*, 1998). These proteins share high sequence similarity in the carboxy-terminal region referred to as the UFD2-homology domain or U-box (Koegl *et al.*, 1999).

2.6. The 26S proteasome

In eukaryotes, the 26S proteasome consists of two 19S regulatory particles which enclose either end of a hollow, cylindrical 20S proteolytic core particle. The regulatory complexes are thought to function in the recognition and unfolding of ubiquitylated substrates in an ATP dependent manner prior to their degradation in the core particle (reviewed in Voges *et al.*, 1999). The crystal structure of the *S. cerevisiae* 20S particle revealed that it contains four stacked rings each consisting of 7 subunits. The two outer rings consist of 7 unique α-type subunits (α1-7) while the two inner rings consist of 7 unique β-type subunits (β1-7). The side chains of the amino-terminal ends of α-type subunits prevent non-specific entry of proteins into the proteolytic core compartment. Three β-type subunits possess the chymotrypsin-like, trypsin-like, or peptidyl-glutamyl peptide proteolytic activities (Groll *et al.*, 1997). Interestingly, in mammals, γ-interferon induces expression of new β-type subunits which replace three of the existing β-type subunits, thus effecting a change in proteolytic cleavage to allow the production of peptides for MHC class I presentation (Monaco, 1995; Pamer *et al.*, 1998). Furthermore, γ-interferon induces expression of PA28, which associates with the 20S particle to increase the production of antigenic peptides (Dick *et al.*, 1996; Gray *et al.*, 1994).

In *S. cerevisiae*, each 19S regulatory complex consists of 17 unique subunits which exhibit ATPase, polyubiquitin binding, or deubiquitylating activity. Six of these subunits are AAA family ATPases (Rpt1 to Rpt6) (Glickman *et al.*, 1998). The

S5a/Rpn10 subunit interacts with lysyl-48-linked polyubiquitin chains no less than four ubiquitin moieties in length (Devereaux *et al.*, 1994). In mammalian cells, subunits of the 19S particle have been identified which participate in deubiquitylation (Usp14 and a 37 kDa protein) (Borodovsky *et al.*, 2001; Lam *et al.*, 1997).

Recent evidence has demonstrated that the 26S proteasome interacts with components of the ubiquitin pathway. Various E2s, including UBC4, have been shown to associate with the proteasome, although it is not clear whether this interaction is direct or requires other factors (Tongaonkar *et al.*, 2000). The ability of UBC4 to bind the proteasome is not contigent on its ubiquitylation activity. Since non-functional UBC4 can bind the proteasome, it is thought that ubiquitylation activity of UBC4 occurs after it binds the proteasome. A model has been proposed whereby the association of E2s with the proteasome may promote efficient degradation of substrate proteins by decreasing the possibility of multiubiquitin chain disassembly through the action of ubiquitin proteases (Ubps) and constraining ubiquitin conjugation to the site of protein degradation. The idea that multiubiquitin assembly takes place *in situ* at the proteasome is supported by the fact that ubiquitylation activity of UBC4 is not required for interaction with the proteasome. Interestingly, heat shock induces UBC4 expression and also enhances its ability to bind to the proteasome (Seufert and Jentsch, 1990; Tongaonkar *et al.*, 2000). Thus, heat shock or other events may change the type of E2 associated with the proteasome in order to alter the selectivity of protein degradation by the proteasome (Tongaonkar *et al.*, 2000). Other ubiquitin pathway enzymes with a demonstrated interaction with components of the 19S particle of the 26S proteasome include the E3s: Ubr1p, a RING finger domain protein, and a HECT domain protein called Ufd4p (Xie and Varshavsky, 2000). Close association of E2s and E3s with the proteasome may therefore prove to be a general theme in the degradation of select target proteins via the ubiquitin pathway.

3. UBC4/5-like E2s: diverse examples and functions in ubiquitylation

E2s similar to UBC4/5 in *S. cerevisiae* have been isolated in other eukaryotes and have been shown to exhibit multiubiquitylation activity. UBC4/5-like E2s have been cloned from various other organisms including: wheat ($E2_{15K}$) (Girod and Vierstra, 1993); *Arabidiopsis thaliana* (AtUBC8-12) (Girod *et al.*, 1993); cow ($E2_{25K}$) (Chen *et al.*,

1991; Chen and Pickart, 1990); rat (UBC4-1, UBC4-2 and UBC4-testis) (Rajapurohitam *et al.*, 1999; Wing and Jain, 1995; Wing *et al.*, 1996), *Schizosaccharomyces pombe* and *Candida albicans* (Damagnez *et al.*, 1995); *Drosophila* (UbcD1) (Treier *et al*, 1992); *C. elegans* (Zhen *et al.*, 1993); and human (UbcH5A-C) (Jensen *et al.*, 1995; Scheffner *et al.*, 1994).

Interestingly, it has become apparent that proteins similar to yeast UBC4/5 have distinct *in vivo* functionality depending on the organism in which they are expressed. Mutations in UbcD1 appear to promote the fusion of chromosomal telomeres (Cenci *et al.*, 1997). It has been proposed that the association of chromosomes through their telomeres may enhance chromosome breakage and recombination, both of which may be linked to tumor development (Hastie and Allshire, 1989). It has been proposed that UbcD1 may be involved in the degradation of proteins interacting with telomeres to prevent abnormal telomere behaviour (Cenci *et al.*, 1997).

The elimination of mutated tumor suppressors through the ubiquitin pathway is believed to be linked to the development of various human cancers. For instance, human UbcH5a-c are responsible for the degradation of tumor suppressor transcription factors, Smad2 and Smad4, when they are mutated (Xu and Attisano, 2000). In addition, Smad2 and Smad3 promote the ubiquitylation and subsequent degradation of SnoN mediated by UbcH5 and the anaphase-promoting complex (APC) ubiquitin ligase (Stroschein *et al.*, 2001). SnoN is a protein which represses genes induced in response to transforming growth factor-β (TGF-β) and is found in elevated levels in a variety of human cancers.

In rat, the UBC4/5 homologues UBC4-2 and UBC4-testis exhibit specific patterns of expression in the testis germ cells (Rajapurohitam *et al.*, 1999; Wing and Jain, 1995; Wing *et al.*, 1996). Since UBC4-2 and UBC4-testis are present in the rat testis and exhibit increased expression and ubiquitylation activity in spermatids, it is thought that these E2s play a major role in the degradation of cellular proteins required for production of haploid spermatids during the process of spermatogenesis.

Proteins similar to budding yeast UBC4 have demonstrated roles in nervous system development in higher eukaryotes and have been linked to diseases affecting the nervous system in humans. Analysis of UBC-2 in *C. elegans* revealed ubiquitous expression in early developmental stages which became specific to the nervous system in

L4 stage larvae and adults (Zhen *et al.*, 1996). In addition, expression of UBC-2 appeared to be more crucial for viability in early stages of development. UbcD1 has a demonstrated role in the neuronal differentiation of the R7 photoreceptor precursor cell through elimination of the transcriptional repressor of photoreceptor differentiation called Tramtrack (TTK88) (Tang *et al.*, 1997). A model has been proposed for photoreceptor cell fate decision whereby stimulation of Sevenless, a receptor tyrosine kinase, causes a RAS1/MAPK signaling cascade resulting in: the transcription of PHYL, the association of PHYL with TTK88 and SINA, and the interaction of UbcD1 with SINA to promote the degradation of TTK88 and differentiation of the R7 photoreceptor cell (Tang *et al.*, 1997). In addition, another yeast UBC4-like protein in human called E2-25K exhibits higher expression levels in the caudate nucleus and putamen regions of the brain which also demonstrate neural degeneration in Huntington disease (HD) affected individuals (Kalchman *et al.*, 1996). Interestingly, these affected regions of the brain in HD also reveal concomitant expression of a protein linked to HD called Huntingtin which interacts with E2-25K and is ubiquitylated (Kalchman *et al.*, 1996).

Ubiquitin conjugation to membrane receptor proteins appears to signal receptor endocytosis. For instance, in the presence of excess extracellular zinc, the zinc transporter (ZRT1) in *S. cerevisiae* appears to be targeted for endocytosis and degradation in the vacuole by ubiquitylation, mediated by UBC4/5 and the E3 RSP5 (Gitan and Eide, 2000). In addition, the a-factor (Ste3) and α-factor pheromone receptors (Ste2), required for inter-cellular communication prior to mating of a and α haploid cells types, and the ABC transporter Ste6, required for a-factor pheromone secretion, are ubiquitylated in a UBC4/5 dependent manner prior to endocytosis (Hicke and Riezman, 1996; Roth and Davis, 1996). In rabbit reticulocyte lysate, herbimycin A treated epidermal-growth factor (EGF) receptors are polyubiquitylated *in vitro* in the presence of recombinant human UBC4 and other ubiquitylation factors (Mori *et al.*, 1997). It is thought that herbimycin A, a tyrosine kinase inhibitor, binds and induces conformational changes in the tyrosine kinase EGF receptor which are recognized as signals for degradation by components of the ubiquitin pathway.

In *S. cerevisiae*, UBC4 can mediate the monoubiquitylation of calmodulin in a calcium and E3 dependent manner *in vitro* (Parag *et al.*, 1993). When calcium binds to

calmodulin, structural changes are induced that enhance its interaction with other proteins (O'Neil and De Grado, 1990). Since it has not been proven that monoubiquitylation leads to degradation of calmodulin *in vivo*, it has been proposed that the addition of ubiquitin may be necessary to modify the ability of calmodulin to interact with other proteins (Parag *et al.*, 1993). It is also possible that multiubiquitylation and degradation of calmodulin may be promoted only at certain stages of the cell cycle when intracellular calcium levels increase.

4. Ubiquitylation mediated by UBC4 family E2s and associated E3s

4.1. Ubiquitylation mediated by UBC4 family E2s and HECT domain E3s

A variety of E3s containing homology to the E6-AP C-terminus have been identified and grouped into an E3 family termed the HECT domain proteins (Huibregtse *et al.*, 1995). Human papillomavirus (HPV) 16, believed to be a causative agent in cervical carcinoma, produces a protein called E6 that binds and targets the p53 tumor suppressor protein for destruction via the ubiquitin pathway, thus, paving the way for proliferative tumor growth (Scheffner *et al.*, 1990). Human UbcH5, a homologue of budding yeast UBC4, acts as an E2 while the viral E6 protein and the host E6-associated protein (E6-AP), in combination, act as an E3 in the ubiquitylation and degradation of p53 (Rolfe *et al.*, 1995; Scheffner *et al.*, 1993; Scheffner *et al.*, 1994). E6-AP is the final receptor of ubiquitin in the E1-E2-E3 ubiquitin thiolester cascade prior to transfer of ubiquitin to p53 (Rolfe *et al.*, 1995).

The ability to bind ubiquitin through a conserved cysteinyl residue, located in the approximately 350 amino acid HECT domain, appears to be a common feature of proteins containing homology to the C-terminal portion of E6-AP (Huibregtse *et al.*, 1995; Scheffner *et al.*, 1995). An *in vitro* E2-E3 interaction between UbcH5 and the HECT domain proteins E6-AP and *S. cerevisiae* RSP5 is mediated by the UbcH5 C-terminal region surrounding the active site cysteinyl residue and a phenylalanine residue (Nuber and Scheffner, 1999). These conserved regions appear in many members of the UBC4 branch of E2s. It is thought that the N-terminal portion of HECT domain proteins may mediate interaction with the proteasome and substrate proteins targeted for degradation via the ubiquitin pathway (You and Pickart, 2001; Wang *et al.*, 1999). It

should be noted that while *in vitro* experiments do indicate that Rsp5 and E6-AP interact specifically with the UbcH5 E2, *in vivo* yeast two-hybrid experiments reveal that E6-AP interacts with UbcH7 and not UbcH5 (Kumar *et al.*, 1997; Schwarz *et al.*, 1998). Therefore, a yeast two-hybrid screen for UBC-2 interacting proteins may preclude the identification of HECT domain proteins.

4.2. Ubiquitylation mediated by UBC4 family E2s and the APC E3

Exit from mitosis and entry into anaphase is mediated by the degradation of cyclins through the activity of E2s and a multicomponent E3 called the anaphase-promoting complex (APC). For instance, in *Xenopus* egg extracts, the ubiquitylation of cyclin B requires the presence of the ubiquitin conjugating enzyme UBC4, a homolog of *S. cerevisiae* UBC4, and the APC in association with an activator of cyclin B degradation called Cdh1 (King *et al.*, 1995; Visintin *et al.*, 1997). Many substrates of the APC, including cyclin B, exhibit an N-terminal nine amino acid destruction box (D-box) motif required for degradation mediated by the APC (Glotzer *et al.*, 1991; reviewed in Page and Hieter, 1999).

It is estimated that the APC contains 11 subunits in both budding yeast and vertebrates (Gmachl *et al.*, 2000; reviewed in Page and Hieter, 1999). Some subunits of the budding yeast APC include: a RING-H2 finger protein called APC11 and a cullin protein called APC2 (Yu *et al.*, 1998; Zachariae *et al.*, 1998). The similarity of APC2 and APC11 to proteins found in the Skp1-cullin-F box protein complex (SCF) and the von Hippel Lindau complex (VHL) might indicate the importance of both subunits in mediating the E3 activity of the APC; however, it has been demonstrated that APC11 is the only portion of the APC required for promoting ubiquitylation of cyclin B (Gmachl *et al.*, 2000). Nevertheless, it is thought that efficient activity of the APC likely requires all subunits of the complex including APC2 (Leverson *et al.*, 2000). For instance, E3 activity reconstituted by APC2 and APC11 demonstrates non-specificity in that it catalyzes ubiquitylation of a cyclin B lacking a D-box (Tang *et al.*, 2001). Furthermore, the RING-H2 finger domain of APC11 was determined to be necessary for ubiquitin ligase activity and for interaction with the ubiquitin conjugating enzyme UBC4 (Leverson *et al.*, 2000). Since an *in vitro* interaction between APC11 and UBC4 has been demonstrated, it is

possible that UBC-2 may exhibit a yeast two-hybrid interaction with the RING finger domain of a *C. elegans* APC11 budding yeast E3 homologue.

4.3. Ubiquitylation mediated by UBC4 family E2s and SCF complex E3s

The SCF complex family is composed of a group of E3s which contain the following components in *S. cerevisiae*: Skp1, Cdc53, and an F-box containing protein (reviewed in Patton *et al.*, 1998). In metazoans, homologues of budding yeast Cdc53 are termed cullins (Kipreos *et al.*, 1996). The F-box motif enables interaction with Skp1 while the remaining portion of the F-box protein is thought to be involved in recognizing and recruiting target proteins to the SCF for ubiquitylation (Bai *et al.*, 1996; reviewed in Patton *et al.*, 1998). F-box proteins are classified according to the type of protein-protein interaction domain present in the non-F-box portion of the protein that promotes recognition of ubiquitin pathway substrates. The F-box protein classes include: Fbws which contain WD-40 domains, Fbls which contain leucine-rich domains, and Fbxs which contain leucine zippers, ring fingers, helix-loop-helix domains, proline-rich repeats or Src homology 2 (SH2) domains (Cenciarelli *et al.*, 1999). The most recently identified component of the SCF is a RING-H2 finger protein, called Rbx1 or Hrt1, which stimulates E3 activity of the complex (Kamura *et al.*, 1999; Seol *et al.*, 1999; Skowyra *et al.*, 1999). Providing further clues as to how the subunits of the SCF assemble, in *Drosophila*, homologues of human Skp1 and Rbx1 have demonstrated an ability to interact with homologues of Cul1 and the F-box protein Slmb (Bocca *et al.*, 2001).

Individual SCF complexes are distinguished by the F-box that they contain. Some examples of substrates targeted by the $SCF^{\beta TrCP}$ complex include: phosphorylated IκBα, an inhibitor of a transcription activator of pro-inflammatory genes called NFκB, in response to pro-inflammatory signals (Winston *et al.*, 1999) and phosphorylated β-catenin, a transcription factor which stimulates the activity of the oncogene c-MYC in colorectal cancers and is required for regulation of development via the Wnt/Wingless pathway (He *et al.*, 1999; Winston *et al.*, 1999). Interestingly, members of the UBC4 branch of E2s, such as budding yeast UBC4 and human UbcH4, UbcH5b and UbcH5c, function in conjunction with the $SCF^{\beta TrCP}$ complex to mediate ubiquitylation of IκBα (Gonen *et al.*, 1999; Kawakami *et al.*, 2001; Strack *et al.*, 2000). In addition, human

NEDD8, a ubiquitin-like protein, binds the Cul1 subunit to stimulate interaction of monoubiquitylated UbcH4 with the $SCF^{\beta TrCP}$ complex (Kawakami *et al.*, 2001). For this reason, the possibility exists that UBC-2 may exhibit a yeast two-hybrid interaction, in a Nedd8 dependent fashion, with components of a putative homologous $SCF^{\beta TrCP}$ complex in *C. elegans*. This possibility seems plausible given the fact that homologues of human $SCF^{\beta TrCP}$, including the cullin dCul1 and the F-box protein Slmb, have been identified in *Drosophila* and have demonstrated an interaction with UbcD1 (Bocca *et al.*, 2001).

4.4. Ubiquitylation mediated by UBC4 family E2s and the VHL E3 complex

The von Hippel-Lindau (VHL) tumor suppressor complex associates with the Elongin BC complex in mammals (Kibel *et al.*, 1995). This large complex interacts with another complex of Cul1 (a cullin) and Rbx1 to promote Ubc5 ubiquitylation activity (Kamura *et al.*, 2000). Specifically, the Elongin BC complex acts as an adaptor to bridge the VHL complex with the Cul1-Rbx1 complex to allow ubiquitylation of the α subunits of hypoxia inducible factors HIF1 and HIF2. The VHL complex interacts with the HIF substrates through an oxygen-dependent degradation domain when a specific prolyl residue is hydroxylated (Ivan *et al.*, 2001). Since the Elongin BC complex can bind various BC box-containing proteins, such as VHL, and since Elongin BC complex binds Cul1/Rbx1 to promote ubiquitylation activity, it is thought that Elongin BC may function to link a particular substrate recognizing BC-box containing protein with Cul1/Rbx1 to promote assembly of a functional E3 (Kamura *et al.*, 2001). Since the VHL-Elongin BC complex can activate ubiquitylation activity of Ubc5, it is possible that UBC-2 may interact in a yeast two-hybrid screen with a component of this complex.

4.5. Ubiquitylation mediated by UBC4 family E2s and RING finger domain E3s

RING (for Really Interesting New Gene) finger domains are defined by a sequence containing eight cysteinyl and histidyl residues that chelate two zinc atoms E2s (reviewed in Borden, 2000). Proteins containing RING domains exhibit the following consensus sequence: $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}C/HX_2CX_{(4-48)}CX_2C$ where the cysteinyl and histidinyl residues represent zinc binding residues and the X represents any amino acid (reviewed in Freemont, 2000). The RING proteins are classified further as RING-

H2 or RING-HC if a histidyl residue or a cysteinyl residue, respectively, is present at the 5th zinc coordination site. RING domains in E3s appear to be necessary for ubiquitylation of substrates and for mediating interaction with E2s (reviewed in Joazeiro and Weissman, 2000; Lorick *et al.*, 1999). For instance, *in vitro* experiments have demonstrated interactions between UbcH5, a homologue of *S. cerevisiae* UBC4, and RING finger proteins including: A07, BRCA1, Siah-1, TRC8, NF-X1, kf-1 and Praja1 (Lorick *et al.*, 1999). In addition, human homologues of *S. cerevisiae* UBC4 appear to be required for the E3 activity of the following RING proteins: the membrane protein Rma1, the receptor protein-tyrosine kinase adaptor c-Cbl, and the apoptosis inhibitors c-IAP1 and XIAP (Joazeiro *et al.*, 1999; Matsuda *et al.*, 2001; Yang *et al..*, 2000).

RING domains are found in a diversity of E3 multicomponent complexes in addition to single protein E3s (reviewed in Jackson *et al.*, 2000). Examples of RING finger proteins that exist in E3 complexes include the previously described Apc11 subunit of the APC complex and Rbx1/Hrt1 of the SCF complex (Kamura *et al.*, 1999; Leverson *et al.*, 2000; Seol *et al.*, 1999; Skowyra *et al.*, 1999). As already mentioned, the RING-H2 finger domain of APC11 is required for ubiquitin ligase activity and for interaction with UBC4 (Leverson *et al.*, 2000). Another RING-H2 finger protein, called Rbx1 or Hrt1, stimulates E3 activity of the SCF complex (Kamura *et al.*, 1999; Seol *et al.*, 1999; Skowyra *et al.*, 1999). Since UbcH4 interacts with an SCF complex (Kawakami *et al.*, 2001), and since a variety of RING fingers exhibit an ability to interact with E2s, it is possible that Rbx1 binds and recruits UbcH4 to the SCF complex. Therefore, it is plausible that a yeast two-hybrid screen for proteins that interact with *C. elegans* UBC-2 may identify multiple RING finger proteins.

4.6. Ubiquitylation mediated by UBC4 family E2s and the U-box E3s

Proteins containing U-box domains are thought to represent a new class of E3s due to their ability to catalyze multiubiquitin chain formation in the presence of E1 and E2 (Hatakeyama *et al.*, 2001). CHIP (carboxyl terminus of Hsc70-interacting protein) is a U-box protein which interacts with UbcH5c and catalyzes multiubiquitylation of heat denatured protein in a U-box dependent manner (Jiang *et al.*, 2001; Murata *et al.*, 2001). The E3 activity of CHIP and the recognition of misfolded substrate protein by molecular

chaperones Hsc70 or Hsp90 indicates that molecular chaperones exhibit dual roles in protein folding and in targeting misfolded proteins for degradation via the ubiquitin pathway (Murata *et al.*, 2001). *In vitro* experiments demonstrated that U-box containing murine proteins including: UFD2a, UFD2b, KIAA0860, and CHIP all exhibit the greatest ability to catalyze multiubiquitylation in the presence of UbcH5c, a member of the UBC4 family (Hatakeyama *et al.*, 2001). Therefore, the possibility exists that a yeast two-hybrid screen for proteins that interact with *C. elegans* UBC-2 may identify U-box proteins.

5. Study objectives

Given that UBC4 branch proteins have been implicated in a diversity of cellular roles and are responsible for the selective degradation of many cellular proteins, and since UBC-2 plays an essential role in the development of *C. elegans*, a study was initiated to identify proteins that function in conjunction with UBC-2 in order to increase our knowledge about this vital component of the ubiquitin pathway. As discussed in the previous sections, a variety of potential UBC-2 interacting proteins have been identified, including HECT domain proteins, components of the APC and SCF multi-subunit complexes, RING finger domain proteins, and U-box containing proteins. In order to identify proteins that interact with UBC-2 in *C. elegans*, a yeast two-hybrid screen was employed.

II. MATERIALS AND METHODS

1. DNA techniques

1.1 Standard polymerase chain reaction (PCR)

Polymerase chain reactions were performed in a total reaction volume of 50 µl using 1 µl of DNA template, 50 pmoles of each oligonucleotide primer, 50 µM each deoxyribonucleotide triphosphate, and 1 unit of Vent DNA polymerase, all in 1X Vent DNA polymerase buffer as defined by the manufacturer (New England Biolabs). PCR conditions followed the same general protocol: 3 minutes at 94°C; 30 cycles of 45 seconds at 94°C, 45 seconds at 60°C, 1 minute at 72°C; 10 minutes at 72°C. The annealing temperature or each primer was in the range of 58°C to 62°C depending on the GC content and length of the oligonucleotide. The TwinBlock™ system thermal cycler (ERICOMP Inc.) was used to carry out PCR reactions.

1.2 Restriction endonuclease digestion of DNA

Various restriction enzymes acquired from GIBCO/BRL, Pharmacia and New England Biolabs were used to digest DNA samples. Digested samples were analyzed on horizontal agarose gels. For cloning purposes, alkaline phosphatase (Amersham Pharmacia) was used to dephosphorylate appropriately restriction digested vectors prior to ligation to restriction digested DNA fragments. Alkaline phosphatase treatment of DNA was performed according to standard protocols (Sambrook *et al.*, 1989).

1.3 Agarose gel electrophoresis

Agarose powder was added to TBE buffer (10.8 mg/ml Tris base, 5.5 mg/ml boric acid, 0.9 mg/ml EDTA) to make gels in the range of 0.7% and 2.0% agarose. After heating the solution to solubilize the agarose and cooling the solution to approximately 55°C, ethidium bromide was added to a final concentration of 0.5 µg/ml. Concentrated gel loading buffer was added to the DNA samples prior to gel loading in order to achieve a final concentration of 0.04% Bromophenol blue, 0.04% Xylene cyanol FF, 2.5% Ficoll Type 4000, and 20 mM EDTA.

1.4 DNA extraction from agarose

The Qiaex II kit (Qiagen, Inc.) was used to purify DNA bands of interest from agarose gels.

1.5 Ligation of restriction endonuclease digested DNA

To perform DNA ligations of cut insert and plasmid DNA, T4 DNA ligase was used (Amersham Pharmacia). DNA was performed according to standard protocols (Sambrook *et al.*, 1989).

1.6 Transformation of DNA into *Escherichia coli*

Ligation reactions and purified plasmids were transformed into competent *E. coli* DH5α (GIBCO/BRL) according to the manufacturer's instructions. Purified plasmids were transformed into $CaCl_2$ treated competent *E. coli* BL21(DE3) cells according to a standard protocol (Sambrook et al., 1989).

A volume of 2 µl of plasmid DNA isolated from yeast was used to transform 50 µl of electrocompetent *E. coli* HB101 cells. 0.1 cm electroporation cuvettes (BioRad, Hercules, California) were used in the BioRad electroporator to transform cells according to the manufacturer's instructions with instrument settings at 1.8 V, resistance at 200 Ω and capacitance at 25 F. After electroporation, 1ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 20mM $MgSO_4/MgCl_2$, 20mM glucose, pH 7.0) was added to the cells followed by an incubation at 37°C for one hour and subsequently spreading on antibiotic selection plates for overnight incubation.

1.7 Transformation of plasmid DNA into *S. cerevisiae*

For 10 transformations, yeast cells from a 10 ml overnight culture were harvested by centrifugation at 500xg for 5 minutes and the cell pellet was resuspended in 1 ml TL (10mM Tris-HCl pH 7.5, 1mM EDTA, 100mM lithium acetate, pH 7.5). Each transformation reaction consisted of a 100 µl aliquot of cells, 44 µl DMSO, 60 µg sheared salmon sperm DNA (Eppendorf), 4 µg plasmid DNA and 400 µl TLP (10mM Tris-HCl pH 7.5, 1mM EDTA, 100mM lithium acetate (LiAc), 44% polyethylene glycol (PEG) 4000, pH 7.5). The cell suspensions were mixed, incubated at 30°C for 1 hour, and

mixed again. Cells were then heat shocked at 42°C in a water bath for 10 minutes and plated on selection media.

For high efficiency transformations (used in the transformation of Y190 with the *C. elegans* plasmid cDNA library), a 10 ml overnight culture was counted using a hematocytometer and used to inoculate 50 ml of synthetic complete medium to a cell density of $5x10^6$ cells/ml. After the culture had reached a cell density of $2x10^7$ cells/ml, cells were harvested at 3000xg for 5 minutes and the cell pellet was resuspended in 1 ml of 100 mM LiAc. Cells were pelleted and resuspended again to a final cell density of $2x10^9$ cells/ml. Transformation reactions consisted of a 50 µl aliquot of cells, 240 µl 50% w/v PEG 4000, 36 µl 1.0 M LiAc, 100 µg sheared salmon sperm DNA, x µl of plasmid DNA and 34-x µl of water. Transformation reactions were mixed for 1 minute (Fisher Scientific Vortex Genie 2), incubated at 30°C for 30 minutes, and heat shocked at 42°C for 25 minutes prior to plating on selection media.

1.8 Plasmid preparation from *E. coli*

Plasmids were prepared for DNA sequencing and other manipulations requiring high purity DNA using the Qiagen kits according to the manufacturer's instructions. The alakaline lysis method of DNA purification was also employed (Birnboim and Doly, 1979).

1.9 Plasmid preparation from *S. cerevisiae*

Yeast cells from a 1.5 ml overnight culture harbouring the pACT::*C. elegans* cDNA plasmid of interest were harvested at 12,000 rpm in a microcentrifuge and resuspended in 200 µl of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0) in 1.5 ml microfuge tubes. After adding 0.3 g acid washed 0.45 to 0.55 mm glass beads (Sigma) and 200 µl 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol, samples were mixed at high speed in a Vortex multitube head for 3 minutes. Following a 5 minute centrifugation at 12,000 rpm, the aqueous layer was saved for transformation of electrocompetent *E. coli* HB101.

1.10. DNA concentration determination

A Pharmacia Ultraspec 3000 spectrophotometer was used to determine the concentration of DNA samples knowing that 1 OD_{260} = 50 μg/ml dsDNA.

1.11. DNA sequencing

DNA samples suspended in EB buffer, prepared using the Qiagen Miniprep kit, were sequenced and analyzed by the Nucleic Acid Protein Services (NAPS) Unit at the University of British Columbia, Vancouver, B.C., Canada. DNA samples were supplied at a concentration of 100 ng/μl and primers were supplied at a concentration of 3.2 pmoles/μl. Samples were sequenced by technicians at NAPS using the enzyme Sequenase" Version 2.0 T7 DNA polymerase and the PRISM Dye Terminator Cycle Sequencing kit. Extension products were isolated using a Centri-Sep column and were analyzed on either the Applied Biosystems (ABI) Model 373 Stretch sequencer or the ABI Prism 377 DNA sequencer.

1.12. Preparation of the yeast two-hybrid bait plasmid

ubc-2 was cloned from pRSET-c-*ubc-2* using primer oligonucleotides MG1 and MG2 (Appendix I) and inserted into the *Nde*I and *Bam*HI restriction endonuclease sites of the pAS2 vector (Matchmaker).

1.13. Conversion of bacteriophage library λACT-RB2 into plasmid library pACT-RB2

A *C. elegans* cDNA library called λACT-RB2 was previously constructed by Robert Barstead in the lambda vector ACT (kindly provided by Steve Elledge) using cDNA primed with random hexamers (Durfee *et al.*, 1993; Elledge *et al.*, 1991). The titer of the library λACT-RB2 previously amplified and frozen at -80 C by E. Crowe was determined to be 1.2 plaque forming units (pfu)/ml. For phage titer determination, TMG buffer (10 mM Tris-HCl, pH 7.5; 10 mM $MgSO_4$; 0.01% gelatin) was used to dilute the bacteriophage and LB top agar was prepared with 0.7% agarose. *E. coli* was cultured in LB liquid media with 50 μg/ml kanamycin and 0.2% maltose. $1.2x10^9$ pfu were used to infect a total of $7.2x10^9$ log phase cells (OD_{600} = 0.6 $3x10^8$ cells/ml at log phase for *E. coli* RB4E) in the presence of 10 mM $MgSO_4$. Logarithmic phase cells, in 2 ml aliquots,

were infected with bacteriophage and grown at 28 C for 30 minutes. 2 ml LB medium was added to the bacteriophage infected culture and the infected cells were then agitated for one hour at 30 C. Infected cells were concentrated at 2,000 rpm in a clinical centrifuge for 8 minutes, resuspended in 200 µl, and plated on 150 mm LB plates containing 50 µg/ml ampicillin. A total of $5.5x10^7$ cells were infected, plated and grown overnight at 37 C. Colonies were resuspended in 30 ml LB and used to inoculate 3 L Terrific Broth containing 50 µg/ml ampicillin. Cells were harvested and the pACT-RB2 plasmid library was isolated using the Qiagen Maxi DNA preparation kit for low copy plasmids (pACT is a low copy pBR322 based plasmid).

2. Culturing *C. elegans*

As a source of wild type nematodes for the RNAi experiments, *C. elegans* Bristol (N2) was cultured on NG plates (0.3% NaCl, 0.25% tryptone, 5 mg/ml cholesterol, 1mM $CaCl_2$, 1mM $MgSO_4$, 25mM KH_2PO_4, 1.7% agarose, pH 6.0) spread with a lawn of *Escherichia coli* OP50 (Brenner, 1974). L4 stage larvae were transferred to new OP50 seeded plates to support the growth of the next generation.

3. Maintenance and mating of *S. cerevisiae*

Yeast strains Y187 (MATα *gal4 gal80 his3 trp1*-901 *ade2*-101 *ura3*-52 *leu2*-3,112 + URA3::GAL *lacZ* LYS2::GAL(UAS) HIS3 *cyh*^{r}) and Y190 (MATa *gal4 gal80 his3 trp1*-901 *ade2*-101 *ura3*-52 *leu2*-3,-112 *met-* URA3::GAL *lacZ*) were maintained on YEPD plates (10 mg/ml Bacto yeast extract, 20 mg/ml Bacto tryptone peptone, 20 mg/ml glucose and 2% agarose). Yeast strains were cultured on plates and in liquid media at 30 C. Selection media contained 20 mg/ml glucose, yeast nitrogen base without amino acids (Difco), 2% agarose, and a portion of a synthetic complete (SC) mixture of amino acids lacking leucine (-Leu) (0.7 mg/ml), lacking tryptophan (-Trp) (0.7 mg/ml), lacking leucine and tryptophan (-Leu-Trp) (0.6mg/ml), or lacking leucine, tryptophan and histidine (-Leu-Trp-His) (0.6 mg/ml) (Bio101). SC-Leu media was used to select for yeast transformed with pACT::*cDNA* plasmids or pSE1111 control plasmid. SC-Trp media was used to select for yeast transformed with pAS2::*ubc-2* or pSE1112 control plasmid. SC-Leu-Trp was used to select for the presence of yeast doubly

transformed with plasmids containing these nutrient selection markers. SC-Leu-Trp-His was used to select for yeast colonies containing two plasmids containing these nutrient selection markers and to select for yeast colonies expressing interacting proteins. This SC dropout medium was supplemented with 25 mM 3-aminotriazole to inhibit growth of colonies expressing non-interacting proteins due to potential weak activation of the reporter genes while still permitting growth of colonies expressing interacting proteins. Following an attempt to lose the bait plasmid by non-selection by streaking colonies transformed with the bait plasmid (pAS2::*ubc-2*) and a particular pACT::*cDNA* plasmid on SC-Leu media, colonies were plated on SC-Leu containing 2.5 μg/ml cycloheximide. This drug was used to eliminate colonies transformed with pAS2::*ubc-2* (which contains a marker for cycloheximide sensitivity) and a particular pACT::*cDNA* plasmid while permitting growth of colonies containing only a single pACT::*cDNA* plasmid. This facilitated isolation of the cDNA plasmid away from the bait plasmid to permit isolation of a single cDNA plasmid species for sequencing and manipulation.

Yeast matings were used as a means to eliminate non-specific interactors of UBC-2. Y190 (MATa) transformed with a particular pACT::*cDNA* plasmid was streaked in patches on SC-Leu media grown at 30 C for 1 day. Y187 (MATα) transformed with pAS1::*snf1* or pAS1::*cdk2* was streaked on SC-Trp plates and grown at 30 C for 3 days. One Y187 yeast colony was resuspended in 1 ml YEPD and 250 μl of this mixture was spread on a YEPD plate and grown at 30 C for 3 hours. Sterilized velvet was used to replica plate the patched Y190 from the SC-Leu plate onto a lawn of Y187 on the YEPD plate and yeast was grown at 30 C overnight. Yeast colonies were replica plated from the YEPD plate onto a SC-Trp-Leu plate and were grown at 30 C until colonies appeared. An X-gal filter lift assay (described below) was performed to detect the activation of the reporter gene *lacZ*, and hence, a non-specific interaction between a UBC-2 interacting protein and SNF1 or CDK2.

4. X-gal filter lift assay

Schleicher and Schuell BA85 45 μm circular nitrocellulose filters were used to lift yeast colonies expressing interacting proteins (i.e. they grew on SC-His media) from the plate media. Liquid nitrogen was used to permeabilize the cells to Z buffer (60 mM

Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 40 mM 2-mercaptoethanol, pH 7.0) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethyl formamide. Filters were incubated at 30°C until a blue colour developed *in situ* in the positive controls (i.e. Y190 yeast strain transformed with pSE1111 and pSE1112).

5. Computer analysis

DNA Strider was used to determine cloning strategies and to design oligonucleotide primers. BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) was employed to positively identify cDNA sequences encoding putative UBC-2 interacting proteins from the *C. elegans* plasmid cDNA library. Protein sequences of UBC-2 interacting proteins were obtained from the GenBank web site (http://www.ncbi.nlm.nih.gov/Database/index.html). The Simple Modular Architecture Research Tool (SMART) was used to search protein sequences for protein domains (http://smart.embl-heidelberg.de/) (Schultz *et al.*, 1998; Schultz *et al.*, 2000). The WormPD was used to find homologues for UBC-2 interacting proteins (Bateman *et al.*, 1999; Genome sequence of the nematode *C. elegans*: a platform for investigating biology, 1998)

6. Protein and immunological techniques

6.1 TNT® Quick Coupled Transcription/Translation of radiolabeled DSK-2

The cDNA fragment encoding DSK-2 was excised from plasmid pACT::F15C11.2 (*dsk-2*) and cloned into the *Xho*I site of the multiple cloning site of pcDNA3.1/Hygro(+) (Invitrogen). ^{35}S-labeled DSK-2 was produced using [^{35}S]methionine and a TNT® Quick Coupled Transcription/Translation System (Promega). Translation was performed according to the manufacturer's instructions using 40 µl TNT® Quick Master Mix, 20 µCi [^{35}S]methionine, and 1 µg plasmid DNA template (pcDNA3.1::*dsk*-2).

6.2 Overexpression and purification of 6XHIS-UBC-2

C-terminal 6XHIS-UBC-2 fusion protein was expressed from a pRSET-c-*ubc-2* (Invitrogen) construct which had been previously made by M. Zhen (Zhen, Ph.D. thesis,

1995). An N-terminal 6X HIS-UBC-2 fusion protein was also prepared from the pET28-a (+) expression vector (Novagen). *ubc-2* was cloned from pAS2::*ubc-2* using primer oligonucleotides MG7 and MG10 and inserted into the *Nco*I and *Xho*I sites of the pET28-a (+) vector.

The pRSETC::*ubc-2* and pET28-a(+)::*ubc-2* constructs were transformed into competent BL21 (DE3) STAR (Invitrogen) *E. coli*. Fresh pRSETC::*ubc-2* and pET28-a(+)::*ubc-2* transformed colonies were used to inoculate 10 ml of LB containing 100 μg/ml ampicillin or 50 μg/ml kanamycin, respectively. These cultures, grown overnight at 37°C, were used to inoculate 1 L of the same medium. After the OD_{600} reading had reached 0.7, cells were induced with 1 mM IPTG, grown for another 3 hours and cell pellets were obtained following centrifugation at 2,000xg for 15 minutes at 4°C. Each pellet was resuspended in 40 ml of extraction/wash buffer (300 mM NaCl, 50 mM $NaPO_4$, pH 7.0). Cell suspensions were sonicated using a 10 second pulse on and 20 second pulse off for 5 min. The cell extract was centrifuged at 11,000xg for 20 minutes at 4°C to pellet the insoluble portion. The soluble portion was incubated for 20 minutes at room temperature with 4 ml of a 50% slurry of TALON® resin equilibrated in extraction/wash buffer. The resin was pelleted at 700xg for 5 minutes and washed three times. 500 μl fractions were collected after adding the resin to a column and applying elution buffer (extraction/wash buffer with 150 mM imidazole). Fractions were pooled and dialyzed overnight at 4°C in UBC-2 buffer (300 mM NaCl, 50 mM NaPO4, pH 7.4) using MWCO 3350 dialysis tubing to remove imidazole. During dialysis, the C-terminal 6XHIS-UBC-2 precipitated out of solution. The soluble portion of the protein preparation was removed as the supernatant following centrifugation for 10 minutes at 12,000 rpm in a microcentrifuge. The protein concentration was determined using a Bio-Rad microassay. One litre of culture yielded 12 mg of soluble C-terminal 6XHIS-UBC-2 fusion protein and 2 mg of N-terminal 6XHIS-UBC-2. To reduce the total reaction volume for the cross-linking experiments, N-terminal 6XHIS-UBC-2 was concentrated 10 fold on a Millipore Ultrafree-4 centrifugal filter device (Biomax 5K NMWL Membrane 4 ml volume).

6.3 Overexpression and purification of GST::DSK-2

N-terminal GST::DSK-2 fusion protein was expressed using a pGEX-4T-2 (Pharmacia Biotech.) construct containing the *dsk-2* gene which was subcloned from the pACT vector (Matchmaker) into the *Xho*I site of the pGEX-4T-2 vector in the correct orientation and reading frame. The pGEX-4T-2 construct was transformed into BL21 (DE3) STAR *E. coli*. Fresh pGEX-4T-2::*dsk-2* transformed colonies were used to inoculate 10 ml of LB containing 100 μg/ml ampicillin. These cultures grown overnight at 37°C were used to inoculate 1 L of the same medium. After the culture had reached an OD_{600} reading of 0.4, cells were induced with 1 mM IPTG and grown for an additional 3 hours. The preparation of bacterial sonicates and the batch purification of GST::DSK-2 fusion protein was performed using procedure 11 and 12 from the GST Gene Fusion System (Third Edition, Revision 1; Amersham Pharmacia Biotech). The buffer used during protein purification included 1 mM DTT and 1 protease inhibitor cocktail minitablet (Complete™ Mini, EDTA free; Roche) for every 10 ml of buffer.

The GST tag of N-terminal GST::DSK-2 was removed using Thrombin Protease (Amersham Pharmacia Biotech). Thirty μg of GST::DSK-2 were incubated with one unit (50 ng) of Thrombin Protease in 1X phosphate buffered saline [PBS; 0.58 M Na_2HPO_4, 0.17 M NaH_2PO_4, 0.68 M NaCl, pH 7.4] overnight at room temperature.

6.4. Preparation of *S. cerevisiae* protein extract

To confirm that the GAL4DBD::UBC-2 fusion protein was expressed from the pAS2::*ubc-2* construct in yeast strain Y190, protein extracts were prepared from two 50 ml, 48 hour Y190 cultures, of which one expressed the GAL4DBD::UBC-2 fusion protein. Cell pellets were obtained after centrifugation for 10 minutes at 1500 rpm in a clinical centrifuge. Cells were washed with water, pelleted and resuspended in 200 μl of 2X SDS sample buffer. An equal volume of glass beads (Sigma) was added before vigorous mixing for 10 minutes using a Vortex mixer. Twenty μl of this extract was boiled and separated on an SDS-PAGE gel prior to Western blot analysis using an anti-UBC-2 antibody to detect the GAL4DBD::UBC-2 fusion protein.

6.5. Protein concentration determination

The concentration of protein samples was determined using a BioRad microassay according to the manufacturer's instructions. A BSA standard curve was used to determine the protein concentration of unknown protein samples.

6.6. SDS-polyacrylamide gel electrophoresis

Discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to separate proteins according to size (Laemmli, 1970). Protein samples were suspended in loading sample buffer (LSB) (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.1% bromophenol blue) and boiled for 5 minutes prior to gel loading. Gels (0.75 mm X 7.5 cm X 8.0 cm) were poured and run using the Mini-PROTEAN II apparatus (BioRad) according to the manufacturer's instructions.

6.7. Western blot analysis

Methanol soaked Immobilon-P membranes (Millipore) and SDS-PAGE gels containing separated proteins were soaked in transfer buffer (25mM Tris-HCl pH 8.5, 192 mM glycine, 20% methanol) for 5 minutes prior to transfer. A sandwich of filter paper-membrane-gel-filter paper was prepared prior to assembly in the BioRad transfer apparatus. Transfer of proteins took place at 250 mA for one hour.

Western blots were agitated at room temperature in TBS-Tween (140 mM NaCl, 20 mM Tris-HCl, 0.05% Tween-20, pH 7.5) containing 10% milk powder for one hour. One hour of primary antibody incubation was followed by one hour of secondary antibody incubation. Blots were washed three times for 5 minutes in TBS-Tween after blocking and antibody incubations. Polyclonal primary antibodies used in Western blot analysis in this study were used at the following dilutions in TBS-Tween: anti-GST (a kind gift from I. J. Sadowski, The University of British Columbia, Canada) at 1:3,000, anti-HIS at 1:5,000 (Santa Cruz), anti-UBC-2 at 1:5,000 (prepared by T.A. Stevens), anti-DSK2 (a kind gift from M. Rose, Princeton University, U.S.A.), and anti-ubiquitin at 1:500 (Santa Cruz). A 1:10,000 dilution of peroxidase-labelled donkey anti-rabbit antibody (Amersham Pharmacia Biotech) was used for secondary antibody incubation.

The enhanced chemiluminescence kit (ECL™-Amersham Pharmacia Biotech) was used to visualize the protein-antibody complexes.

6.8. Coprecipitation of UBC-2 and DSK-2*

Ten µl or 30 µl of radiolabelled DSK-2* synthesized in a TNT kit was incubated with 0.5 µg C-terminal 6XHIS-UBC-2 in 200 µl UBC-2 buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.1% Triton X-100, pH 8.0) on a Nutator (Clay Adams® Brand) for 1.5 hours at 4°C. As a negative control to demonstrate the absence of non-specific interactions between DSK-2 and the resin, 10 µl DSK-2* was incubated with TALON® resin under the same reaction conditions. As a positive control to demonstrate that N-terminal 6XHIS-UBC-2 was interacting with the TALON® resin, 0.5 µg C-terminal 6XHIS-UBC-2 was incubated with TALON® resin under the same reaction conditions. Twenty µl of 50% TALON® resin in UBC-2 buffer was then added for 1 hour. Reactions were pelleted at 2,000 rpm in a microcentrifuge. Twenty µl of the supernatant was saved for analysis. TALON® resin pellets were washed twice in UBC-2 buffer before resuspension in 50 µl LSB. The supernatant and pellet fractions were divided and analyzed by autoradiography to detect DSK-2* and Western blotting to detect UBC-2.

6.9. Coprecipitation of UBC-2 and DSK-2 fusion proteins

Seven µg N-terminal 6XHIS-UBC-2 or C-terminal 6XHIS-UBC-2 was incubated for two hours on a Nutator at 4 C with 40 µl 50% TALON® resin and 30 µg GST::DSK-2 in 200 µl of UBC-2 buffer supplemented with 1% Triton X-100 and 10 mM imidazole. Reactions were pelleted at 2,000 rpm in a microcentrifuge and pellets were washed three times in UBC-2 buffer. The same amounts of the UBC-2 fusion proteins and GST::DSK-2 were incubated individually with the TALON® resin to demonstrate, respectively, positive and negative interaction of these proteins with the resin under co-precipitation reaction conditions. Twenty µl of the supernatant, 20 µl of the wash, and the pellet fractions of the co-precipitation experiments were separated on 10% and 12.5% SDS-PAGE gels and respectively analyzed via Western blotting with anti-GST and anti-UBC-2 primary antibodies.

6.10. Cross-linking UBC-2 and DSK-2 fusion proteins

Three μg GST::DSK-2 (10 μl) and 0.2 μg C-terminal 6XHIS-UBC-2 (10 μl) were incubated together for 1 hour at room temperature followed by one hour on ice. BS^3 (Pierce) cross-linker was added to a final concentration of 1 mM. Cross-linking reactions were quenched with 100 mM Tris-HCl pH 7.5 after 3, 5, 10, 15, 20, 25, and 30 minutes. As a control, three μg GST::DSK-2 were incubated in 10 μl UBC-2 buffer with cross-linker. Reactions were divided and subjected to Western blot analysis using anti-GST or anti-UBC-2 primary antibodies.

6.11. Coprecipitation of multi-ubiquitin and GST::DSK-2

Forty μl of a 50% slurry of TALON® resin was incubated with 2 μg poly-ubiquitin chains (Ub_{2-7}) (Affiniti) and 7 μg C-terminal 6XHIS-UBC-2 in 200 μl UBC-2 buffer supplemented with 0.5% Triton X-100 for 2 hours on a Nutator at 4 C. Forty μl of a 50% slurry of glutathione agarose was incubated with 2 μg polyubiquitin chains and 30 μg GST::DSK-2 in 200 μl UBC-2 buffer supplemented with 0.5% Triton X-100 for 2 hours on a Nutator at 4 C. Negative control precipitation reactions involved the incubation of polyubiquitin and GST::DSK-2 individually with TALON® resin. Positive control precipitation reactions involved the incubation of C-terminal 6XHIS-UBC-2 with TALON® resin and GST::DSK-2 with glutathione agarose. Reactions were pelleted at 2,000 rpm in a microcentrifuge and washed three times in UBC-2 buffer. Twenty μl of the supernatant, 20 μl of the wash, and the pellet fractions of the co-precipitation experiments were analyzed via SDS-PAGE and Western blotting with anti-GST or anti-UBC-2 primary antibodies.

7. RNA interference

7.1.Overexpression of double stranded RNA

cDNAs with *Xho*I site cohesive ends, isolated from the Robert Barstead *C. elegans* cDNA plasmid library in the screen for UBC-2 interacting proteins, were cloned non-directionally into the Timmons and Fire RNAi feeding vector (L4440) (Timmons and Fire, 1998). L4440 is an altered pBluescript vector with T7 promoters on either side of the polylinker region. The following cDNAs were cloned into L4440: B0432.9,

C25D7.8, F15C11.2, R160.7, W02A11.3, *mca-1*, *ubc-2*, and *ubiA*. The *rad-23* cDNA, a homologue of human Rad23, was cloned from yk81e3 (a kind gift from Y. Kohara, National Institute of Genetics, Japan) into the *Xba*I and *Kpn*I sites of L4440.

Constructs were transformed into HT115(DE3) *E. coli*, which lacks RNase III activity and exhibits IPTG inducible T7 polymerase activity, and grown overnight on LB plates containing 100 μg/ml ampicillin. Freshly transformed colonies were used to inoculate 5 ml of LB containing 100 μg/ml ampicillin. Cultures grown overnight at 37°C for 8 to 16 hours were pelleted at 5,000 rpm in a microcentrifuge, resuspended in 100 μl and used to seed NGM plates supplemented with 25 μg/ml carbenicillin and 1mM IPTG. Plates were left overnight at room temperature to induce T7 polymerase activity. L4 stage hermaphrodites were placed on these RNAi treatment plates for 72 hours at 15°C. Subsequently, treated nematodes were transferred to a second RNAi treatment plate for 24 hours at 15°C, after which adults were removed and the phenotypes of the progeny were recorded [Ahringer Lab RNAi Feeding Protocol (Version 10.8.00)] (Timmons and Fire, 1998).

7.2. Scoring phenotypes of RNAi treated nematodes

The number of progeny per RNAi treated nematode (fecundity) and the average length of RNAi treated progeny was recorded. To measure the progeny, approximately 10 nematodes were picked from each RNAi treatment plate into 5 drops of M9 buffer (22mM KH_2PO_4, 22mM Na_2HPO_4, 85mM NaCl, 1mM $MgSO_4$) on a slide. The slides were heated for 30 seconds on a hot plate at 100 C. This treatment effectively killed the worms and straightened their bodies enabling more accurate body length measurement. Nematodes were measured at 25X magnification using an ocular micrometer. The measurements were converted to millimeter units using a conversion factor 1 unit=0.4 mm. A Zeiss microscope was used to analyze and photograph RNAi treated worms.

III. RESULTS AND DISCUSSION

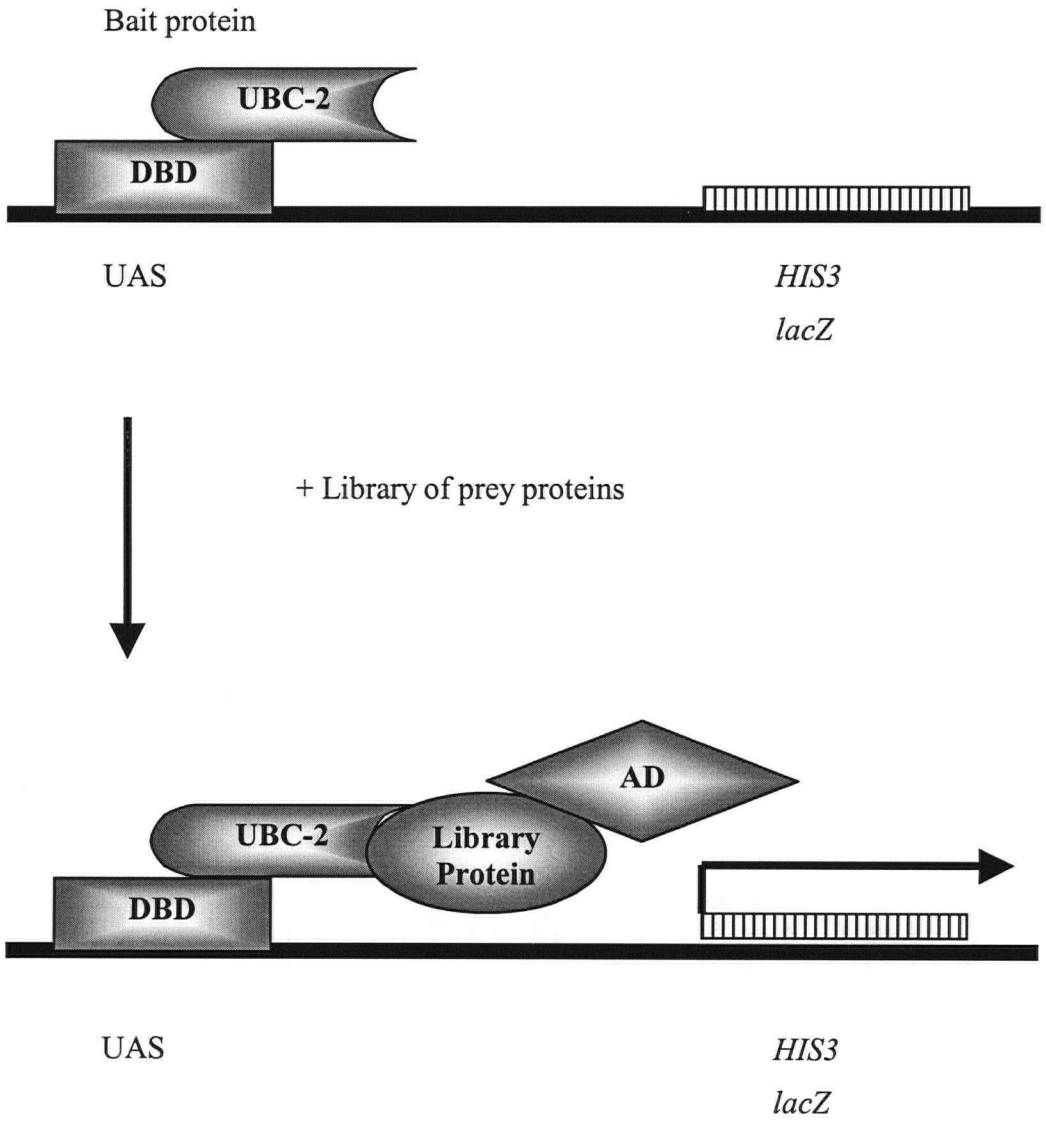
1.a. The yeast two-hybrid screen to identify interacting proteins

To identify protein partners for UBC-2 in *C. elegans,* a yeast two-hybrid screen was employed. This method, performed in yeast strain Y190, involved expressing a bait protein consisting of the DNA binding domain (DBD) of the GAL4 transcription factor fused to UBC-2, and expressing a library of prey proteins consisting of *C. elegans* library proteins fused to the activation domain (AD) of the GAL4 transcription factor (Figure 2). The key idea of the yeast two-hybrid screen is that if a protein, such as UBC-2, interacts with a particular library protein, the GAL4 transcription factor will be reconstituted and allow transcription of the yeast strain reporter genes under GAL4 transcriptional control, such as *his3* and *lacZ* in Y190 (Fields and Song, 1989). Yeast colonies containing interacting proteins grew on synthetic complete media lacking histidine and turned blue in the presence of X-gal. Prior to initiating the screen, it was determined that the bait protein was expressed in yeast strain Y190, and that it was the appropriate size (Figure 3).

Various control tests were performed to confirm that neither the bait plasmid pAS2::*ubc-2* nor the vectors pAS2 or pACT alone or in combination, caused autoactivation of the reporter genes (Table 1). It was determined that supplementation of the synthetic complete medium lacking histidine with 25 mM 3-aminotriazole (3-AT) was sufficient to prevent growth of colonies transformed with the pAS2::*ubc-2* construct. Colonies transformed with pAS2 and pACT together unexpectedly turned blue in the X-gal filter lift assay probably due to leakiness of the *lacZ* reporter gene. It has been reported that once sequences are cloned into the pAS2 plasmid, this weak activation disappears, possibly due to sequences beyond the polylinker that are of no consequence once cDNAs are cloned in it (Elledge, 1993).

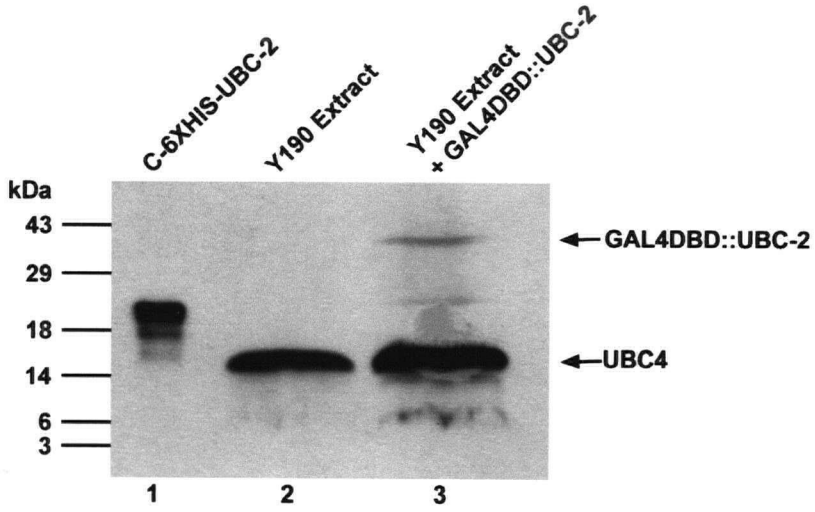
In addition, positive control tests were performed to ensure that reporter gene activation could be detected using the known interacting GAL4DBD fusion proteins, SNF4 (encoded by plasmid pSE1111) and SNF1 (encoded by plasmid pSE1112). Yeast colonies containing both of these interacting proteins grew on synthetic complete media

Figure 2. The yeast two-hybrid assay for interacting proteins
The yeast two-hybrid assay involved making a bait protein consisting of the DNA binding domain (DBD) of the GAL4 transcription factor fused to UBC-2 and making a library of prey proteins consisting of *C. elegans* library proteins fused to the activation domain (AD) of the GAL4 transcription factor. If UBC-2 interacts with a particular library protein, the GAL4 transcription factor will be reconstituted and allow transcription of the yeast strain Y190 reporter genes, *his3* and *lacZ*. Yeast colonies containing interacting proteins grow on synthetic complete medium lacking histidine and turn blue in the presence of X-gal.



(Adapted from Fields and Bartel, 1997)

Figure 3. GAL4DBD::UBC-2 fusion protein is expressed in yeast strain Y190
Yeast extract was prepared from cultures of yeast strains Y190 and Y190 transformed with the yeast two-hybrid bait encoding plasmid pAS2::*ubc-2*. Proteins were separated by electrophoresis on a 12.5% SDS-PAGE gel and subjected to Western blot analysis using anti-UBC-2 antibody (1:5,000). Note the higher molecular weight band in the Y190 extract + GAL4DBD::UBC-2 (lane 3) which likely corresponds to the 34 kDa bait fusion protein, GAL4DBD::UBC-2 The latter was used in the yeast two-hybrid screen for interacting proteins of the pACT-RB2 *C. elegans* cDNA library. To ensure the antibody could detect the bait protein, C-terminal 6XHIS-UBC-2 (23 kDa) was included as a positive control (lane 1). Endogenous UBC4/5 in yeast strain Y190 was detected by anti-UBC-2 antibody as seen in the lower molecular weight band in both Y190 extracts (lanes. 2 and 3)



lacking leucine and tryptophan to select for the vectors' nutrient marker and to select for activation of the protein interaction reporter genes *his3* and *lac*Z, by virture of their ability to grow in the absence of histidine and turn blue in the presence of X-gal (Table 1).

To ensure that the *C. elegans* library was sufficiently screened for proteins that interact with UBC-2, it was necessary to screen at least 600,000 double transformants containing both the bait and prey proteins. In total, 980,000 double transformants were screened for activation of the *his3* and *lac*Z reporter genes. Initially, 56 colonies expressing interacting proteins were isolated. From the putative positive colonies, plasmids encoding putative UBC-2 interacting proteins were separated from plasmids encoding the bait protein. This was accomplished through selection solely for the prey encoding plasmids, resulting in eventual loss of the bait encoding plasmid. Additionally, cycloheximide sensitivity selection against colonies hosting plasmids encoding the bait protein was employed. Subsequently, the prey encoding plasmids were electroporated into and amplified in *Escherichia coli*.

2. Yeast two-hybrid false positive elimination

Four tests were employed to eliminate false positive interactors of UBC-2 (Table 1). The first test involved transforming yeast strain Y190 with pACT::cDNA plasmids encoding putative UBC-2 interacting GAL4AD fusion proteins. Colonies expressing putative interacting prey proteins grew on synthetic complete media lacking leucine to select for the prey encoding plasmid, pACT, but did not grow in the absence of histidine with 25 mM 3-AT present or turn blue in the X-gal filter lift assay. Therefore, it was concluded that putative UBC-2 interacting proteins do not independently activate the reporter genes *lac*Z and *his*3 in the yeast two-hybrid system.

The second test involved reconfirming the interaction between the putative UBC-2 interacting GAL4AD fusion proteins and the GAL4DBD::UBC-2 fusion protein. This test involved double transforming yeast strain Y190 with the pAS2::ubc-2 plasmid and one of the pACT::cDNA plasmids and selecting for activation of the reporter genes. Colonies expressing the bait protein and a putative UBC-2 interacting prey protein grew on synthetic complete medium lacking tryptophan and leucine to select for the bait

Table 1. Yeast Two-Hybrid Assay Control Tests

DNA used for Y190 transformation	Growth on -TRP	Growth on -LEU	Growth on -TRP -LEU	Growth on -HIS + 25 mM 3-AT	Growth on -TRP -LEU -HIS + 25 mM 3-AT	Positive X-gal Assay
pAS2	Yes	No	No	No	n.d.	No
pAS2::*ubc-2*	Yes	No	No	No	n.d.	No
pACT	No	Yes	No	No	n.d.	No
pAS2 + pACT	n.d.	n.d.	Yes	n.d.	No	Yes*
pAS2::*ubc-2* + pACT	n.d.	n.d.	Yes	n.d.	No	No
pSE1111	No	Yes	No	No	n.d.	No
pSE1112	Yes	No	No	No	n.d.	No
pSE1111 + pSE1112	n.d.	n.d.	Yes	n.d.	Yes	Yes

* The colonies unexpectedly turned blue in the X-gal filter lift assay probably due to leakiness of the *lacZ* reporter gene. It has been reported that once sequences are cloned into the pAS2 plasmid, the weak activation appears to go away probably due to sequences beyond the polylinker that are of no consequence once cDNAs are cloned in it (Elledge, 1993).

pSE1111 = pACT::*snf1*
pSE1112 = pAS1::*snf4*
n.d. = no data

encoding plasmid, pAS2, and the prey encoding plasmid, pACT. Twenty-nine of these double transformant colonies grew on synthetic complete medium lacking histidine supplemented with 25 mM 3-AT and turned blue in the X-gal filter lift assay. However, a yeast two-hybrid interaction could not be confirmed for the rest of the original pool of 56 putative UBC-2 interacting GAL4AD fusion proteins.

The third test involved confirming that the putative UBC-2 interacting protein portion of the bait protein, not the GAL4 DNA binding domain portion, mediates the yeast two-hybrid interaction. Yeast strain Y190 was transformed with pAS2 and one of the pACT::cDNA plasmids. Colonies expressing the GAL4 DNA binding domain portion and a putative interacting prey protein grew on synthetic complete media lacking tryptophan and leucine to select for the bait encoding plasmid, pAS2, and the prey encoding plasmid, pACT. Doubly transformed colonies did not grow in the absence of histidine indicating that the *his3* reporter gene was not activated; however, the colonies did unexpectedly turn blue in the X-gal filter lift assay probably due to leakiness of the *lacZ* reporter gene. It has been reported that once sequences are cloned into the pACT plasmid, the weak activation appears to be suppressed, probably due to sequences beyond the polylinker that are inactivated once cDNAs are cloned into it (Elledge, 1993).

The fourth false positive test involved a yeast mating assay to eliminate putative UBC-2 interactors that bind the GAL4DBD::UBC-2 fusion protein nonspecifically. After mating yeast strain Y187(MATα)/pAS2::*cdk2* or Y187(MATα)/pAS2::*snf1* with yeast strain Y190(MATa)/pACT::cDNA and assaying for activation of the reporter gene *lacZ*, it was found that the UBC-2 interacting proteins did not bind to the CDK2 or SNF1 GAL4DBD fusion proteins (Tables 2.A. and B.). Therefore, the prey proteins identified in the yeast two-hybrid screen appear to be specific in their interaction with UBC-2. As a positive control for the mating assay, yeast strain Y187(MATα)/pAS2::*ubc-2* was mated with yeast strain Y190(MATa)/pACT::cDNA. The positive results of the X-gal assays revealed that the mating assay was optimized to replicate most of the yeast two-hybrid interactions between the GAL4DBD::UBC-2 fusion protein and the putative GAL4AD::UBC-2 interacting proteins which were discovered in the initial screen.

Table 2.A. UBC-2 interacting GAL4AD fusion proteins do not interact with GAL4DBD::CDK2 fusion protein

[Y187(MATα) + pAS2::*cdk2*] X [Y190 (MATa) + pACT::*cDNA*]

cDNA in pACT	Growth on –TRP, -LEU	Positive X-gal Assay
W02A11.3	Yes	No
F15C11.2	Yes	No
B0432.9	Yes	No
mca-1	Yes	No
R160.7	Yes	No

Table 2.B. UBC-2 interacting GAL4AD fusion proteins do not interact with GAL4DBD SNF1 fusion protein

[Y187(MATα) + pAS2::*snf1*] X [Y190 (MATa) + pACT::*cDNA*]

cDNA in pACT	Growth on –TRP, -LEU	Positive X-gal Assay
W02A11.3	Yes	No
F15C11.2	Yes	No
B0432.9	Yes	No
mca-1	Yes	No
R160.7	Yes	No

3. Proteins that exhibit a yeast-two hybrid interaction with UBC-2

Plasmids (pACT::cDNA) encoding putative UBC-2 interactors were analyzed by restriction digests, sequencing, and BLAST. In conclusion, six different proteins that interact with UBC-2 were identified using the yeast-two hybrid assay. It was determined that UBC-2 interacts with the following proteins of interest: ubiquitin (encoded by *Ubi*A); a ubiquitin-like protein encoded by F15C11.2 hereafter referred to as DSK-2 due to its similarity to *S. cerevisiae* DSK2; two RING finger proteins (encoded by B0432.9 and W02A11.3), a calcium ATPase (encoded by *mca*-1), and a FYVE finger protein (encoded by R160.7) (Figure 4). Four clones of the 29 clones encoding UBC-2 interacting proteins identified in the yeast two-hybrid screen, which passed false positive testing, did not exhibit homology to any known gene sequences. A protein encoded by the C25D7.8 gene and ubiquitin represented 1 and 3 clones, respectively, of the 29 clones encoding UBC-2 interacting proteins identified in the yeast two-hybrid screen which passed false positive testing. A search for protein domains using the Simple Modular Architecture Research Tool (SMART) revealed that the amino acid sequence of the protein encoded by C25D7.8 does not exhibit any previously characterized domains (Schultz *et al.*, 1998; Schultz *et al.*, 2000). The interaction between UBC-2 and ubiquitin was expected due to a previous experiment by M. Zhen which showed that UBC-2 can form a thiolester bond with ubiquitin (Zhen, Ph.D. thesis, 1995).

3.1. UBC-2 interacts with a plasma membrane calcium ATPase

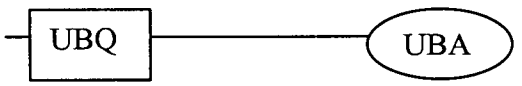
A plasma membrane calcium ATPase with 10 alpha-helical transmembrane domains, exhibits a yeast two-hybrid interaction with *C. elegans* UBC-2. MCA-1 represented 1 of 29 clones encoding UBC-2 interacting proteins identified in the yeast two-hybrid screen which passed false positive testing. A search for protein domains using the Simple Modular Architecture Research Tool (SMART) reveals that the 1228 amino acid sequence of MCA-1 contains ten transmembrane domains from residues 99-116, 155-177, 357-379, 399-421, 856-878, 888-905, 935-957, 972-989, 1010-1032, and 1037-1059 (Schultz *et al.*, 1998; Schultz *et al.*, 2000). A WormPD database search reveals homologues of *C. elegans* MCA-1 in other organisms including: *S. cerevisiae* (the calcium ATPase Pmc1p is 35% similar to MCA-1); *Mus musculus* (the calcium ATPase

Figure 4. Predicted domains of *C. elegans* proteins identified in the yeast two-hybrid screen for proteins that interact with UBC-2

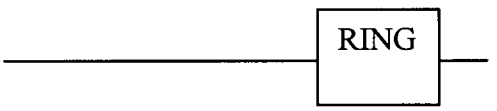
Gene/Size/
Number of clones identified
in the yeast two-hybrid screen

Predicted domains

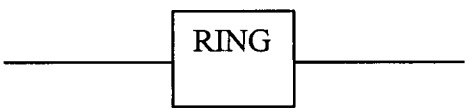
F15C11.2 (DSK-2);
60 kDa;
17



W02A11.3;
59 kDa;
1

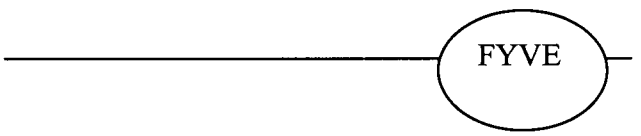


B0432.9;
51 kDa;
1



mca-1;
147 kDa;
1

R160.7;
79 kDa;
1



ATP2B2 is 55% similar to MCA-1), and *H. sapiens* (the calcium ATPases ATP2B1, ATP2B2, ATP2B3 are all 55% similar to MCA-1) (Bateman *et al.*, 1999; Genome sequence of the nematode *C. elegans*: a platform for investigating biology, 1998).

Calcium-ATPases in eukaryotes pump calcium outside of the cell coupled with ATP hydrolysis. Previous analysis of this protein called MCA-1 in *C. elegans* revealed that it is a functional calcium ATPase, it has a serine-rich stretch of 11 residues between the 2nd and 3rd transmembrane domains and it interacts with human calmodulin through a C-terminal calmodulin-binding domain (Kraev *et al.*, 1999). Perhaps monoubiquitylation of calmodulin mediated by UBC4, albeit demonstrated only *in vitro*, (Parag *et al.*, 1993) is enabled through interaction of UBC4 with MCA-1.

In *S. pombe*, calmodulin is a calcium binding protein that localizes to the spindle pole body (SPB), stimulates plasma membrane calcium ATPases, and is required for chromosome segregation (Moser *et al.*, 1997). Due to the fact that MCA-1 can bind calmodulin, it is possible that MCA-1 may be associated in some way with the centrosome. Recently, it has been determined that calcium, calmodulin, and calcium/calmodulin-dependent protein kinase II (CaMKII) are necessary for the first duplication of the centrosome in *Xenopus* (Matsumoto and Maller, 2002). CaMKII is activated by calcium to stimulate cyclin B degradation via the APC to allow exit from mitosis (Lorca *et al.*, 1993). Since a sudden increase in calcium is required for centrosome duplication at the G_1-S transition of the cell cycle (Matsumoto and Maller, 2002), the maintenance of low levels of intracellular calcium, possibly through export by a calcium ATPase such as MCA-1, may provide a link between duplication of the centrosome and MCA-1 function. It is intriguing to note that two detected interactors of UBC-2, DSK-2 (discussed below), and MCA-1, have homologues in other organisms that are linked to duplication of microtubule organizing centres such as the SPB in yeast and the centrosome in metazoans.

3.2. UBC-2 interacts with a FYVE finger domain protein

A FYVE finger domain protein called R160.7 exhibits a yeast two-hybrid interaction with UBC-2. R160.7 represented 1 of 29 clones encoding UBC-2 interacting

proteins identified in the yeast two-hybrid screen which passed false positive testing. A search for protein domains using SMART revealed that the 658 amino acid sequence of R160.7 includes a FYVE domain from residues 561 to 616 (Schultz *et al.*, 1998; Schultz *et al.*, 2000). A WormPD database search revealed homologues of *C. elegans* R160.7 in other organisms including: *S. cerevisiae* (Fab1p is 50% similar to R160.7); *M. musculus* (PIKFYVE is 58% similar to R160.7), and *H. sapiens* (KIAA0993 is 58% similar to R160.7) (Bateman *et al.*, 1999; Genome sequence of the nematode *C. elegans*: a platform for investigating biology, 1998). The FYVE finger domain is a cysteine-rich conserved sequence that has a demonstrated ability to bind zinc and the membrane lipid phosphatidylinositol-3-phosphate (PtdIns(3)P) (Gaullier *et al.*, 1998; Stenmark *et al.*, 1996).

3.3. UBC-2 interacts with RING finger proteins

The B0432.9 and W02A11.3 genes encode RING finger proteins that exhibit yeast two-hybrid interactions with UBC-2. B0432.9 and W02A11.3 each represented 1 of 29 clones encoding UBC-2 interacting proteins identified in the yeast two-hybrid screen which passed false positive testing. Interestingly, many E3s contain RING finger domains. It seems plausible that UBC-2 can interact with RING proteins since, as mentioned previously, RING finger domains in E3s appear to be necessary for mediating interaction with E2s and for ubiquitylation of substrates (reviewed in Joazeiro and Weissman, 2000; Lorick *et al.*, 1999).

A search for protein domains using SMART revealed that the 425 amino acid sequence of the B0432.9 protein includes a RING domain from residues 192 to 232 and that the 489 amino acid sequence of the W02A11.3 protein includes a RING domain from residues 433 to 473 (Schultz *et al.*, 1998; Schultz *et al.*, 2000). A WormPD database search revealed that *S. cerevisiae* Hrd1p is 27% similar to *C. elegans* B0432.9 and that *S. cerevisiae* Apc11p and San1p are respectively 34% and 49% similar to *C. elegans* W02A11.3 (Bateman *et al.*, 1999; Genome sequence of the nematode *C. elegans*: a platform for investigating biology, 1998). Interestingly, Hrd1p/Der3p in *S. cerevisiae* is an E3 which catalyzes ubiquitylation and endoplasmic reticulum-associated degradation (ERAD) of misfolded lumenal and membrane proteins in association with its interacting

E2s, UBC1 and UBC7 (Bays *et al.*, 2001; Boradallo, 1998). Since UBC-2 interacts with a protein similar to Hrd1p, it is possible that UBC-2 may also play a previously unrecognized role in the ERAD of misfolded proteins. Furthermore, the RING-H2 finger domain of APC11 was determined to be necessary for E3 activity in the ubiquitylation of cyclin B to allow exit from mitosis and for interaction with the ubiquitin conjugating enzyme UBC4 in *S. cerevisiae* (Gmachl *et al.*, 2000; Leverson *et al.*, 2000). Since an *in vitro* interaction between APC11 and UBC4 has been demonstrated, it is plausible that UBC-2 exhibits a yeast two-hybrid interaction with an APC11-like protein to promote progression of the cell cycle. Nevertheless, it should be noted that B0432.9 and W02A11.3 belong to the RING-HC class while their respective counterparts Hrd1p and APC11 in *S. cervisiae* belong to the RING-H2 class. Whether these differences preclude the involvement of these UBC-2 interacting RING finger proteins in ERAD or cyclin B degradation remains to be determined.

3.4. UBC-2 interacts with an ubiquitin-domain protein

An ubiquitin-domain protein was identified in the yeast two-hybrid screen for UBC-2 interacting proteins. This *C. elegans* protein encoded by F15C11.2 was given the name DSK-2 due to a 32% similarity to *S. cerevisiae* DSK2 (dominant suppressor of *kar1*). DSK-2 represented 17 of the 29 clones encoding UBC-2 interacting proteins identified in the yeast two-hybrid screen which passed false positive testing. A search for protein domains using SMART revealed that the 502 amino acid sequence of DSK-2 includes an ubiquitin-like domain from residues 8 to 79 and an ubiquitin associated domain from residues 462 to 500 (Schultz *et al.*, 1998; Schultz *et al.*, 2000). A WormPD database search revealed homologues of *C. elegans* DSK-2 in other organisms including: *S. cerevisiae* (DSK2 is 32% similar to DSK-2); *S. pombe* (Dph1p is 23% similar to DSK-2), *M. Musculus* (PLIC2 is 35% similar to DSK-2), and *H. sapiens* (UBQLN3 is 29% similar to DSK-2) (Bateman *et al.*, 1999; Genome sequence of the nematode *C. elegans*: a platform for investigating biology, 1998). In *S. cerevisiae*, the requirement of RAD23 and DSK2 for spindle pole body (SPB) duplication potentially links the ubiquitin-proteasome pathway to processes involved in cell cycle progression (Biggins *et al.*, 1996). It is interesting to note that *in vivo* expression of UBQLN3, the human

homologue of DSK-2, is restricted to the testis, as are UBC4-2 and UBC4-testis (Rajapurohitam *et al.*, 1999; Wing and Jain, 1995; Wing *et al.*, 1996), rat homologues of UBC-2. The co-expression of DSK-2 and UBC-2 homologues in mammals provides further support for the possibility of an *in vivo* interaction between UBC-2 and DSK-2.

DSK-2 is a member of the ubiquitin-domain protein (UDP) class which is characterized by a ubiquitin-like domain and an inability to covalently bind other proteins through their C-termini, unlike the ubiquitin-like protein (UBL) class which exhibit this capability (reviewed in Jentsch and Pyrowolakis. 2000). Jentsch and Pyrowolakis have classified the following proteins as members of UDP class: *S. cerevisiae* RAD23, UBP6, and DSK2; *Xenopus* XDRP1 and Scythe; and mammalian HHR23A, HHR23B, PLIC1, PLIC2/Chap1, BAG-1L, BAG-1S, BAT3/Chap2, Parkin, UIP28/RBCK1, Elongin B, and Gdx.

Ubiquitin-domain proteins demonstrate an ability to physically interact with components of the ubiquitin pathway. For example, *S. cerevisiae* DSK2 and RAD23, human BAG-1S, hHR23a, hHR23b, hPLIC-1 and hPLIC-2 can associate with the proteasome (Hiyami *et al.*, 1999; Funakoshi *et al.*, 2002; Lűders *et al.*, 2000; Kleijnen *et al.*, 2000; Schauber *et al.*, 1998). In particular, hHR23a, hHR23b, and hPLIC-2 exhibit a specific interaction with the S5a subunit of the 19S regulatory complex of the proteasome (Hiyami *et al.*, 1999; Walters *et al.*, 2002). Furthermore, hPLIC-2 interacts with ubiquitin ligase E6-AP and associates with a large complex containing the proteasome and E6-AP, indicating the possibility that ubiquitin-domain proteins link the ubiquitylation machinery with the proteasome (Leijnen *et al.*, 2000).

Interestingly, the association of a molecular chaperone cofactor UDP called BAG-1S with the proteasome promotes the interaction of heat shock inducible Hsp70 and constitutively expressed Hsc70 molecular chaperones with the proteasome, thus, providing a relationship between protein degradation and folding processes (Lűders *et al.*, 2000). It is intriguing to note that there is also a relationship between protein folding, protein degradation, and eukaryotic microtubule organizing centres in that the molecular chaperones Hsp70 and Hsp90 and active proteasomes associate with the centrosome, a homologue of the SPB in budding yeast, which is thought to serve a scaffolding function for protein degradation and folding (Fabunmi *et al.*, 1999; Wigley *et al.*, 1999).

Since budding yeast DSK2 is involved in SPB duplication and can bind the proteasome, perhaps DSK2 is responsible for recruiting the proteasome to the SPB, possibly through a direct or indirect interaction between DSK2 and the SPB that has not yet been demonstrated. Although an *in vitro* interaction between DSK2 and CDC31 has not been proven, DSK2 expression is required for proper localization of CDC31 to the SPB, perhaps indicating that DSK2 and CDC31 may interact transiently (Biggins *et al.*, 1996) or require other factors for their interaction. It is possible that proteasomes localized at the SPB, perhaps through DSK2, may be involved in the degradation of specific proteins to allow SPB separation after SPB duplication. In disagreement with this idea, budding yeast lacking both DSK2 and RAD23 exhibit a defect in SPB duplication rather than separation, although, the SPBs were often enlarged in this double mutant strain (Biggins *et al.*, 1996).

Some UDPs exhibit an UBA (ubiquitin associated) domain in addition to an ubiquitin-like domain. The UBA domain appears to function in promoting the association of UDPs with multiubiquitin chains and possibly in stabilizing substrates of the ubiquitin pathway. Proteins containing UBA domains which bind multiubiquitin chains include: *S. cerevisiae* DSK2 and RAD23; *S. pombe* homologues of *S. cerevisiae* RAD23 and DSK2; and a protein encoded by the *mud1*$^{+}$ gene in *S. pombe* (Chen *et al.*, 2001; Funakoshi *et al.*, 2002; Wilkinson *et al.*, 2001). In particular, the UBA domains of RAD23, DDI1, and DSK2 appear to selectively bind lysyl-48 and lysyl-29 linked mulitubiquitin chains and preferentially bind multiubiquitin chains at least four ubiquitin moieties in length over monoubiquitin (Rao and Sastry, 2002). *In vitro* and *in vivo* experimentation has revealed that RAD23 inhibits the assembly of multiubiquitin chains on substrates (Chen *et al.*, 2001; Ortolan *et al.*, 2000). Furthermore, a UBA containing UDP called ubiquilin, which localizes to neurofibrillary tangles and Lewy bodies in brains affected with Parkinson's disease and Alzheimer's disease (AD), interacts with and promotes the stability of proteins presenilin-1 and presenilin-2 which are implicated in the development of early-onset AD (Mah *et al.*, 2000). Intriguingly, *Xenopus* XDRP1, a UDP homologue of *S. cerevisiae* DSK2, binds to and prevents the calcium induced degradation of cyclin A through its ubiquitin-like domain (Funakoshi *et al.*, 1999). These

examples suggest the possibility that UBA domains UDPs inhibit degradation of certain substrates via the ubiquitin pathway.

In contrast, the UBA domains of RAD23 and DSK2 are also required for promoting the degradation of substrates of the ubiquitin fusion degradation (UFD) pathway (Rao and Sastry, 2002). It is possible that UDP proteins which contain UBA domains bind multiubiquitin chains through their UBA domain to prevent chain degradation by deubiquitylating enzymes, and function as an adapter to link ubiquitylated substrate proteins to the proteasome (Rao and Sastry, 2002). It is possible that the preference of RAD23, DDI1, and DSK2 for lysyl-48 and lysyl-29 linked multiubiquitin chains provides a means for accomplishing selective protein degradation by the proteasome (Rao and Sastry, 2002). Since UBA-containing UDP protein RAD23 interacts with DNA repair proteins and the proteasome, it has been suggested that the UBA-containing UDP protein DSK2 may link the proteasome to another cellular role such as cell cycle progression in the same manner that RAD23 links protein degradation to DNA repair by virtue of separate domains mediating separate protein-protein interactions in the same protein (Rao and Sastry, 2002). The fact that DSK2 is involved in SPB duplication (Biggins *et al.*, 1996), binds the proteasome (Funakoshi *et al.*, 2002), and binds multiubiquitin chains may indeed indicate that DSK2 functions in linking a progression of events leading to cell division with protein degradation.

UBA domains have also demonstrated an ability to promote protein-protein interactions. For instance, the UBA domain mediates interaction of RAD23 with itself; interaction of RAD23 and DDI1; and interaction of RAD23 and DSK2 (Bertolaet *et al.*, 2001). Since dimerization of RAD23 abrogates the interaction of RAD23 with multiubiquitin (Bertolaet *et al.*, 2001), Bertolaet *et al.* have suggested that dimerization may be a means to eliminate the ability of RAD23 to prevent substrate degradation by interacting with multiubiquitin (Bertolaet *et al.*, 2001; Ortolan *et al.*, 2000). It is thought that UBA domains are prevented from binding multiubiquitin when they are involved in mediating protein-protein interactions (Bertolaet *et al.*, 2001). It remains to be determined if dimerization of other UBA domain proteins eliminates their ability to bind multiubiquitin.

Interestingly, the ability of an ubiquitin-domain protein to bind an ubiquitin conjugating enzyme has not been previously demonstrated. It appears that the interaction of the ubiquitin-domain protein, DSK-2, with the ubiquitin conjugating enzyme, UBC-2, in *C. elegans* is a novel result which provides further evidence for the involvement of ubiquitin-domain proteins in the ubiquitin pathway. It remains to be determined if the UBA domain, UBL domain, or another region of DSK-2 mediates interaction with UBC-2. Further experimentation could also determine whether or not DSK-2 binds the proteasome and is involved in centrosome duplication in *C. elegans*. Since DSK-2 represented 60% of the UBC-2 interacting proteins identified by the yeast-two hybrid screen, further effort was concentrated on studying this interaction *in vitro*.

4. Confirming the interaction of UBC-2 with DSK-2 *in vitro*

4.1. Attempt to coprecipitate radiolabeled DSK-2 with 6XHIS-UBC-2

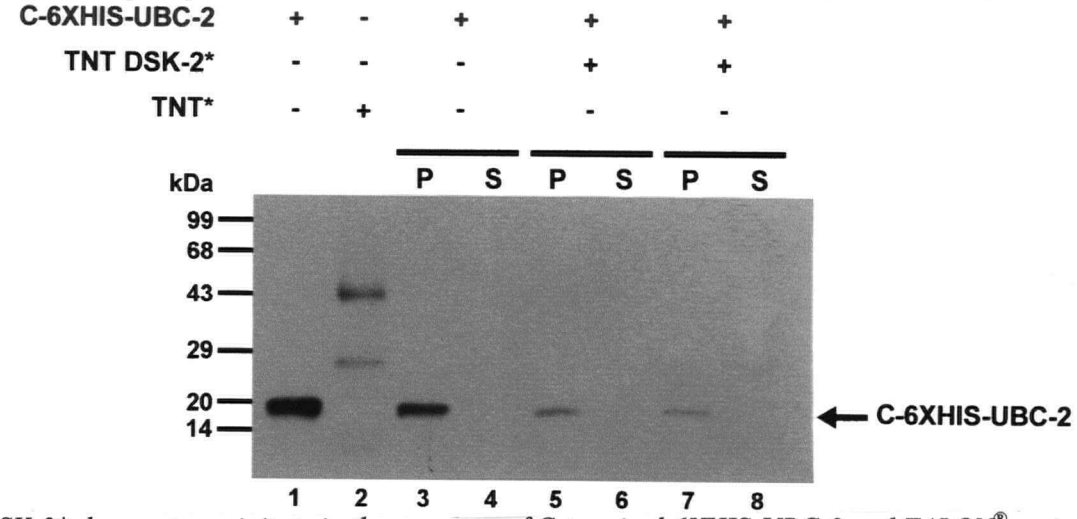
Radiolabeled DSK-2 (DSK-2*) synthesized in a TNT kit was incubated with 6XHIS-UBC-2. Control tests confirmed that 6XHIS-UBC-2 binds TALON® resin (Figure 5.A. lane 4) and that DSK-2* alone does not bind TALON® resin (Figure 5.B. lane 3). Due to the fact that DSK-2* did not precipitate in the presence of 6XHIS-UBC-2 and TALON® resin, it appeared that DSK-2* could not bind 6XHIS-UBC-2. The possibility existed that conditions such as pH, salt concentration, temperature, time of incubation, and/or the presence of additional factors or modifications of the proteins normally present *in vivo* were not favorable to promote the interaction of DSK-2* and 6XHIS-UBC-2. Interestingly, a reduction in binding of 6XHIS-UBC-2 to the TALON® resin was observed in the presence of increasing amounts of DSK-2* (Figure 5.B. lanes 5 and 7). It was considered possible that the binding of DSK-2* to 6XHIS-UBC-2 might prevent TALON® resin from binding to the C-terminal 6XHIS-tag. For this reason, a N-terminal 6XHIS-UBC-2 fusion protein was prepared and tested (Section 4.5, 4.6, and 4.7) in an attempt to prove an *in vitro* interaction between UBC-2 and DSK-2. It was also considered possible that a component of the TNT kit was preventing either the interaction of C-terminal 6XHIS-UBC-2 with the TALON® resin or the interaction of UBC-2 with DSK-2. To avoid this possibility, a different tagged version of DSK-2, a GST::DSK-2 fusion, was constructed and tested.

Figure 5. Attempt to coprecipitate radiolabeled DSK-2 with C-terminal 6XHIS-UBC-2

Radiolabeled DSK-2* synthesized in a TNT® Quick Coupled Transcription/Translation System Quick Master Mix was incubated with C-terminal 6XHIS-UBC-2 in UBC-2 buffer on a Nutator for 1.5 hours at 4°C. TALON® resin was added to this mixture for 1 hour. Resin pellets were isolated and washed in UBC-2 buffer. The pellet (P) and supernatant (S) fractions were separated on 12.5% SDS-PAGE gels, and analyzed by Western blotting to detect UBC-2 and autoradiography to detect DSK-2*.

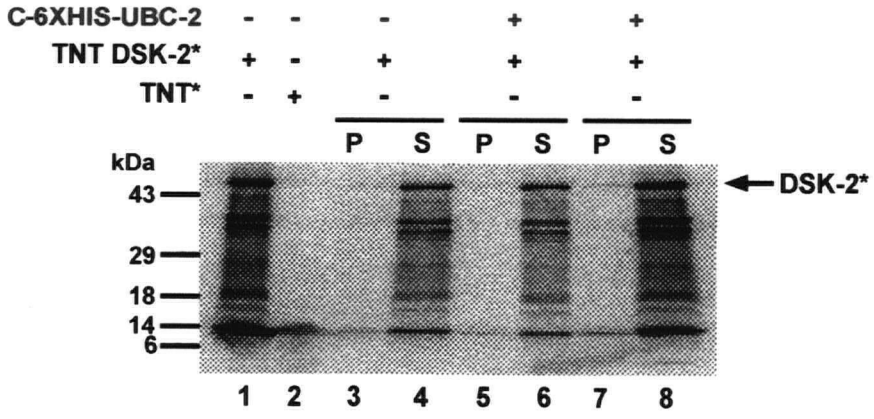
*A. C-terminal 6XHIS-UBC-2 precipitates with TALON® resin less efficiently in the presence of increasing amounts of DSK-2**

Western blot analysis [anti-HIS antibody (1:5,000)] showed that 23 kDa C-terminal 6XHIS-UBC-2 binds TALON® resin efficiently. It was present in the P fraction (lane 3) but absent in the S fraction (lane 4). In the presence of radiolabeled DSK-2* (10 µl and 30 µl), less C-terminal 6XHIS-UBC-2 precipitated with TALON® resin as seen in the P fractions (lanes 5 and 7).



B. DSK-2 does not precipitate in the presence of C-terminal 6XHIS-UBC-2 and TALON® resin*

An autoradiograph to detect ^{35}S-labeled DSK-2* showed that 60 kDa DSK-2* was produced at the correct size in the TNT® Quick Master Mix (lane 1). DSK-2* was not found in the TALON® resin P fractions in the absence or presence of C-terminal 6XHIS-UBC-2 and TALON® resin (lanes 3, 5, and 7).



4.2. Expression and Purification of GST::DSK-2

A *Xho*I fragment which contained the entire open reading frame of *dsk-2* was isolated from a pACT::cDNA plasmid and subcloned directly into pGEX-4T-2 (Pharmacia Biotech). This fragment, encoding a 60.3 kDa protein, was inserted in-frame with a sequence encoding a 26 kDa N-terminal glutathione S-transferase (GST) tag. After transforming this construct into *E. coli* BL21 (DE3) STAR (Invitrogen), a culture was grown to an OD_{600} of 0.4 and expression of the 86.3 kDa protein was induced with the addition of 1 mM IPTG. GST::DSK-2 protein was batch purified using glutathione agarose after determining that the fusion protein could be expressed in soluble form. Purified fusion protein migrated according to the predicted size on a Coomassie stained SDS-PAGE gel (Figure 6 lane 8). A 2 L *E. coli* culture yielded 9.3 mg of fusion protein.

4.3. Confirming the identity of GST::DSK-2 fusion protein

In addition to sequencing the pGEX-4T-2::*dsk-2* construct to confirm the reading frame, I carried out thrombin protease cleavage and Western blot analysis to confirm the identity of the expressed and purified fusion protein. Thrombin was successful in removing the GST tag from the fusion protein (Figure 7.A.). The GST tag permitted facile purification of the protein and the ability to eliminate this tag could prove useful in the production and purification of antibody against DSK-2 in the future. Since an antibody against *C. elegans* DSK-2 was not available for studies of the interaction between DSK-2 and UBC-2, other available antibodies were tested for recognition of GST::DSK-2. A polyclonal anti-GST antibody and a polyclonal antibody against *S. cerevisiae* DSK2 both recognized the 86.3 kDa GST::DSK-2 fusion protein following Western blot analysis (Figures 7.B. and C.).

4.4. Co-precipitation of GST::DSK-2 with an C-terminal 6XHIS-UBC-2 preparation

To confirm the yeast two-hybrid interaction of UBC-2 and DSK-2, an *in vitro* co-precipitation experiment was performed using equimolar amounts of GST::DSK-2 and C-terminal 6XHIS-UBC-2. The reaction buffer, UBC-2 buffer, was supplemented with 1% Triton X-100 to inhibit non-specific protein interactions with the TALON® resin. TALON® resin was pre-incubated with C-terminal 6XHIS-UBC-2 for 1 hour prior to

Figure 6. Expression and Purification of GST::DSK-2

GST::DSK-2 fusion protein expression was induced by the addition of 1 mM IPTG to a culture of *E. coli* BL21 (DE3) STAR grown to an OD_{600} of 0.4. Glutathione agarose affinity chromatography was used to purify the fusion protein. Lane 1: total *E. coli* protein extract before IPTG induction; lane 2: total *E. coli* protein extract after IPTG induction; lane 3: total *E. coli* protein extract after sonication; lane 4: insoluble pellet of the post-sonication extract; lane 5: soluble portion of the post-sonication extract; lane 6: supernatant from the glutathione agarose batch incubation with soluble post-sonication extract; lane 7: wash of the glutathione agarose incubated with soluble extract; and lane 8: eluate of the glutathione agarose incubated with soluble extract. Purified GST::DSK-2 migrated in a heavy band that approximated the predicted size of 86 kDa on a Coomassie stained 10% SDS-PAGE gel (arrow, lane 8).

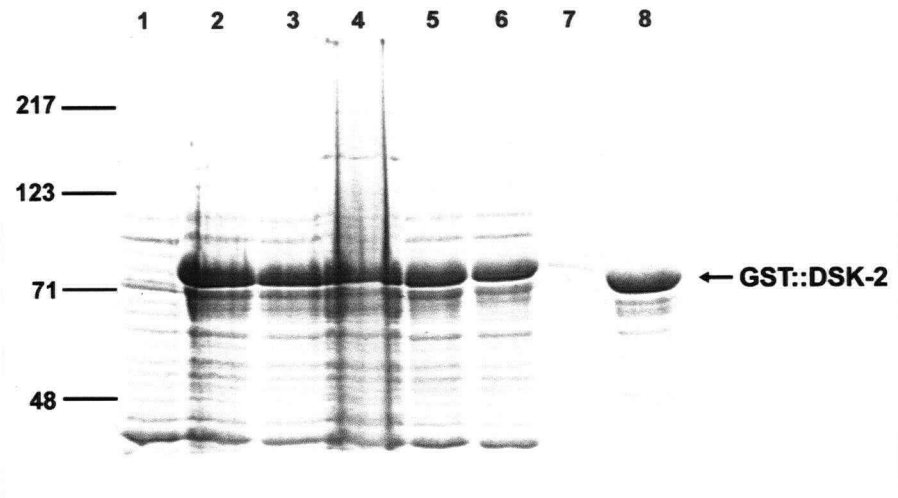
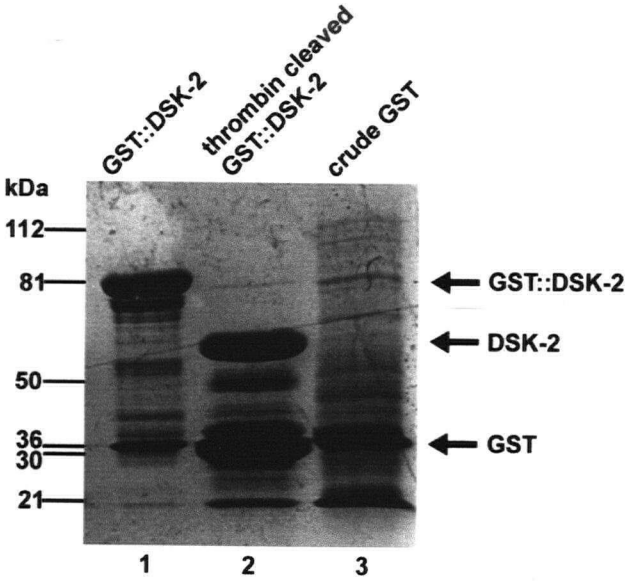
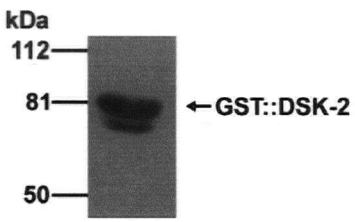


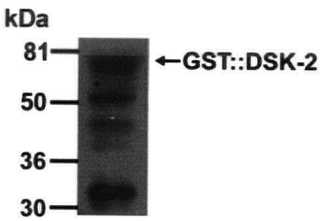
Figure 7. Confirming the identity of GST::DSK-2 fusion protein
A. Thrombin protease cleavage of GST::DSK-2 (86 kDa)
GST::DSK-2 (lane 1) incubation with Thrombin overnight at 22 C produced two bands on a Coomassie stained 10% SDS-PAGE gel (lane 2). These bands approximated the predicted sizes of fragments resulting from a GST::DSK-2 cleavage event: a 60 kDa DSK-2 portion and a 26 kDa GST portion of size similar to unpurified GST in *E. coli* lysate (lane 3).



B. GST::DSK-2 is recognized by anti-GST antibody
3 µg of GST::DSK-2 was separated on a 10% SDS-PAGE gel and subjected to Western blot analysis using anti-GST antibody (1:3,000 dilution in TBS-Tween).



C. GST::DSK-2 is recognized by anti-DSK2 antibody
3 µg of GST::DSK-2 was separated on a 10% SDS-PAGE gel and subjected to Western blot analysis using anti-DSK2 antibody (1:3,000 dilution in TBS-Tween).



incubation with GST::DSK-2 overnight on a Nutator at 4 C. The addition of 1% Triton X-100 to the co-precipitation buffer proved to be crucial in preventing non-specific interaction of GST::DSK-2 with the TALON® resin. The detergent concentration was low enough, however, to permit the interaction of GST::DSK-2 with C-terminal 6XHIS-UBC-2 *in vitro*. Analysis of the supernatant, wash and pellet fractions of the co-precipitation experiments via SDS-PAGE and Western blotting with anti-GST antibody revealed that GST::DSK-2 was found in the pellet fraction only in the presence of C-terminal 6XHIS-UBC-2 and TALON® resin (Figure 8). Thus, this co-precipitation experiment confirmed the yeast two-hybrid interaction of UBC-2 and DSK-2. However, the *in vitro* interaction did not seem particularly strong as very little of the input GST::DSK-2 was found to precipitate with 6XHIS-UBC-2 in the presence of TALON® resin (lane 6). This preparation of C-terminal 6XHIS-UBC-2 had been previously frozen. Therefore, newly prepared UBC-2 fusion protein was prepared in the hope of improving the *in vitro* interaction of 6XHIS-UBC-2 and GST::DSK-2.

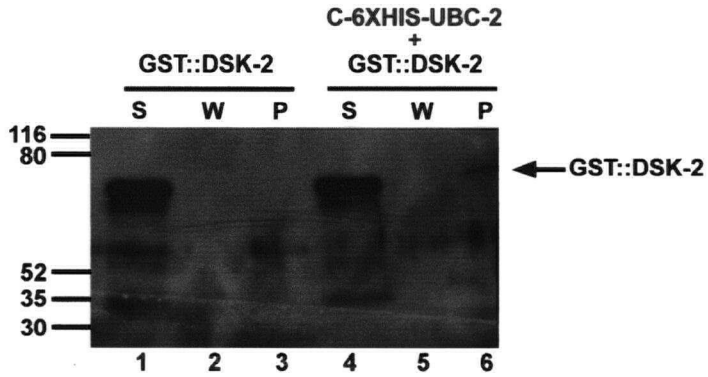
4.5. Expression and Purification of UBC-2

The potential negative effect of freezing on native folding of C-terminal 6XHIS-UBC-2 protein was considered as a possible cause for the weak interaction of UBC-2 with DSK-2 in the co-precipitation experiment. Therefore, new 6XHIS-UBC-2 was expressed and purified using the pRSETC-*ubc-2* construct as previously described (Zhen, PhD. thesis, 1995).

Previously, the possibility was considered that DSK-2* binding to 6XHIS-UBC-2 might occlude TALON® resin binding to the C-terminal 6XHIS tag. For this reason, a C-terminal 6XHIS-UBC-2 fusion protein was prepared in an attempt to prove an *in vitro* interaction between UBC-2 and DSK-2. A fragment which contained the entire open reading frame of *ubc-2* was amplified from pRSETC::*ubc-2*. The restriction digested fragment was cloned into pET28-a (+) in frame with sequence encoding an N-terminal 6XHIS tag. After transforming this construct into *E. coli* BL21 (DE3) STAR (Invitrogen), a culture was induced to express the 86.3 kDa protein with the addition of 1 mM IPTG. 6XHIS-UBC-2 protein was batch purified using TALON® resin. Purified

Figure 8. Co-precipitation of GST::DSK-2 with 6XHIS-UBC-2

Co-precipitation experiments were performed using equimolar amounts of GST::DSK-2 and 6XHIS-UBC-2 in UBC-2 buffer supplemented with 0.1% Triton X-100 in an overnight incubation on a Nutator at 4 C. Co-precipitation #1 included the GST::DSK-2 and TALON® resin while co-precipitation #2 included 6XHIS-UBC-2, GST::DSK-2, and TALON® resin. The supernatant (S), wash (W), and resin pellet (P) fractions of the co-precipitation reactions were separated on a 10% SDS-PAGE gel and analyzed by Western blotting using anti-GST antibody (1:3,000). Lanes 1, 2, and 3 contain the respective S, W, and P fractions of co-precipitation #1. Lanes 4, 5, and 6 contain the respective S, W, and P fractions of co-precipitation #2. GST::DSK-2 (arrow) was localized to the TALON® resin P only in the presence of 6XHIS-UBC-2 indicating a positive interaction between GST::DSK-2 and 6XHIS-UBC-2 (lane 6, arrow).



fusion protein migrated according to the predicted size, as detected on a Western blot probed with anti-UBC-2 antibody.

4.6. N-terminal 6XHIS-UBC-2 and C-terminal 6XHIS-UBC-2 bind TALON® resin

The TALON® resin was incubated with a fresh preparation of either N-terminal 6XHIS tagged UBC-2 or C-terminal 6XHIS tagged UBC-2 in UBC-2 buffer containing 1% Triton X-100 for 2 hours on a Nutator at 4 C. Subsequently, the supernatant, wash and pellet fractions from the UBC-2 incubations with TALON® resin were analyzed by SDS-PAGE and Western blotting with anti-UBC-2 antibody. Both N-terminal and C-terminal 6XHIS tagged UBC-2 fusion proteins were precipitated by the TALON® resin (Figure 9.A. and B.).

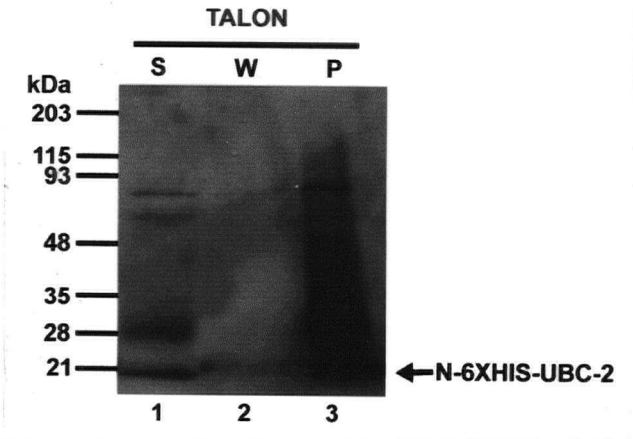
4.7. Co-precipitation of GST::DSK-2 with fresh protein preparations of N-terminal 6XHIS-UBC-2 and C-terminal 6XHIS-UBC-2

In the place of frozen 6XHIS-UBC-2 prepared by Tracy Stevens, fresh protein preparations of 6XHIS tagged UBC-2 fusion proteins were utilized in a co-precipitation experiment in the hope of promoting a stronger *in vitro* interaction between UBC-2 with DSK-2. N-terminal 6XHIS tagged UBC-2 or C-terminal 6XHIS-UBC-2 was incubated for two hours on a Nutator at 4 C with TALON® resin and GST::DSK-2 in UBC-2 buffer supplemented with 1% Triton X-100 and 10 mM imidazole to eliminate non-specific interactions of the proteins with the resin. Equimolar amounts of DSK-2 and UBC-2 fusion protein were used. Analysis of the supernatant, wash and pellet fractions of the co-precipitation experiments via SDS-PAGE and Western blotting with anti-GST antibody revealed that GST::DSK-2 is found in the pellet fraction only in the presence of TALON® resin and either N-terminal 6XHIS tagged UBC-2 or C-terminal 6XHIS tagged UBC-2 (Figure 10.A. and B.). Thus, these co-precipitation experiments confirmed the yeast two-hybrid interaction of UBC-2 and DSK-2. GST::DSK-2 degradation, visible in lanes 4 and 6 (versus in lane 1), is likely due to protease activity in the UBC-2 preparations (Figure 10.A. and B.).

Figure 9. UBC-2 binds TALON® resin

N-terminal 6XHIS-UBC-2 or C-terminal 6XHIS-UBC-2 was incubated for two hours on a Nutator at 4 C with TALON® resin in UBC-2 buffer supplemented with 1% Triton X-100 and 10 mM imidazole to eliminate non-specific protein-resin interactions. Resin pellets were isolated and washed three times. The supernatant (S), wash (W), and pellet (P) fractions of the precipitation reactions were separated on 12.5% SDS-PAGE gels and were subsequently analyzed via Western blotting using anti-UBC-2 antibody (1:5,000).

A. N-terminal 6XHIS-UBC-2 binds TALON® resin
23 kDa N-terminal 6XHIS-UBC-2 is present in the P fraction (lane 3).



B. C-terminal 6XHIS-UBC-2 binds TALON® resin
23 kDa C-terminal 6XHIS-UBC-2 is present in the P fraction (lane 3).

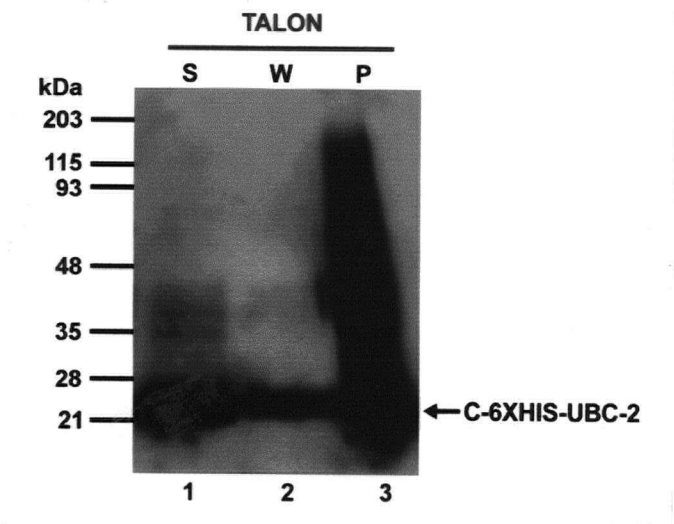
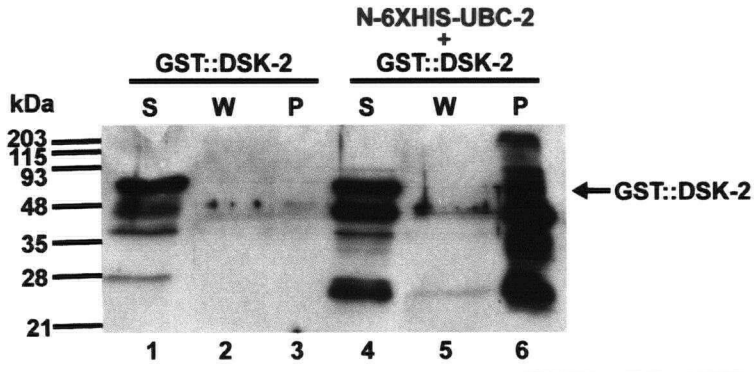
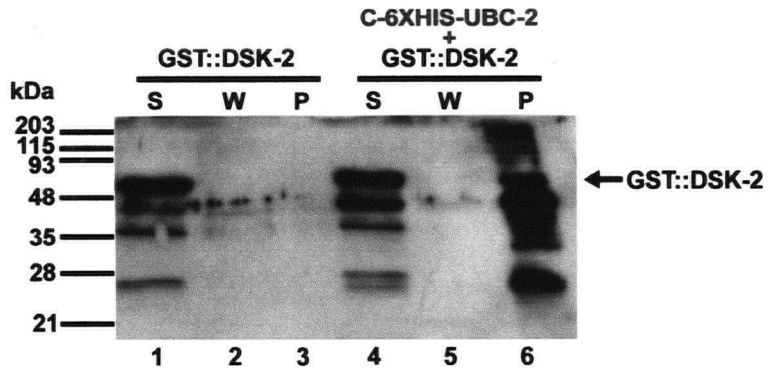


Figure 10. Co-precipitation of DSK-2 with UBC-2
Equimolar amounts of GST::DSK-2 and N-terminal 6XHIS-UBC-2 or C-terminal 6XHIS-UBC-2 were incubated together for two hours at 4 C on a Nutator. Co-precipitation reaction #1 (lanes 1-3) included GST::DSK-2 and TALON® resin while co-precipitation reaction #2 (lanes 4-6) included N-terminal or C-terminal 6XHIS-UBC-2, GST::DSK-2, and TALON® resin. The supernatant, wash and resin pellet fractions of the co-precipitation experiments were analyzed via SDS-PAGE and Western blot analysis with anti-GST antibody (1:3,000). Lanes 1, 2, and 3 contain the respective supernatant (S), wash (W), and pellet (P) fractions of co-precipitation #1. Lanes 4, 5, and 6 contain the respective S, W, and P fractions of co-precipitation #2 (N-terminal 6XHIS-UBC-2 in A. and C-terminal 6XHIS-UBC-2 in B.). Fractions of the precipitation reactions were separated on 10% SDS-PAGE gels and were subsequently analyzed via Western blotting using anti-GST antibody (1:3,000).

A. GST::DSK-2 interacts with N-terminal 6XHIS ::UBC-2 bound to TALON® resin
GST::DSK-2 (86 kDa) localized to the TALON® resin pellet only in the presence of N-terminal 6XHIS-UBC-2 (P, lane 6).



B. GST::DSK-2 interacts with C-terminal 6XHIS::UBC-2 bound to TALON® resin
GST::DSK-2 (86 kDa) localized to the TALON® resin pellet only in the presence of C-terminal 6XHIS-UBC-2 (P, lane 6).



5. GST::DSK-2 self-associates

To demonstrate the interaction of UBC-2 and DSK-2 using another *in vitro* method, an attempt was made to cross-link GST::DSK-2 to C-terminal 6XHIS tagged UBC-2. Proteins were incubated for 1 hour at room temperature followed by one hour on ice before BS^3 cross-linker (Pierce) was added to a final concentration of 1 mM. Cross-linking reactions were quenched with 100 mM Tris-HCl pH 7.5 at various time points. Cross-linked proteins were analyzed via SDS-PAGE and Western blot analysis using anti-GST or anti-UBC-2 antibody. No difference was observed in the protein banding pattern of C-terminal 6XHIS-UBC-2 cross-linked to itself versus C-terminal 6XHIS-UBC-2 cross-linked in the presence of GST::DSK-2 on a Western blot probed with anti-UBC-2 antibody (data not shown). In addition, no difference was observed in the protein banding pattern of GST::DSK-2 cross-linked to itself versus GST::DSK-2 cross-linked in the presence of C-terminal 6XHIS-UBC-2 on a Western blot probed with anti-GST antibody. Therefore, cross-linking failed to demonstrate an *in vitro* interaction between C-terminal 6XHIS tagged UBC-2 and GST::DSK-2. Interestingly, it was observed that GST::DSK-2 forms higher molecular weight conjugates with itself, perhaps in the form of homodimers or higher order multimers (Figure 11.A. and B.).

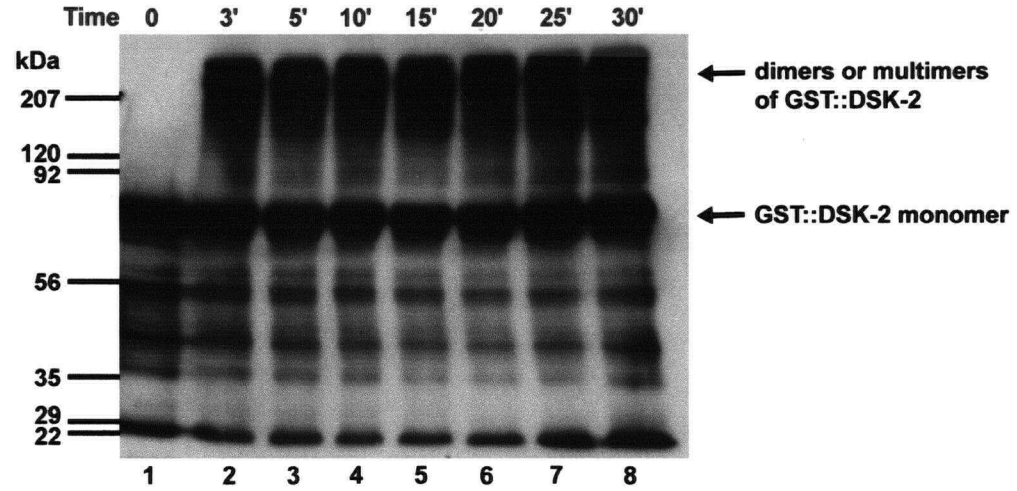
UBA domains have been implicated in promoting protein-protein interactions. As mentioned previously, the UBA domain mediates the interaction of RAD23 with itself, with DDI1, and with DSK2 (Bertolaet *et al.*, 2001). However, the interaction of DSK2 with itself has not previously been demonstrated. Therefore, it appears that the self-association of DSK-2, a *C. elegans* homologue of budding yeast DSK2, is a novel result. It remains to be determined if DSK-2 dimerization eliminates interaction with multiubiquitin in the same manner that dimerization of RAD23 eliminates the interaction of RAD23 with multiubiquitin (Bertolaet *et al.*, 2001).

6. GST::DSK-2 interacts with polyubiquitin chains

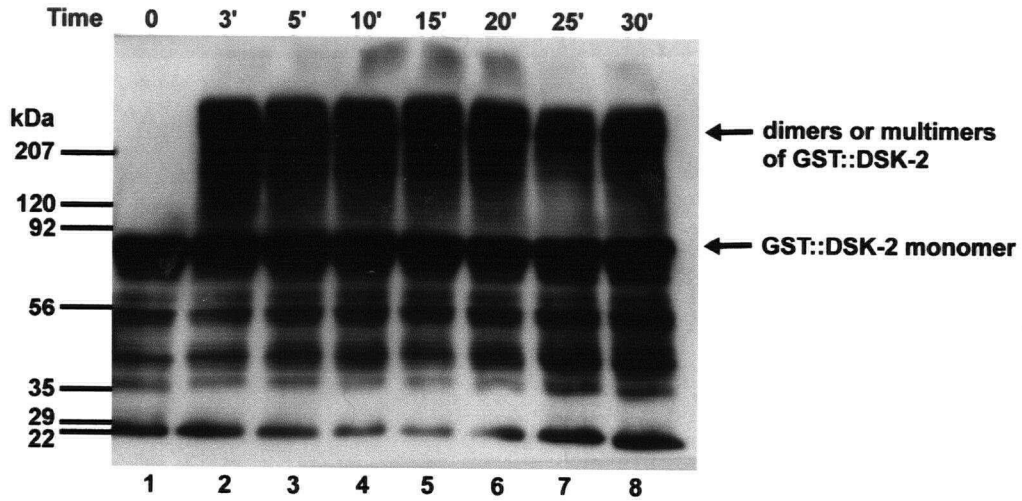
Recently, it was discovered that *Schizosaccharomyces pombe* proteins with UBA domains are capable of binding polyubiquitin chains (Wilkinson *et al.*, 2001). In particular, it was found that *S. cerevisiae* DSK2 can bind to polyubiquitin chains

Figure 11. GST::DSK-2 self-associates in the presence of cross-linker
Equimolar amounts of GST::DSK-2 and C-terminal 6XHIS tagged UBC-2 were incubated together and then cross-linked with 1 mM BS^3 for various time periods (cross-linking time in minutes is indicated above each lane). Cross-linking reactions were separated on 10% SDS-PAGE gels and were subsequently analyzed via Western blotting using anti-GST antibody (1:3,000). There appears to be no difference in the protein band pattern between GST::DSK-2 cross-linked to itself (A.) versus GST::DSK-2 cross-linked in the presence of C-terminal 6XHIS-UBC-2 (B.). Interestingly, GST::DSK-2 forms higher molecular weight conjugates with itself only in the presence of BS^3 cross-linker (lanes 2-8, A. and B.).

A. GST::DSK-2 cross-linked with BS^3



B. GST::DSK-2 cross-linked in the presence of 6XHIS-UBC-2 with BS^3



(Funakoshi *et al.*, 2002). Since *C. elegans* DSK-2 exhibits 32% similarity to *S. cerevisiae* DSK2 and possesses a C-terminal UBA domain, a co-precipitation experiment was performed to determine if DSK-2 also binds polyubiquitin. Since UBC-2 was found to interact with DSK-2 *in vivo* and *in vitro*, the ability of UBC-2 to interact with polyubiquitin chains (Ub_{2-7}) was also tested. Co-precipitation experiments were performed in UBC-2 buffer supplemented with 0.5% Triton X-100 to eliminate non-specific binding of proteins to TALON® resin or to glutathione agarose. Negative controls for the co-precipitation experiments revealed that polyubiquitin does not bind to TALON® resin or glutathione agarose in a non-specific fashion (Figure 12). Additional negative control tests showed that 6XHIS-UBC-2 binds TALON® resin and that GST::DSK-2 binds glutathione agarose in the presence of polyubiquitin (Figure 13.A. and B.). Co-precipitation experiments indicated that 6XHIS-UBC-2 bound to TALON® resin does not interact with polyubiquitin (Figure 14.A.). However, GST::DSK-2 bound to glutathione agarose did interact with polyubiquitin in a co-precipitation experiment (Figure 14.B.). This result confirmed the hypothesis that UBA domain containing proteins interact with polyubiquitin (Wilkinson et al., 2001).

7. RNA interference of genes encoding UBC-2 interacting proteins

RNA interference experiments were performed to determine the biological function of genes encoding UBC-2 interacting proteins. Previously, it was demonstrated that injection of double stranded RNA (dsRNA) into *C. elegans* interferes with gene function (Fire *et al.*, 1998). This method, termed RNA interference (RNAi), is used to determine the *in vivo* function of genes by generating a temporary null phenotype which is often identical to the null mutant phenotype. Evidence suggests that RNAi occurs by a post-transcriptional mechanism, although the exact mechanism has not yet been determined. A gene called *dicer-1* in *Drosophila* is believed to encode a protein responsible for cleavage of the introduced dsRNA into 21 to 23 nucleotide long fragments to mediate an RNAi effect (Knight and Bass, 2001). Recently, it has been demonstrated that RNAi can be induced when *C. elegans* is fed with bacteria expressing target gene dsRNA (Timmons and Fire, 1998). Conveniently, this feeding method

Figure 12. Polyubiquitin does not bind to TALON® resin or glutathione agarose
Polyubiquitin chains (Ub_{2-7}) were incubated with TALON® resin or glutathione agarose in UBC-2 buffer supplemented with 0.5% Triton X-100 for 2 hours on a Nutator at 4 C. The supernatant (S), wash (W), and pellet (P) fractions were analyzed via SDS-PAGE and Western blotting using a 1:500 dilution of anti-ubiquitin antibody (Santa Cruz). Lanes 1-3: S, W, and P fractions from an incubation of Ub_{2-7} with TALON® resin. Lanes 4-6: S, W, and P fractions from an incubation of Ub_{2-7} with glutathione agarose. Polyubiquitin was not visible in the pellet fractions indicating that it does not bind non-specifically to TALON® resin or glutathione agarose.

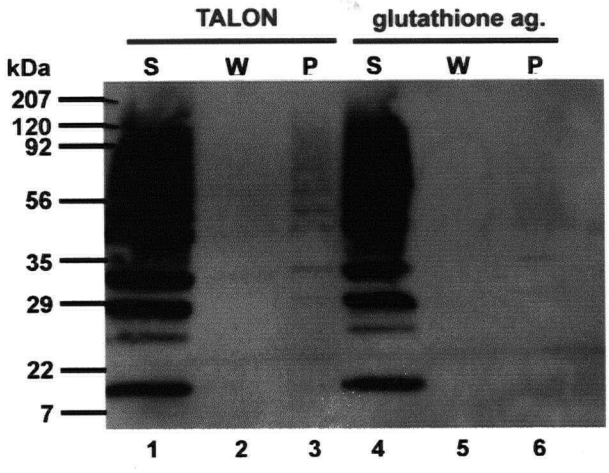
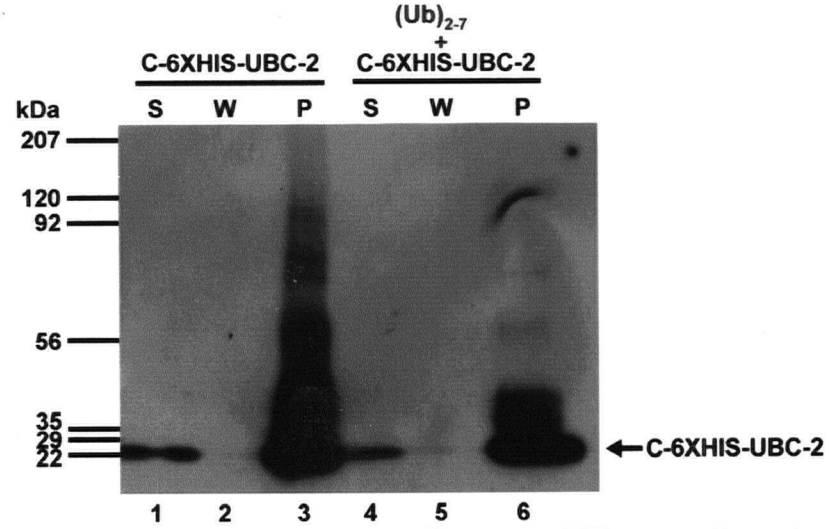


Figure 13. Polyubiquitin does not interfere with protein interaction with affinity resin
Negative control precipitation experiments demonstrated that the presence of polyubiquitin (Ub_{2-7}) did not prevent the interaction of C-terminal 6XHIS::UBC-2 with the TALON® resin or GST:: DSK-2 with glutathione agarose in UBC-2 buffer supplemented with 0.5% Triton X-100 for 2 hours on a Nutator at 4 C. The supernatant (S), wash (W), and pellet (P) fractions were analyzed via SDS-PAGE and Western blotting using a 1:5,000 dilution of anti-UBC-2 antibody.
A. C-terminal 6XHIS-UBC-2 binds TALON® resin in the presence of polyubiquitin
Lanes 1-3: S, W, and P fractions from an incubation of C-terminal 6XHIS-UBC-2 with TALON® resin. Lanes 4-6: S, W, and P fractions from an incubation of Ub_{2-7}, C-terminal 6XHIS-UBC-2, and TALON® resin. C-terminal 6XHIS-UBC-2 is found in the pellet fraction (lanes 3 and 6).



B. GST:: DSK-2 binds glutathione agarose in the presence of polyubiquitin
Lanes 1-3: S, W, and P fractions from an incubation of GST::DSK-2 with glutathione agarose. Lanes 4-6: S, W, and P fractions from an incubation of Ub_{2-7}, GST:: DSK-2, and glutathione agarose. GST:: DSK-2 is found in the pellet fraction (lanes 3 and 6).

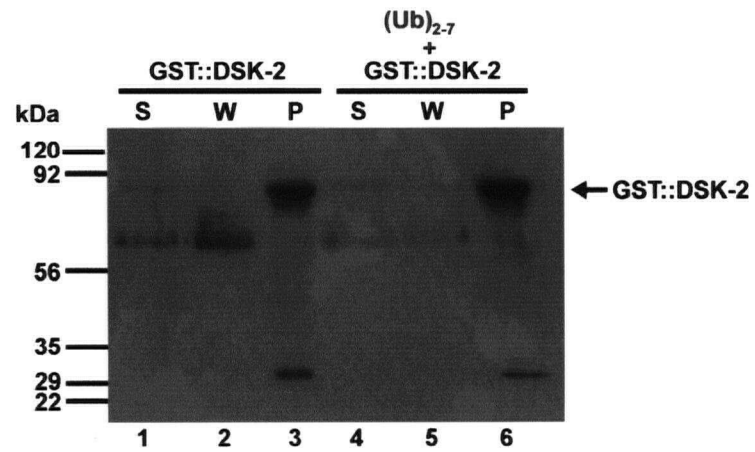
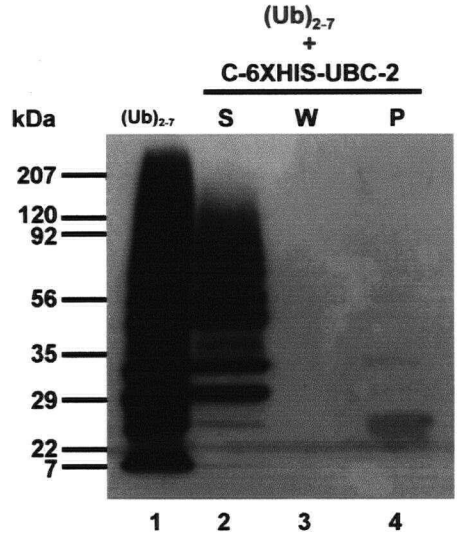


Figure 14. Polyubiquitin co-precipitation experiments

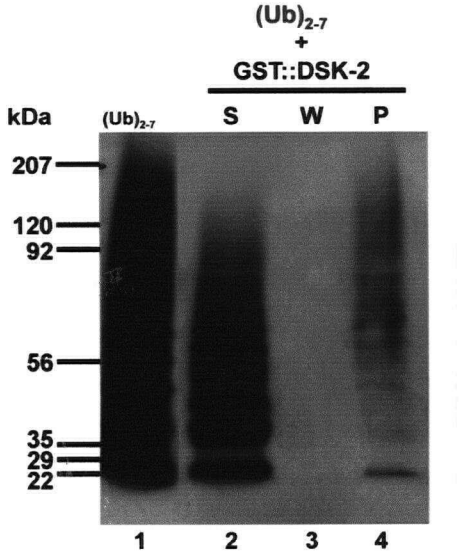
A. C-terminal 6XHIS-UBC-2 does not interact with polyubiquitin

After attempting to co-precipitate Ub_{2-7} with C-terminal 6XHIS-UBC-2 and TALON® resin in UBC-2 buffer supplemented with 0.5% Triton X-100 for 2 hours on a Nutator at 4 C, the supernatant (S), wash (W), and pellet (P) fractions were analyzed via SDS-PAGE and Western blotting using a 1:500 dilution of anti-ubiquitin antibody (Santa Cruz). Lane 1: 1 μg Ub_{2-7}, lanes 2-4: S, W, and P fractions from an incubation of Ub_{2-7}, C-terminal 6XHIS-UBC-2, and TALON® resin. The absence of Ub_{2-7} in lane 4 indicated that 6XHIS-UBC-2 does not interact with polyubiquitin.



B. GST::DSK-2 interacts with polyubiquitin

After attempting to co-precipitate Ub_{2-7} with GST::DSK-2 and glutathione agarose, the S, W, and P fractions were analyzed via SDS-PAGE and Western blotting using a 1:500 dilution of anti-ubiquitin antibody (Santa Cruz). Lane 1: 1 μg Ub_{2-7}, lanes 2-4: S, W, and P fractions from an incubation of Ub_{2-7}, GST::DSK-2, and glutathione agarose. The presence of Ub_{2-7} in lane 4 indicated that GST::DSK-2 interacts with polyubiquitin.



overcomes the difficulty and expense of single worm injections and enhances the efficiency of determining the function of many genes (Kamath *et al.*, 2000).

This method involved non-directional cloning of genes encoding UBC-2 interacting proteins, and genes for RNAi positive controls, into a double T7 promoter vector (pPD129.36). Genes cloned into the pPD129.36 included: *ubi*A (encodes ubiquitin); F15C11.2 (encodes a ubiquitin-like protein referred to as DSK-2); B0432.9 and W02A11.3 (encodes two RING finger proteins), *mca*-1 (encodes a calcium ATPase), R160.7 (encodes a FYVE finger protein) and C25D7.8 (encodes a *C. elegans* protein lacking any previously characterized protein domains). HT115 (DE3), a strain of *E. coli* with inducible T7 polymerase activity and an RNase III deficiency, was transformed with one of these plasmids and induced with IPTG to express T7 polymerase which uses both T7 promoters to generate dsRNA against the target gene. Late larval L4 stage *C. elegans* hermaphrodite nematodes were transferred onto the induced plates for RNAi treatment. The rationale for using L4 stage nematodes was to induce RNAi in the developing eggs prior to egg shell formation (D. Jones, pers. comm.). The treated nematodes were removed and the progeny were scored for phenotypes. The fecundity per RNAi treated L4 stage worm was determined. The number of progeny produced per RNAi treated worm after the 72 hour treatment period varied between 70 and 119.

Interestingly, the only RNAi treatment which reduced the fecundity below that of the negative control population was the one directed at *mca*-1 (Table 3). This gene encodes a calcium ATPase which pumps calcium outside of the cell through ATP hydrolysis (Kraev *et al.*, 1999). Since *dsk*-2 function, in combination with *rad23*, is required for SPB duplication, and calcium spikes appear to promote duplication of the metazoan microtubule organizing center (Biggins et al., 1996; Matsumoto and Maller, 2002), I investigated the effect of calcium chloride depletion on RNAi treatments for *mca*-1, R160.7, and *dsk-2*. It was reasoned that this might enhance subtle RNAi phenotypes. RNAi treatments of other genes encoding UBC-2 interacting proteins were also performed in this calcium chloride-deficient background. In these experiments, only RNAi treatment of *mca*-1 reduced the fecundity below that of the negative control population (Table 4).

Table 3.
Fecundity of RNAi treated *C. elegans*

Plasmid Used for HT115(DE3) *E.coli* Transformation	Number of treated L4 stage nematodes	Number of viable progeny	Progeny per RNAi treated nematode	Number of RNAi progeny as percent difference from number of 'no plasmid' progeny [(A-74)/74]X100
no plasmid	3	222	74	0
pPD129.36::B0432.9	5	594	119	+61%
pPD129.36::C25D7.8	4	395	99	+34%
pPD129.36::*dsk-2*	2	200	100	+35%
pPD129.36::*mca-1*	4	281	70 *	-5%
pPD129.36::R160.7	4	304	76	+3%
pPD129.36::*ubc-2*	4	0	0 **	--
pPD129.36	3	255	85	+15%

* RNAi treated nematodes vs. *mca*-1 resulted in progeny appearing to be at the L2 stage of development in comparison to the rest of the RNAi treated nematodes which were at the L4 stage.

** Positive control RNAi vs. *ubc*-2 is known to result in arrested development of fertilized eggs.

Table 4
Fecundity of RNAi treated *C. elegans* grown in the absence of an environmental supply of calcium chloride

HT115(DE3) transformation for RNAi feeding method treatment	Number of treated L4 stage nematodes	Number of viable progeny	Progeny per RNAi treated nematode (A)	Number of RNAi progeny as percent difference from number of 'no plasmid' progeny [(A-86)/86]X100
no plasmid	5	432	86	0
pPD129.36::B0432.9	4	350	88	+3%
pPD129.36::C25D7.8	4	489	122	+42%
pPD129.36::*dsk-2*	4	541	135	+60%
pPD129.36::*mca-1*	5	371	74 *	-14%
pPD129.36::R160.7	4	444	111	+29%
pPD129.36::*ubc-2*	4	0	0 **	--
pPD129.36	5	435	87	+1%

* RNAi treated nematodes vs. *mca*-1 resulted in progeny appearing smaller than the other RNAi treated nematodes populations.
** RNAi treated nematodes vs. *ubc*-2 resulted in arrested development of fertilized eggs.

Results indicated that nematodes treated with dsRNA against *dsk*-2 appeared normal. In agreement with this result, *dsk*2 is a non-essential gene in *S. cerevisiae* (Biggins *et al.*, 1996). Interestingly, *rad*23 in *S. cerevisiae* is also non-essential. However, it has been shown that a double *dsk*2*rad*23 mutation leads to cell arrest due to a block in spindle pole body duplication (Biggins *et al.*, 1996). To determine if *S. cerevisiae dsk*2 and *rad*23 homologues in *C. elegans* have a similar essential cellular function, nematodes were treated with dsRNA against *dsk*2 and *rad*23 in combination. This double RNAi treatment did not produce a detectable phenotype. However, since experiments have demonstrated that RNAi treatments of multiple genes in combination result in a dramatic reduction in the phenotypes induced independently by each RNAi treatment (Kamath *et al.*, 2000), this result remains inconclusive.

Negative controls for the RNAi testing included feeding the nematodes with HT115 (DE3) *E. coli* alone or HT115 (DE3) *E. coli* transformed with the empty double T7 promoter vector. These negative RNAi controls did not produce a detectable phenotype. As a positive control, RNAi against *ubc*-2 produced an embryonic lethal phenotype as previously demonstrated via the injection method (Jones *et al.*, 2002; T.A. Stevens, Ph.D. thesis, 1999). RNAi versus *ubi*A also produced an embryonic lethal effect, and the treated adult worms exhibited a decrease in motility in comparison to negative control nematodes (data not shown). In addition, the small number of eggs laid by *ubi*A RNAi treated nematodes after the 72 hour treatment period did not hatch (data not shown). These two RNAi treatments provided good positive controls to demonstrate that the RNAi feeding method procedure was functioning as expected.

mca-1 RNAi treated nematodes appeared to exhibit a reduction in body length in comparison to the negative control populations (Figure 15). To determine the existence or extent of body length reduction caused by RNAi of genes encoding UBC-2 interacting proteins, approximately ten progeny worms were picked from each RNAi treatment plate onto a slide containing five drops of M9 buffer. The slides were heated to effectively fix the nematodes in a straightened conformation and enable accurate body length measurement. RNAi versus the *mca*-1 gene produced the most pronounced phenotype of all the genes encoding UBC-2 interacting proteins. Adult *mca*-1 RNAi treated nematodes

Table 5.
Average length of RNAi treated *C. elegans*
Approximately 10 worms were picked from each RNAi treatment plate into five drops of M9 buffer on a slide. The slides were heated for 30 seconds on a hot plate at 100C. This treatment effectively killed the nematodes and straightened their bodies to enable accurate body length measurement. Nematodes were measured at 25X magnification using an ocular micrometer. The measurements were convert to millimeters using the conversion factor 1 unit=0.4 mm.

Gene target of RNAi treatment	Average nematode length (mm)	Percent of control length (%)
pPD129.36 control	1.42±.04	100±2.8
B0432.9	1.38±.05	97±3.5
C25D7.8	1.37±.07	99±4.9
dsk-2	1.29±.04	91±2.8
mca-1	1.17±.05	82±3.5
R160.7	1.34±.06	94±4.2
W02A11.3	1.32±.08	93±5.6

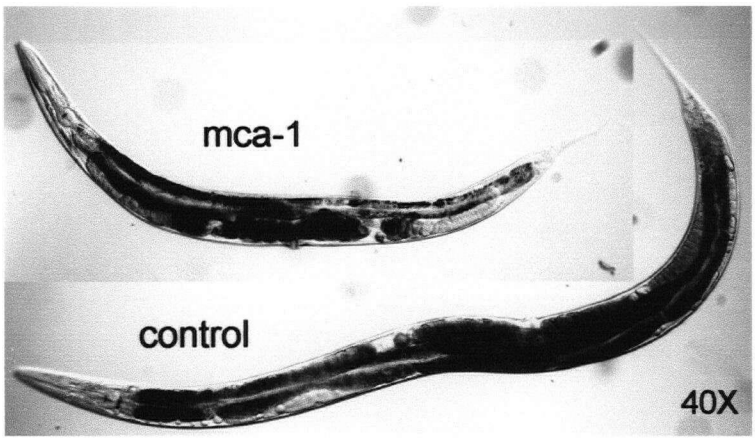


Figure 15. RNAi vs. *mca-1*: Adult *mca-1* RNAi treated nematodes exhibit a reduction in body length
RNAi versus the *mca-1* gene produced the most pronounced phenotype of all the genes encoding UBC-2 interacting proteins. Adult *mca-1* RNAi treated nematodes are 82±3.5% of the control adult population (100±2.8%) which were fed HT115 (DE3) *E. coli* containing the empty feeding vector pPD129.36.

were 82±3.5% of the control adult nematodes (100±2.8%) which were fed HT115 (DE3) *E. coli* containing the empty feeding vector pPD129.36 (Table 5).

9. Conclusions and future directions

In summary, a yeast two-hybrid screen of a *C. elegans* cDNA library indicated that UBC-2 interacts with ubiquitin, a calcium ATPase, a protein containing a FYVE domain, two RING finger proteins, and a ubiquitin-like protein called DSK-2, which represented the majority of the UBC-2 interactions detected. The interaction between DSK-2 and UBC-2 was confirmed biochemically *in vitro.* The interaction of DSK-2 with multiubiquitin chains was demonstrated *in vitro*, in agreement with other studies using the budding yeast homologue of DSK-2. The self-association of DSK-2 *in vitro* was also demonstrated. RNAi experimentation revealed that genes encoding UBC-2 interacting proteins studied here are non-essential for viability.

Future studies could determine the precise domains involved in mediating the interaction of UBC-2 with DSK-2, DSK-2 with itself, and DSK-2 with polyubiquitin chains. It would also be interesting to determine if DSK-2 exhibits a specific preference for ubiquitin chain linkages mediated by certain lysyl residues. The interaction of DSK-2 with the proteasome could also be investigated.

It would be helpful to confirm the interaction of UBC-2 with other proteins identified in the screen using *in vitro* methods. Immunolocalization analysis of UBC-2 interacting proteins in the cell and in *C. elegans* might confirm their proposed roles in various cellular pathways as discussed previously. Finally, it would be intriguing to determine if UBC-2 interacting proteins interact with each other and/or other proteins, to further elucidate their precise involvement in the ubiquitin mediated pathway of selective protein degradation.

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APPENDIX I

A. LIST OF OLIGONUCLEOTIDES AND THEIR SEQUENCES

MG1	5' GCGCTTGCATATGATGGCTCTCAAAAGAATCCAG 3' (*Nde*I)
MG2	5' CGGGATCCTCACATAGCATACTTTTGCGTC 3' (*Bam*HI)
MG3 (pAS2 sequencing primer)	5' TCGGAAGAGAGTAGTAA 3'
MG6 (pcDNA3.1 sequencing primer)	5' TGGGAGTGGCACCTTCC 3'
MG 7	5' ATATACCATGGCTCTCAAAAGAAT 3' (*Nco*I)
MG10	5' ATATGCTCGAGCATAGCGACTTTT 3' (*Xho*I)
SEQEC1 (5' pACT sequencing primer)	5' TTCGATGATGAAGATACCCC 3'
SEQEC2 (3' pACT sequencing primer)	5' AGGCAAAACGATGTATAA 3'
pGex5' sequencing primer	5' GGGCTGGCAAGCCACGTTTGGTG 3'

B. SUMMARY OF BACTERIA AND YEAST STRAINS AND THEIR GENOTYPES

1. *E. coli* strains

DH5α™	F- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k-, m_k+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1 (GIBCOBRL® LIFE TECHNOLOGIES™)
BL21(DE3)	*hsdS gal* (λc*Its857 ind1 Sam7 nin5 lacUV5-T7 gene1*)
HT115(DE3)	F- *mcr*A *mcr*B IN(*rrn*D-*rrn*E)1 lambda-*rnc*14::*Tn*10(DE3 lysogen: *lav*UV5 promoter -T7polymerase) (IPTG-inducible T7 polymerase) (RNase III minus)
HB101	*D*(gpt-proA)*62 leuB6 thi-1 lacY1 hsdSB20 recA rpsL20 (Strr) ara-14 galK2 xyl-5 mtl-1 supE44 mcrBB*
OP50	*E. coli* strain B uracil auxotroph

2. Yeast strains

Y187	MATα *gal4 gal80 his3 trp1*-901 *ade2*-101 *ura3*-52 *leu2*-3, -112 + URA3::GAL *lac*Z LYS2::GAL(UAS) HIS3 *cyh*^{r}
Y190	MATa *gal4 gal80 his3 trp1*-901 *ade2*-101 *ura3*-52 *leu2*-3,-112 *met*- URA3::GAL *lac*Z