Characterisation of Caenorhabditis elegans RING Finger Protein 1 (RFP-1), a Binding Partner of Ubiquitin-Conjugating Enzyme 1 (UBC-1)

by

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ABSTRACT

Ubiquitin-mediated proteolysis is an ATP-dependent pathway that targets proteins for degradation in eukaryotes. The conjugation of a multiubiquitin chain to a target protein requires a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and often a ubiquitin-protein ligase (E3). While specificity for target protein recognition resides in the E2 and E3 components, very little is known about their roles in multicellular organisms.

In a yeast two-hybrid screen, RING Finger Protein 1 (RFP-1), UBR1, and SCA-1 were identified as potential binding partners of *C. elegans* UBC-1, a ubiquitin-conjugating enzyme with a high degree of identity to *S. cerevisiae* UBC2/RAD6. The interaction between RFP-1 and UBC-1 was confirmed by co-immunoprecipitation experiments in buffer and *C. elegans* extract. The RFP-1 and UBC-1 interaction was not dependent on the presence of ubiquitin or ATP. Yeast interaction trap experiments mapped the region of interaction to the basic N-terminal 313 residues of RFP-1 and the UBC core domain of UBC-1. Western blot analysis and indirect immunohistochemical staining showed that RFP-1 is present in embryos, larvae, and adults, where it is found in intestinal, nerve ring, pharyngeal, gonadal, and oocyte cell nuclei. Limited UBC-1 indirect immunohistochemical staining experiments showed that UBC-1 is localised to oocyte nuclei.

Double stranded RNA interference experiments against *rfp-1* indicate that this gene is not essential, but is required for proper vulval development and for egg-laying. By contrast, RNA interference experiments against *ubc-1* gave no obvious phenotype, suggesting that *ubc-1* is non-essential or is functionally redundant. The Class A and Class B Synthetic Multivulva pathways define two functionally redundant genetic pathways in *C. elegans* that antagonise vulval development. RNA interference experiments against *rfp-1* in *lin-15A* (*n767*) or *lin-15B* (*n744*) animals suggest that *rfp-1* is neither a Class B nor a Class A Synthetic Multivulva gene. Similarly, RFP-1 failed to directly interact with LIN-35 (467-961), a Class B Synthetic Multivulva protein and the putative retinoblastoma protein in *C. elegans*. The *rfp-1* RNA interference-induced phenotypes were exacerbated in the *lin-15B* (*n744*) background. Based on the sequence similarity to *R. norvegicus* Staring and *S. cerevisiae* Bre1p, RFP-1 may be a RING finger ubiquitin-protein ligase required for UBC-1-dependent ubiquitylation of nuclear proteins.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>APC/C</td>
<td>anaphase-promoting complex/cyclosome</td>
</tr>
<tr>
<td>β-TrCP</td>
<td>β-transducin repeat-containing protein</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>BRR</td>
<td>basic residue rich</td>
</tr>
<tr>
<td>Cdc</td>
<td>cell division cycle</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CHIP</td>
<td>carboxyl terminus of Hsc70-interacting protein</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded ribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>E6-AP</td>
<td>E6-associated protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>egl</td>
<td>egg-laying defective</td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial Na⁺ channel</td>
</tr>
<tr>
<td>evl</td>
<td>everted vulva</td>
</tr>
<tr>
<td>F</td>
<td>farad</td>
</tr>
<tr>
<td>FOA</td>
<td>5-fluoro-orotic acid</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>hect</td>
<td>homologous to E6-AP carboxy-terminus</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HM</td>
<td>silent mating-type</td>
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HSN  hermaphrodite-specific neuron
Hsp  heat shock protein
IPTG  isopropyl-β-D-thiogalactopyranoside
kDa  kiloDalton
L  larval
mes  maternal effect sterile
mRNA  messenger ribonucleic acid
muv  multivulva
nt  nucleotide
OD  optical density
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PCR  polymerase chain reaction
pfu  plaque forming units
PMSF  phenylmethylsulfonyl fluoride
PVDF  polyvinylidene difluoride
RFP  RING finger protein
RING  really interesting new gene
RISC  RNA-induced silencing complex
RNA  ribonucleic acid
RNAi  RNA interference
SCF  Skp1, Cdc53, F box protein
SDS  sodium dodecyl sulphate
siRNA  small interfering ribonucleic acid
SMART  simple modular architecture research tool
TPEN  N,N,N',N'-tetraakis (2-pyridylmethyl) ethylenediamine
ubc  ubiquitin-conjugating enzyme
V  volt
VCB  von Hippel-Lindau tumour suppressor Elongin C Elongin B
X-gal  5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
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I dedicate this thesis with love to my parents, who taught me to persevere and to be true to myself.

"Nothing great was ever done without much enduring"

St. Catherine of Siena
INTRODUCTION

1. The Ubiquitin Proteasome Proteolytic Pathway

Rapid and selective degradation of cellular proteins is accomplished by the ubiquitin proteasome pathway (reviewed by Pickart, 2001; Hershko et al., 2000). Ubiquitin is a highly conserved 76-residue protein that is conjugated to other proteins to target them for degradation in the 26 S proteasome, a multisubunit ATP-dependent protease. Ubiquitylation is the process of conjugating a ubiquitin moiety to a substrate protein and this highly regulated process involves an enzymatic cascade.

Figure 1 summarises the enzymatic reactions in the ubiquitylation of a substrate protein and its subsequent degradation by the 26S proteasome. In an ATP-dependent step, a ubiquitin-activating enzyme (E1) forms a thiol ester with the carboxyl group of glycine 76 of ubiquitin, thus activating the C-terminus for nucleophilic attack. Next, a ubiquitin-conjugating enzyme (also called a UBC or E2) forms a thiol ester bond with ubiquitin via the active site cysteinyl residue of the ubiquitin-conjugating enzyme. Finally, a ubiquitin-protein ligase (E3) transfers the activated ubiquitin from the ubiquitin-conjugating enzyme to a lysyl residue in the substrate. Some E3s make a thiolester bond to ubiquitin and then conjugate the ubiquitin to the substrate, while other E3s recognise target proteins and bring them in close proximity to the ubiquitin-conjugating enzyme for ubiquitylation. Regardless of whether the E3 makes a thiolester bond to ubiquitin first or simply serves as a scaffold to bring the UBC and substrate together, an isopeptide bond is formed between the carboxyl group of glycine 76 of ubiquitin and the ε-amino group of a lysyl residue in the target protein.

An important feature of ubiquitylation as a degradation signal is the conjugation of a multiubiquitin chain to a target protein. This is accomplished by successive rounds of ubiquitylation on lysine 48 of the previously conjugated ubiquitin moiety (Chau et al., 1989). Once a substrate becomes multiubiquitylated, it is recognised by a multisubunit ATP-dependent protease called the 26S proteasome. The target protein is cleaved into small peptides and free ubiquitin is released by ubiquitin isopeptidases.
Figure 1: The Ubiquitin-Proteasome Pathway

The conjugation of ubiquitin to a target protein involves an enzymatic cascade. (1) A ubiquitin-activating enzyme (E1) conjugates ubiquitin (Ub) via a thiolester bond (\(-\)) in an ATP-dependent reaction. (2) Ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2) with a thiolester bond between the active site cysteinyl residue of the E2 and the carboxy terminal glycine of ubiquitin. (3) Ubiquitin may be transferred via a thiolester bond to a cysteinyl residue in a ubiquitin-protein ligase enzyme (E3). (4a) A substrate bearing specific recognition sequences is recognised and bound by the substrate-specific E3. (4b) Ubiquitin may be transferred directly from the E2 to a substrate bound to a substrate-specific E3. (5) Ubiquitin is conjugated to a substrate via an isopeptide bond (\(-\)) between the carboxy-terminal glycine of ubiquitin and the \(\varepsilon\)-amino group of an internal lysyl residue of the substrate. Multiubiquitin chains are produced by rounds of ubiquitin conjugation to a lysyl residue in the previously conjugated ubiquitin moiety. (6) The multiubiquitylated substrate binds to the ubiquitin receptor subunit of the 19S cap of the 26S proteasome. The substrate is fed into the 20S core, where it is degraded into peptides. (7) Ubiquitin-specific isopeptidases recycle ubiquitin. This figure was adapted from Ciechanover, 1998.
2. Ubiquitin

Ubiquitin is a highly conserved 76-residue protein found in all eukaryotes from yeast to humans. Ubiquitin is encoded either as a polyubiquitin precursor protein consisting of several ubiquitin repeats fused head to tail (Ozkaynak et al., 1984; Graham et al., 1989) or as a ubiquitin precursor fused to a downstream ribosomal protein of either the large or small ribosomal subunits (Finley et al., 1989; Ozkaynak et al., 1987; Jones et al., 1995). In the latter case, ubiquitin appears to promote the assembly of the associated ribosomal protein into ribosomes. The polyubiquitin precursor protein is cleaved post-translationally at the C-terminal of each ubiquitin moiety by ubiquitin C-terminal hydrolases to release free ubiquitin (Liu et al., 1989).

While multiubiquitin chains linked via lysine 48 constitute a degradation signal for target proteins, there are several different types of ubiquitin linkages, involving lysyl residues other than lysine-48 of ubiquitin. A ubiquitin moiety can be conjugated to lysine 6, lysine 11, lysine 29, or lysine 63 of the previously conjugated ubiquitin in a growing multiubiquitin chain (Baboshina and Haas, 1996; You and Pickart, 2001; Arnason and Ellison, 1994). Lysine 63-linked multiubiquitin chains are important for cellular stress and DNA damage responses and probably do not direct a target protein to the proteasome for degradation. Yeast expressing a ubiquitin derivative in which lysine 63 was mutated to arginine were indistinguishable from wild type yeast in their ability to degrade short-lived proteins and abnormal, canavanine-containing proteins (Spence et al., 1995; Arnason and Ellison, 1994).

3. Ubiquitin-Activating Enzymes (E1)

In most organisms, a single ubiquitin-activating enzyme (E1) commences the enzymatic cascade that may result in the proteolysis of a target protein. The E1 binds MgATP and ubiquitin and catalyses the formation of a high energy ubiquitin-adenylate intermediate. This activated ubiquitin is then conjugated to a cysteinyl residue in the E1 via a thiolester bond (reviewed in Pickart, 2001). In S. cerevisiae, the 114 kDa ubiquitin-activating enzyme is encoded by the essential gene UBA1 (McGrath et al., 1991). Similarly, the C. elegans ubiquitin-activating enzyme encoded by uba-1 is essential, as RNA interference (RNAi) against uba-1 produces a severe embryonic lethal phenotype and kills the RNAi-treated adult (Jones et al., 2001). Finley et al. (1984) showed that the mammalian cell cycle mutant ts85 contained a temperature sensitive
mutation in the E1 that rendered extracts of ts85 defective in ubiquitin-conjugating activity. These cells had reduced levels of ubiquitylated histone H2A at the non-permissive temperature.

The \textit{H. sapiens} E1 has two isoforms produced by alternative translational start sites at the first or second methionyl residues in the E1 sequence. The two isoforms, E1a (117 kDa) and E1b (110 kDa), differ in their subcellular localisation and phosphorylation state: E1a is a phosphoprotein and resides predominantly in the nucleus, while E1b is a cytoplasmic protein and is largely unphosphorylated. The first 11 residues of E1a, encompassing a nuclear localisation sequence and a series of seryl residues that could be potentially phosphorylated, were sufficient for both the nuclear localisation and phosphorylation of E1a. Removal of the nuclear localisation sequence KKRR dramatically reduced phosphorylation on E1a, suggesting that phosphorylation occurs within the nucleus. The role of phosphorylation on E1a is unknown, but it may promote stable association with ubiquitin-conjugating enzymes to ensure efficient ubiquitylation of substrates. Phosphorylation may also increase retention of E1a in the nucleus (Stephen \textit{et al.}, 1997).

4. Ubiquitin-Conjugating Enzymes (UBCs)

The ubiquitin-conjugating enzyme (UBC or E2) accepts activated ubiquitin from the E1, resulting in the formation of a UBC-bound ubiquitin thiolester. The UBC gene family shares a conserved catalytic domain containing the active site cysteinyl residue within a 150 residue UBC domain. Members of the family vary due to the presence of N-terminal or C-terminal residue extensions, as well as small (ten residues) insertions between the conserved cysteinyl and tryptophanyl residues in the UBC domain. The N- and C-terminal extensions may serve to direct ubiquitylation towards a particular target protein. The \textit{S. cerevisiae} genome encodes 13 ubiquitin-conjugating enzymes and disruptions in these genes confer various phenotypes on the resulting mutants (reviewed in Scheffner \textit{et al.}, 1998).

The \textit{C. elegans} genome encodes twenty ubiquitin-conjugating enzymes and three ubiquitin E2 variants (uev); the latter lack an active site cysteinyl residue and probably do not conjugate ubiquitin via thiolester bonds. Using double stranded RNA-mediated interference (RNAi) to phenocopy null mutations, Jones \textit{et al.} (2001) showed that only four of the twenty ubiquitin-conjugating enzymes were essential. RNAi against \textit{ubc-2, ubc-9, ubc-12,} or \textit{ubc-14}
resulted in significant embryonic lethality. Secondary phenotypes due to maternal rescue included larval arrest, vulval eversion, and egg-laying deficiencies. Most of the ubiquitin-conjugating enzymes tested had no RNAi-inducible phenotype suggesting that there was a high degree of functional redundancy. As *C. elegans ubc-1* is the focus of this study and since it is the homologue of *S. cerevisiae RAD6*, the diverse functions of *RAD6* and *ubc-1* are discussed in detail.

### 4.1. RAD6

The *S. cerevisiae RAD6* gene was originally identified as a member of a DNA repair pathway and *rad6* mutants have several phenotypes. The *rad6* mutants were hypersensitive to DNA damaging agents such as ultraviolet light, ionizing radiation, and cross-linking and alkylating chemicals. *Rad6* deletion mutants had slower growth rates than wild-type yeast and failed to undergo radiation or chemical mutagen-induced mutagenesis. Homozygous *rad6* diploids do not sporulate, i.e. they do not make ascospores (Haynes and Kunz, 1981; Friedberg *et al.*, 1991). Mutations in *RAD6* promoted an increased rate of mutation due to the transposition of *Ty* elements (Picologlou *et al.*, 1990).

The *RAD6/UBC2* gene encodes a 172 residue ubiquitin-conjugating enzyme (Jentsch *et al.*, 1987). The crystal structure of Rad6 at 2.6 angstrom resolution shows that it adopts the α/β fold characteristic of other ubiquitin-conjugating enzymes. The active site cysteine (C-88) that makes a thiol ester bond with ubiquitin is located in a cleft formed by a conserved coiled region. The residues important for Rad6p to interact with Rad18p or Ubr1p, are on the opposite side of the structure from the active site cleft (Worthylake *et al.*, 1998).

*RAD6* has important roles in sporulation, DNA repair, gene silencing, spermatogenesis in mammals, and protein degradation by the N-end rule pathway. The function of Rad6 in these diverse cellular activities will be discussed in detail below.

### 4.2. The RAD6 DNA Repair Pathway

The *RAD6* DNA repair pathway is responsible for post-replicative DNA repair, when lesions produced by UV irradiation of the template strand cause the replication machinery to stall. This produces gaps in the newly synthesised daughter strand, which must be repaired.
Post-replicative repair comprises two branches: the error-free and the error-prone pathways, both of which are RAD6-dependent. In the error-prone pathway, a DNA polymerase (REV3) makes a mutagenic bypass of the damaged sites, hence accumulating mutations in the repaired DNA. By contrast, the error-free pathway is less understood, but involves RAD6, RAD9, RAD5, RAD18, MMS2, and UBC-13 to enable error-free bypass of a blocked/damaged DNA template strand (Lawrence, 1994). UBC13 encodes a ubiquitin-conjugating enzyme and MMS2, a positive regulator of UBC13, encodes a UBC variant that lacks the active site cysteinyl residue that would confer ubiquitin-conjugating activity. In response to DNA damage, the Ubc13-Mms2 complex builds multiubiquitin chains with lysine 63 linkages (Hofmann and Pickart, 1999).

Rad6p and the RING finger protein Rad18p co-purify as a heterodimer from yeast over-expressing each of these proteins. The Rad6p-Rad18p heterodimer has an ATPase activity, a ubiquitin-conjugating activity and it ubiquitylates the substrate histone H2B in vitro. Furthermore, the Rad6p-Rad18p heterodimer preferentially binds to single stranded DNA in a Rad18p-dependent manner. The ATPase activity of the Rad6p-Rad18p complex is probably dependent on the GKS Walker type A nucleotide binding motif of Rad18 (Bailly et al., 1997).

Ulrich and Jentsch (2000) identified more protein-protein interactions of the RAD6 DNA repair epistasis group by employing yeast interaction trap and co-immunoprecipitation experiments with known members of the RAD6-dependent DNA repair pathway: RAD6, RAD9, RAD5, RAD18, MMS2, and UBC-13. Rad5p, a RING finger protein, interacted with both Ubc13p and Rad18p and the RING finger was required for the interaction with Ubc13p. Using a yeast three-hybrid system, they showed that Rad18p and Rad5p co-ordinate multiple UBC complexes. The Ubc13p-Mms2p ubiquitin-conjugating complex was recruited by Rad5p and Rad6p was recruited by Rad18p. The most interesting finding was that the interaction between Rad5p and Rad18p nucleates a multimeric ubiquitin-conjugating complex. Furthermore, Ulrich and Jentsch identified Rad5p and Rad18p on chromatin spreads, and showed that Rad5p recruited Ubc13p-Mms2p to chromatin in response to DNA damage. Thus, Rad6p and Ubc13p-Mms2p may function in concert to regulate the activity of other repair enzymes and/or to prepare a more accessible DNA template for repair by targeting proteins such as chromatin proteins for destruction.
A potential substrate of the *RAD6* error-free DNA repair pathway is yeast proliferating cell nuclear antigen (PCNA). PCNA binds as a trimeric ring to DNA and functions as a clamp and processivity factor for DNA polymerases. In response to DNA damage, PCNA became multiubiquitylated on lysine 164 in a *RAD6* and *RAD18*-dependent manner. PCNA was only monoubiquitylated in *ubc13, mms2*, or *rad5* mutants, suggesting that Rad6p-Rad18p initiated monoubiquitylation of PCNA, and that multiubiquitylation (via lysine-63 of ubiquitin) was achieved by Ubc13p and Mms2p in a complex with Rad5p. Yeast two-hybrid analysis showed that PCNA interacted with Rad18p and Rad5p. Interestingly, lysine 164 of PCNA was also susceptible to modification by a ubiquitin-like protein, SUMO. This reaction required Ubc9p, the sumo-conjugating enzyme, and sumoylation of PCNA accompanied S phase, suggesting a role for sumoylation of PCNA in normal DNA replication. Sumoylation of PCNA prevented the Rad6p-dependent ubiquitylation at lysine 164 (Hoege *et al.*, 2002).

Figure 2 shows a model of alternative repair complexes in the *RAD6* DNA repair epistasis group proposed by Ulrich and Jentsch (2000). In this model, Rad18p homodimers recruit Rad6p as the only ubiquitin-conjugating activity for error-prone DNA repair. Alternatively, heterodimerization of Rad18p and Rad5p recruits Rad6p (via its interaction with Rad18p) and Ubc13p-Mms2p (via their interaction with Rad5p) to damaged DNA for error-free repair.

### 4.3. *RAD6* and the N-end Rule Pathway of Protein Degradation

The N-end Rule relates the half-life of a protein to the identity of its amino-terminal residue, with destabilising residues conferring rapid degradation upon a protein (Bachmair *et al.*, 1986). N-end Rule substrates have two degradation signals: a destabilising N-terminal amino acid and an internal lysyl residue, to which ubiquitin becomes conjugated (Bachmair and Varshavsky, 1989). Type I primary destabilising residues include the basic residues arginine, lysine, and histidine. Type II primary destabilising residues are the bulky, hydrophobic residues phenylalanine, leucine, tyrosine, tryptophan, and isoleucine. Secondary destabilising residues are aspartate and glutamate, and tertiary destabilising residues are asparagine and glutamine. Secondary and tertiary destabilising residues are enzymatically modified to primary destabilising residues (reviewed in Varshavsky *et al.*, 2000).
Figure 2: Model of *RAD6*-dependent DNA Repair in *S. cerevisiae*

DNA damage results in stalled replication complexes and regions of single stranded DNA. The *RAD6* epistasis DNA repair group consists of *RAD6, RAD9, RAD5, RAD18, MMS2, and UBC13*. In error-prone DNA repair, the RING finger protein Rad18p dimerizes, binds to single stranded DNA and recruits the ubiquitin-conjugating enzyme Rad6p to sites of damaged DNA and stalled replication machinery. Alternative complexes are formed when the RING finger proteins Rad18p and Rad5p dimerize, thereby recruiting the Rad6p, Ubc13p, and Mms2p ubiquitin-conjugating enzymes to the error-free repair complex. Thus, the Rad5p-Rad18p interaction coordinates a multimeric ubiquitin-conjugating complex to ubiquitylate, and perhaps target for destruction, proteins critical for error-free DNA repair. This figure was adapted from Ulrich and Jentsch, 2000.
Using artificial substrates consisting of β-galactosidase fusions bearing various destabilising amino-terminal residues, Dohmen et al. (1991) showed that rad6Δ yeast failed to multiubiquitylate and degrade artificial substrates that were otherwise short-lived in wild type yeast. Rad6p co-immunoprecipitated with the 225 kDa ubiquitin-protein ligase Ubr1p, demonstrating that Rad6p and Ubr1p were essential components of the N-end Rule proteolytic pathway.

4.4. **RAD6 is Required for Gene Silencing and Histone Ubiquitylation in *S. cerevisiae***

In *S. cerevisiae*, gene silencing at telomeres and *HM* (silent mating-type cassette) loci is RAD6-dependent. One silencing assay examined whether particular yeast strains could suppress/silence the expression of *URA3*, and hence survive on plates containing 5-fluoro-orotic acid (FOA). The silencing of telomeric RNA polymerase II-transcribed genes, *URA3* and *ADE2*, was significantly reduced in *rad6Δ* yeast. The *rad6Δ* yeast also had reduced silencing of a telomeric RNA polymerase III-transcribed gene, *SUP4-o*. RAD6 was required for silencing at mating-type loci as mating efficiency was significantly decreased when the MATa strain was *rad6Δ*. Furthermore, silencing of HM-linked *URA3* was reduced in the *rad6Δ* strain, as this strain was unable to grow on media containing FOA (Huang et al., 1997).

Silencing was dependent on the ubiquitin-conjugating activity of RAD6 as silencing of telomeric *URA3* could not be restored in a *rad6Δ* strain carrying plasmids encoding either *rad6 C88A* or *rad6 C88S*. Interestingly, silencing of telomeric *URA3* did not require *UBR1*, the ubiquitin-protein ligase of the N-end rule pathway that interacts with RAD6. Rad18p, the protein that interacts with Rad6p in the DNA repair pathway, was also not required for silencing of telomeric *URA3*. These data indicate that the ubiquitin-conjugating activity of RAD6 is required for gene silencing at telomeric and *HM* loci, and that silencing does not require N-end rule-mediated degradation. One interpretation is that Rad6p is required to target antagonistic silencing regulators for Ubr1p-independent degradation. Another interpretation is that Rad6p is required to remodel chromatin to enable silencing at specific loci (Huang et al., 1997).

Several reports have shown that Rad6p catalyses the covalent attachment of ubiquitin to histone proteins in nucleosomes in yeast. Depending on the purification method used to obtain Rad6 protein, Rad6p can either monoubiquitylate or multiubiquitylate histones. *In vitro,*
recombinant Rad6p can catalyse the transfer of a single ubiquitin from an El to histone H2B or histone H2A (Jentsch et al., 1987). By contrast, Sung et al. (1988) showed that native Rad6p could multiubiquitylate yeast histones H2A and H2B in vitro.

Using a yeast strain that expressed only Flag-tagged histone H2B and a galactose-inducible HA-tagged ubiquitin gene, Robzyk et al. (2000) showed that histone H2B was monoubiquitylated on lysine-123 in vivo. Interestingly, RAD6 was required for histone H2B monoubiquitylation as no ubiquitylated histone H2B species were immunoprecipitated from a rad6Δ strain. Introduction of a plasmid expressing Rad6p restored ubiquitylation on histone H2B in the rad6Δ strain, whereas a plasmid expressing a catalytically inactive rad6-C88A or a plasmid expressing Rad6p lacking the C-terminal tail (rad6-149) was unable to restore histone H2B monoubiquitylation. Moreover, the Rad6p-dependent ubiquitylation of H2B was required for mitotic cell growth and meiosis. These experiments show that Rad6p-dependent ubiquitylation of histones aids chromatin remodelling required for transcriptional regulation and gene silencing. The addition of a bulky ubiquitin moiety to histones in the nucleosome may allow localized “opening” or remodelling of the nucleosome.

Recently, two independent groups showed that RAD6 has an important role in regulating chromatin structure and gene silencing through a series of histone modifications involving H2B and H3. RAD6 was required for methylation of histone H3 on lysine 4, as deletion of the RAD6 locus abolished methylation of histone H3. The H3 methylation was in turn dependent on Rad6p ubiquitylating histone H2B on lysine 123, as H3 methylation was abolished in H2B-K123R mutants. H3 methylation was dependent on the ubiquitin-conjugating activity of Rad6p as plasmid-borne rad6-C88A failed to restore H3 methylation to rad6Δ mutants. The H3 methylation was independent of the N-end rule pathway as ubr1Δ mutants contained wild type levels of methylated H3. Chromatin immunoprecipitation (ChIP) assays of wild type Flag-tagged H2B and Flag-tagged H2B-K123R showed that methylated H3 was associated with chromatin only in the wild type Flag-tagged H2B, again arguing that H2B must be ubiquitylated by Rad6p in order for H3 to be methylated. Thus, Rad6p-mediated H2B ubiquitylation is required for H3 methylation, which then mediates silencing (Sun and Allis, 2002). Dover et al. (2002) also identified Rad6p as a key regulator of gene silencing through its ubiquitylation of
histone H2B, allowing methylation of histone H3 by a large methylation complex called COMPASS (complex of proteins associated with Set1).

RAD6 was required for repressing ARG1 transcription in rich media and this repression was dependent on the ability of Rad6p to ubiquitylate histone H2B. UBR1 was also required for the RAD6-dependent repression of ARG1, suggesting a role for N-end Rule degradation in this particular repression pathway (Turner et al., 2002).

4.5. The Murine Homologue of RAD6 is Required for Spermatogenesis

In recent years, researchers have examined the role of RAD6 homologues in multicellular organisms. Roest et al. (1996) generated a mouse lacking the homologue of RAD6 (mHR6B) and showed that elimination of mHR6B caused male sterility. Several defects in early and late stages of spermatogenesis were observed in adult mHR6B−/− animals. In particular, progression through the elongation and condensation stages of spermatid development was impaired in adult mHR6B−/− animals. More than 90% of the spermatozoa that matured were abnormal and immotile. Roest et al. hypothesised that mHR6B was required during the spermatid elongation stage to ubiquitylate somatic and testis-specific histones, thereby targeting them for degradation, and allowing chromatin to be packaged around transition proteins and protamines for efficient condensation of chromatin during spermatogenesis.

Histone H2A was ubiquitylated in elongating spermatids in both wild type animals and mHR6B−/− mutant animals. This indicated that HR6B was not required for H2A ubiquitylation in elongating spermatids. Clearly, ubiquitylation of histones accompanied the histone-to-protamine transition and was required for spermatogenesis. One hypothesis was that HR6A (another RAD6 homologue) or other ubiquitin-conjugating enzymes were able to rescue the loss of HR6B and maintain H2A ubiquitylation. The male infertility of mHR6B−/− mutant animals was presumably caused by other unidentified interactions with HR6B targets (Baarends et al., 1999).
4.6. The Activity of the Human Homologue of *RAD6* is Regulated via Phosphorylation by Cyclin-Dependent Kinases

The mechanisms regulating the ubiquitin-conjugating activity of a given UBC are largely unknown. The human homologue of Rad6A (hHR6A) was identified as a substrate of cyclin-dependent kinases (CDK) 1 and 2 *in vitro* and *in vivo* during the G₂/M phase of the cell cycle. HHR6A was phosphorylated on Serine 120, a highly conserved residue in all *RAD6* homologues. Moreover, cyclin A/CDK2-dependent phosphorylation on Ser 120 of hHR6A increased the ubiquitin-conjugating activity towards histone H2A four-fold.

Point mutants of hHR6A, in which Ser120 was mutated to alanine or threonine, were unable to ubiquitylate H2A. Conversely, hHR6A S120D had increased H2A ubiquitylation activity compared to wild-type hHR6A, indicating that a negative charge at position 120 was important in the regulation of hHR6A activity. Histone H2B ubiquitylation was increased during the G₂/M phase of the cell cycle, concomitant with phosphorylation of hHR6A. Finally, only plasmids encoding wild-type hHR6A, or S120D hHR6A could rescue the mild growth defect of *rad6Δ* yeast at 37°C, whereas neither S120A hHR6A nor S120T hHR6A could rescue *rad6Δ* yeast. Thus, the growth rate may be regulated by attenuating the ubiquitin-conjugating activity of hHR6A via phosphorylation (Sarcevic *et al.*, 2002).

4.7. *C. elegans* UBC-1

*Ubc-1* encodes a 192 amino acid protein (21.5 kDa) that is the functional homologue of *S. cerevisiae RAD6/UBC2*. *Ubc-1* was able to complement the DNA repair functions of *rad6Δ* yeast, although *ubc-1* failed to rescue the sporulation defect of these cells. UBC-1 contains the conserved UBC domain with the active site cysteinyl residue (C88) as well as a 40 amino acid acidic carboxy-terminal extension or tail. *In vitro* ubiquitin-conjugation assays showed that UBC-1 could conjugate ubiquitin via a thiolester bond that was labile to the reducing agents, DTT and β-mercaptoethanol (Leggett *et al.*, 1995).

Chemical cross-linking studies with ³⁵S-labelled UBC-1 showed that UBC-1 formed homodimers and homotetramers. Deletion of the acidic C-terminal tail significantly reduced, but did not abolish, this self-association. Thus, both the conserved UBC core and the C-terminal tail contribute to homodimerization. The C-terminal tail may be important for specifying protein
targets or modulating interactions with other proteins. \textit{In vitro} assays showed that UBC-1 was conjugated to ubiquitin by the canonical thiolester bond as well as via a peptide bond. The residue that became monoubiquitylated via a peptide bond was mapped to lysine-162 in the C-terminal tail by deletion analysis and ubiquitin-conjugation assays. Moreover, UBC-1 K162R failed to make the peptide bonded ubiquitin-UBC-1 adduct. Clearly, the acidic C-terminal tail has important roles in the self-association and post-translational modification of UBC-1 (Leggett and Candido, 1997).

5. Ubiquitin-Protein Ligases (E3s)

The specificity of the ubiquitin-proteasome pathway towards substrates is achieved by the interaction between the target protein and a ubiquitin-protein ligase (E3). Ubiquitin-protein ligases recognise a degradation motif in a target protein, thereby directing specificity of the ubiquitylation machinery to that particular protein. The ubiquitin-protein ligase can serve as a scaffold to bring a ubiquitin-conjugating enzyme (UBC) and target protein in close proximity for ubiquitin transfer. The ubiquitin-protein ligase may first form a thiolester to ubiquitin, and then subsequently transfer the ubiquitin moiety to the target protein (Pickart, 2001).

There are two superfamilies of ubiquitin-protein ligases, and more E3s are bound to be discovered. Herein, classification of E3s is based on the presence of a common structural domain, either a hect domain or a RING finger domain (Pickart, 2001). For simplicity of discussion, the two superfamilies will be subdivided into five classes comprised of ubiquitin-protein ligases with: 1) a hect domain, 2) a RING finger domain, or 3) a U Box. The other two classes are multisubunit ubiquitin-protein ligases: 4) the SCF and VCB, and 5) the APC/C. The five classes of ubiquitin-protein ligases and their cellular functions will be discussed below. For a comprehensive review of ubiquitin-protein ligases, refer to Jackson \textit{et al.} (2000).

5.1. Hect Domain Ubiquitin-Protein Ligases

The hect domain ubiquitin-protein ligases possess two important characteristics: 1) they make thiolester bonds to ubiquitin and 2) they recognise and bind the target protein for efficient transfer of ubiquitin to the substrate. The hect (homologous to E6-AP carboxy terminus) domain ubiquitin-protein ligases all share a conserved 350 residue domain that contains an active site
cysteinyl residue that accepts ubiquitin from a ubiquitin-conjugating enzyme, forming a thiolester bond with the carboxyl group of glycine 76 in ubiquitin. After formation of the thiolester intermediate, the hect E3 transfers the ubiquitin moiety to the substrate, making an isopeptide bond between the C-terminal glycine of ubiquitin and an ε-amino group of a lysyl residue in the target protein. Thus, there is a thiolester “bucket brigade” transfer of ubiquitin from E1 to the UBC, from the UBC to the hect domain E3, and finally the hect E3 transfers ubiquitin to the target protein (Scheffner et al., 1995).

The first hect ubiquitin-protein ligase studied was E6-AP (E6-associated protein). The oncogenic human papillomavirus (HPV) type 16 or 18 E6 protein interacted with a 100 kDa cellular protein named E6-AP, and together targeted the tumour-suppressor protein p53 for degradation. In vitro, recombinant p53 was ubiquitylated by a recombinant E6-E6-AP complex, providing that E1, UBC8 (from A. thaliana), and ubiquitin were present (Scheffner et al., 1993). This was interesting because it showed how the viral protein E6 could use the cellular ubiquitin-protein ligase E6-AP to directly target and destroy p53, thereby enabling cellular transformation.

Further studies mapped thiolester formation in E6-AP to a cysteinyl residue in the carboxy-terminal domain of E6-AP (hect domain) (Scheffner et al., 1995), and showed that the hect domain of E6-AP was necessary and sufficient for thiolester complexes with ubiquitin (Schwarz et al., 1998). Yeast interaction trap studies determined that UbcH8 and UbcH7 were the cognate E2s of E6-AP and that the hect domain of E6-AP was necessary and sufficient for the interaction with either UbcH8 or UbcH7 (Kumar et al., 1997).

Using the hect domain as a query sequence, data base searches indicate the existence of at least 20 different hect domain proteins in the human genome. Either of the ubiquitin-conjugating enzymes UbcH5 or UbcH7 were capable of interacting with and transferring ubiquitin to a conserved cysteinyl residue in many of these human hect domain-containing proteins (Schwarz et al., 1998). Interestingly, the hect domain of the human E3 KIAA10 was necessary and sufficient to catalyse the assembly of both Lys²⁹- and Lys⁴⁸-linked multiubiquitin chains (You and Pickart, 2001).

The accumulated data on several hect ubiquitin-protein ligases as well as the structure determination of the E6AP hect domain-UbcH7 complex (Huang et al., 1999) is consistent with a bipartite model of catalysis for the hect E3. The C-terminus (hect domain) binds to the
ubiquitin-conjugating enzyme and accepts ubiquitin via a thiolester bond. The N-terminus of the E3 recognises and binds certain motifs in the target protein, enabling ubiquitin transfer to a lysyl residue in the target protein (Wang et al., 1999). Table 1 presents a list of some of the known hect E3s and their substrates.

Table 1: Hect ubiquitin-protein ligases and their substrates

<table>
<thead>
<tr>
<th>Hect E3</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rsp5</td>
<td>RNA pol II large subunit (Rpb1)</td>
<td>(Huibregtse et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>Pheromone receptor (Ste2p)</td>
<td>(Dunn and Hicke, 2001)</td>
</tr>
<tr>
<td>Ufd4</td>
<td>Artificial ubiquitin fusion proteins</td>
<td>(Johnson et al., 1995)</td>
</tr>
<tr>
<td>Tom1</td>
<td>Spt7</td>
<td>(Saleh et al., 1998)</td>
</tr>
<tr>
<td>Higher Organisms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smurf1</td>
<td>Smad1 and Smad5</td>
<td>(Zhu et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Transforming Growth Factor-β Type I Type I</td>
<td>(Ebisawa et al., 2001)</td>
</tr>
<tr>
<td>E6AP</td>
<td>p53 (HPV E6-dependent)</td>
<td>(Scheffner et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>Human homologue of Rad23 (HHR23)</td>
<td>(Kumar et al., 1999)</td>
</tr>
<tr>
<td>Nedd4</td>
<td>α and γ subunits of the epithelial sodium</td>
<td>(Staub et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>channel (ENaC)</td>
<td>(Abriel et al., 1999)</td>
</tr>
<tr>
<td>Itch</td>
<td>Notch</td>
<td>(Qiu et al., 2000)</td>
</tr>
</tbody>
</table>

5.2. RING Finger Ubiquitin-Protein Ligases

The Really Interesting New Gene (RING) finger is a protein-protein interaction domain consisting of several cysteiny1 and histidyl residues with a characteristic spacing that enables coordination of two zinc ions in a cross-brace structure. Figure 3 is a schematic of the RING finger domain showing the two types of RING fingers, the C3H2C3 (also called RING-H2) and the C3HC4 (also called RING-HC) RING fingers.
Figure 3: Consensus Sequence and Structure of RING Finger Domains

The RING finger is a protein-protein interaction domain in which a series of conserved cysteinyl and histidyl residues coordinate two zinc ions and form a stable cross-brace structure. The consensus sequence is listed below the structure with zinc coordinating residues, cysteine and histidine, in bold and their positions are numbered from 1 to 8. X is any residue and subscripts are the number of X residues separating the conserved cysteinyl and histidyl residues. The first, second, fifth and sixth cysteines/histidines coordinate one zinc cation. The third, fourth, seventh, and eighth cysteines/histidines coordinate the other zinc cation. The C3H2C3 RING finger contains a conserved histidyl residue at position 5, whereas a C3HC4 RING finger has a cysteiny1 residue at position 5. This figure was adapted from Pickart, 2001.
RING finger-containing proteins were identified as ubiquitin-protein ligases in yeast-two hybrid screens using the human ubiquitin-conjugating enzyme, UbcH5B, as a bait. A novel murine RING-H2 finger protein named A07 was isolated as a binding partner of UbcH5B. In vitro ubiquitylation assays with recombinant GST-A07, E1, UbcH5B, and 32P-labelled recombinant ubiquitin, showed that A07 was capable of ubiquitylating bacterial lysate proteins in a UbcH5B-dependent manner. Truncation analysis of A07 identified a region containing the RING finger domain to be important for both binding to UbcH5B and ubiquitylation.

Point mutations of cysteinyl residues to serine residues in the RING finger of A07 abolished both the interaction with UbcH5B and the ubiquitylation activity of A07, suggesting an intact RING finger structure was required for E2-binding and ubiquitylation. These results were reproduced in vivo, and A07 itself was ubiquitylated in an E2 and RING-dependent manner. Underscoring the importance of the RING finger, the ubiquitylation of GST-A07 was abolished when it was pre-incubated with divalent cation chelating agents such as EDTA or TPEN. Ubiquitylation of GST-A07 (pre-incubated with TPEN) was restored upon addition of ZnCl₂, suggesting a role for Zn²⁺ in its ubiquitylation (Lorick et al., 1999).

The RING-H2 finger-containing proteins Prajal, NFX-1, Kf-1, TRC8, and the RING-HC finger proteins BRCA1 and Siah-1 were each ubiquitylated in a UbcH5B-dependent manner in vitro. Multiubiquitylation was dependent on an intact RING finger as C → S point mutations in the Zn²⁺-coordinating positions of the RING finger abolished ubiquitylation. This study was intriguing because it identified RING finger domains from several unrelated proteins as regions that interact with ubiquitin-conjugating enzymes and facilitate ubiquitylation. The autoubiquitylation of several of the RING finger proteins tested may be a bona fide event, providing a self-regulatory mechanism, or the autoubiquitylation may simply occur in the absence of substrates.

Unlike hect domain E3s, it is unlikely that cysteinyl residues in the RING finger make thiolester bonds with ubiquitin. Instead, the data are consistent with a model in which the RING finger and surrounding region not only associate with the ubiquitin-conjugated E2, but they provide a favourable environment for ubiquitin transfer to a lysyl residue in a substrate. Thus, RING finger ubiquitin-protein ligases serve as scaffolds and promote E2-dependent ubiquitylation of substrates (Lorick et al., 1999).
The literature on RING finger ubiquitin-protein ligases is extensive, so one particularly interesting example of RING finger ubiquitin-protein ligases, c-Cbl, will be discussed in detail. A list of some of the known single-subunit RING finger E3s and their substrates is presented in Table 2.

The c-Cbl ubiquitin-protein ligase is highly conserved and contains several protein-protein interaction domains including an N-terminal variant SH2 domain, a RING finger, and a C-terminal proline-rich region. Several studies have identified the 120 kDa RING finger protein c-Cbl (and its *C. elegans* ortholog Sli-1) as a ubiquitin-protein ligase that negatively regulates signalling from receptor protein-tyrosine kinases, such as epidermal growth factor receptors (EGFR) and platelet-derived growth factor receptors (PDGFR) (Joazeiro *et al.*, 1999). Overexpression of wild type c-Cbl in CHO cells caused a marked increase in EGF-induced clearance of EGFR, whereas c-Cbl mutants lacking an intact RING finger could not increase receptor disappearance. When immunoprecipitated EGFR was incubated with $^{125}$I-labelled ubiquitin, rabbit reticulocyte lysate, and GST-c-Cbl, EGFR was multiubiquitylated, and this was dependent on an intact RING finger in c-Cbl (Waterman *et al.*, 1999).

A yeast two-hybrid screen identified UbcH7 as the cognate E2 for c-Cbl and truncation studies suggested that the RING finger and some flanking N- and C-terminal sequences of c-Cbl recruited UbcH7. *In vitro* experiments showed that UbcH7 and wild type c-Cbl enhanced ubiquitylation of activated EGFR, whereas ubiquitylation of EGFR was abolished in the presence of the viral-encoded variant 70Z-c-Cbl (contains a deletion of 17 amino acids at the N-terminal boundary of the RING finger, hence a RING finger mutant). It was hypothesised that the SH2 domain of c-Cbl binds to phosphorylated tyrosyl residues in activated EGFR and recruits UbcH7 via the RING finger domain for efficient ubiquitylation of EGFR. The validity of this hypothesis was confirmed when co-expression of 70Z-c-Cbl and UbcH7 inhibited ubiquitylation of EGFR in a dose-dependent manner. 70Z-c-Cbl could still bind to the activated EGFR, but the mutant RING finger failed to recruit UbcH7, thereby inhibiting the wild type UbcH7 and c-Cbl complex from binding to and ubiquitylating EGFR (Yokouchi *et al.*, 1999).

The crystal structure of a portion of c-Cbl (including the variant SH2 domain, linker sequence, and RING finger) complexed with UbcH7 has increased our understanding of how
Table 2: Single-subunit RING finger ubiquitin-protein ligases and their substrates

<table>
<thead>
<tr>
<th>S. cerevisiae</th>
<th>Substrates</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubr1</td>
<td>Cup9</td>
<td>(Turner et al., 2000)</td>
</tr>
<tr>
<td>Scc1</td>
<td></td>
<td>(Rao et al., 2001)</td>
</tr>
<tr>
<td>Hrd1/Der3</td>
<td>HMG-CoA reductase and misfolded proteins;</td>
<td>(Bays et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>misfolded CPY* (carboxypeptidase Y)</td>
<td>(Deak and Wolf, 2001)</td>
</tr>
<tr>
<td>Higher Organisms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLI-1</td>
<td>LET-23 (EGFR)</td>
<td>(Yoon et al., 2000)</td>
</tr>
<tr>
<td>Cbl-b</td>
<td>Phosphatidylinositol 3-Kinase (PI3-K)</td>
<td>(Fang et al., 2001)</td>
</tr>
<tr>
<td>c-Cbl</td>
<td>Epidermal and Platelet-Derived Growth Factor Receptors</td>
<td>(Waterman et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>(EGFR and PDGFR)</td>
<td>(Yokouchi et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Joazeiro et al., 1999)</td>
</tr>
<tr>
<td>Mdm2</td>
<td>p53</td>
<td>(Kubbutat et al., 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Honda et al., 1997)</td>
</tr>
<tr>
<td>Efp</td>
<td>14-3-3σ</td>
<td>(Urano et al., 2002)</td>
</tr>
<tr>
<td>c-IAP2</td>
<td>caspase 3 and caspase 7</td>
<td>(Huang et al., 2000)</td>
</tr>
<tr>
<td>c-IAP1</td>
<td>c-IAP1 (via autoubiquitylation)</td>
<td>(Yang et al., 2000)</td>
</tr>
<tr>
<td>XIAP</td>
<td>XIAP (via autoubiquitylation)</td>
<td>(Yang et al., 2000)</td>
</tr>
<tr>
<td>Parkin</td>
<td>α-synuclein (αSp22)</td>
<td>(Shimura et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Parkin (via autoubiquitylation) and CDCrel-1</td>
<td>(Zhang et al., 2000)</td>
</tr>
</tbody>
</table>

RING finger ubiquitin-protein ligases interact with ubiquitin-conjugating enzymes. The RING domain structure consists of a three-stranded β sheet, an α helix, and two large loops stabilised by two zinc ions. Two loops of UbcH7 extend into a shallow groove on the RING domain of c-Cbl, and this interaction is further stabilised by interactions between UbcH7 and an α helix in a linker region adjacent to the RING domain. Interestingly, the active site cysteinyl residue of UbcH7 is situated on the opposite side of the complex, about 60 angstroms away from the SH2 domain that recruits the substrate. The structure may reconcile this apparent problem because a
deep surface channel running from the SH2 domain to the RING and UbcH7 may position the substrate and E2 for ubiquitin transfer (Zheng et al., 2000).

Figure 4 presents a model of how the RING finger ubiquitin-protein ligase c-Cbl interacts with activated epidermal growth factor receptor and targets the ubiquitylation and down-regulation of this receptor.

5.3. U Box Ubiquitin-Protein Ligases

The U box is an approximately 70 residue domain with a predicted three-dimensional structure similar to the RING finger domain, although the U box does not contain zinc-co­ordinating residues and is stabilised by salt-bridges and hydrogen bonding. Like the RING finger domain, the U box is thought to catalyse ubiquitylation of a target protein by allosterically activating ubiquitin-conjugating enzymes (Aravind and Koonin, 2000). Six mammalian proteins containing U box domains are UFD2a, UFD2b, CHIP, KIAA0860, CYC4, and PRP19. In vitro ubiquitylation assays with recombinant ubiquitin, E1, and E. coli lysate containing the appropriate recombinant E2 (either UbcH5C or Ubc3) showed that all six recombinant mammalian U box containing proteins had ubiquitin-protein ligase activity and could multiubiquitylate E. coli lysate proteins. Deletion studies showed that the U boxes of UFD2a, CHIP, and KIAA0860 were required for ubiquitin-protein ligase activity, and a P → A mutation of a conserved prolyl residue within the U box domain also abolished E3 activity (Hatakeyama et al., 2001). The U box domain of KIAA0860 (also called hUIP5) was necessary and sufficient for the interaction with the murine E2, UbcM4 (UbcH7) (Pringa et al., 2001).

As with the hect ubiquitin-protein ligases, the six U box E3s showed preference for a particular ubiquitin-conjugating enzyme, either Ubc2B, Ubc3, Ubc4, UbcH5C, or UbcH7. In the absence of substrates, UFD2a and KIAA0860 became ubiquitylated in vitro. U box proteins, such as UFD2a, could catalyse multiubiquitylation via K27 or K33 of ubiquitin. This altered specificity for a particular lysyl residue of ubiquitin may serve to direct ubiquitylation towards proteins in times of stress (Hatakeyama et al., 2001).
Figure 4: Model of c-Cbl-mediated Ubiquitylation of the Epidermal Growth Factor Receptor

Epidermal growth factor (EGF) binds to the extracellular domain of the epidermal growth factor receptor (EGFR) and induces autophosphorylation of tyrosyl residues in the receptor. The SH2 domain of c-Cbl binds to phosphorylated tyrosyl residues, thereby recruiting c-Cbl to the EGFR. The RING finger domain of c-Cbl binds to a ubiquitin-conjugating enzyme (UBC), which subsequently transfers the ubiquitin (Ub) it has received from a ubiquitin-activating enzyme (E1) to a lysyl residue in the EGFR. Ubiquitylation of the receptor negatively regulates downstream signalling. This figure was adapted from Yoon et al., 2000.
The U box E3 CHIP (Carboxyl terminus of Hsc70-Interacting Protein) sits at the crossroads of protein re-folding by chaperones and protein degradation by the ubiquitin-proteasome pathway. CHIP interacts with chaperones Hsc70 and Hsp90 and this interaction serves to remodel chaperone complexes and to favour substrate degradation. CHIP ubiquitylated the glucocorticoid receptor (a model Hsp90 substrate) and the U box of CHIP was required for this activity. Thus, CHIP is a co-chaperone that, through its association with Hsp90 and Hsc70, links the chaperone system with the ubiquitin-proteasome pathway (Connell et al., 2001). Hsc70 was also ubiquitylated by CHIP, although Hsc70 was not degraded. Hsc70 was ubiquitylated with non-canonical ubiquitin chains linked via lysine 29 and 63, and these ubiquitin chains may not serve as recognition signals for the proteasome. Instead, CHIP-dependent ubiquitylation of Hsc70 may serve to direct Hsc70 to a certain subcellular location during the stress response or to alter the affinity of Hsc70 for substrates (Jiang et al., 2001).

5.4. Multisubunit Ubiquitin-Protein Ligases: SCF and VCB

The conjugation of multiubiquitin chains to a target protein may involve a multisubunit E3 such as the SCF (Skp1, Cdc53, F box protein) or the VCB (Von Hippel-Lindau tumour suppressor Elongin C Elongin B) ubiquitin-protein ligase. In S. cerevisiae, the SCF ubiquitin-protein ligase consists of the cullin Cdc53, the ubiquitin-conjugating enzyme Cdc34, Skp1, Rbx1, and one of several adaptor proteins called F box proteins. Cdc53 recruits Cdc34 and an F box protein binds to Skp1 and recognises the target protein via interactions with WD40 or leucine rich repeats. There are hundreds of F box proteins and they direct the specificity of the SCF towards phosphorylated substrates. For example, the F box protein Cdc4 binds to phosphorylated Sic1p (an inhibitor of the cyclin-dependent kinase Clb/Cdc28) and stimulates the SCF-dependent ubiquitylation and degradation of Sic1p (Feldman et al., 1997). Another F box protein Grr1 recruits phosphorylated cyclins 1 and 2 to the SCF for ubiquitylation (Skowyra et al., 1997). Finally, the RING finger protein Rbx1 brings the whole SCF complex together, interacting with Cdc53, Cdc34, and several F box proteins. Rbx1 was required for efficient ubiquitylation of phosphorylated Sic1 by the SCF^Cdc4 (Kamura et al., 1999) and phosphorylated Cln1 by SCF^Grr1 (Skowyra et al., 1999). Rbx1 enhanced the autoubiquitylation of Cdc34, suggesting that Rbx1 stimulated the ubiquitin-conjugating activity of Cdc34 (Skowyra et al.,
1999). Rbx1 activated the modification of Cdc53 with Rubl/Nedd8, a ubiquitin-like protein. This modification required the Uba3/Ula1 activating enzyme, Ubc12 conjugating enzyme, ATP, and Rbx1. Rbx1 RING finger mutants were unable to conjugate Rub1 to Cdc53 (Kamura et al., 1999). Thus, a RING finger protein (Rbx1) serves to nucleate a multisubunit E3, recruit and perhaps activate ubiquitin-conjugating enzymes, and promote the modification of Cdc53 with Rub1.

The VCB ubiquitin-protein ligase has a similar architecture to the SCF and consists of Rbx1, the ubiquitin-like protein Elongin B, CUL2 (a homologue of Cdc53), Elongin C (a homologue of Skp1), and VHL (von Hippel-Lindau) (Kamura et al., 1999). VHL, a tumour suppressor protein that is mutated in several cancers (Kaelin and Maher, 1998), recruits substrates for ubiquitin-mediated degradation and has a function analogous to the F box proteins of the SCF. Under normal oxygen levels, VHL binds a transcription factor called hypoxia-inducible factor alpha 1 (HIFα-1), and targets it for ubiquitylation by the VCB complex. Recognition of HIFα-1 is dependent on the hydroxylation of a prolyl residue in HIFα-1. In hypoxic conditions, HIFα-1 is stable because it is not hydroxylated and thus, is not recognised by VHL. HIF can then bind to DNA and activate transcription of certain genes in response to hypoxia (Ivan et al., 2001). The RING finger protein Rbx1 was also shown to activate the Rubl modification of Cul2 in the VCB (Kamura et al., 1999).

5.5. Multisubunit Ubiquitin-Protein Ligases: APC/C

King et al. (1995) fractionated mitotic Xenopus egg extracts and identified a large complex, termed the Anaphase-Promoting Complex (APC) or cyclosome, required for the ubiquitylation of Cyclin B. The APC is a large (36 S) ubiquitin-protein ligase complex required for the metaphase-anaphase transition and for exit from mitosis. In S. cerevisiae, the APC consists of at least twelve subunits including: Cdc16p, Cdc23p, Cdc26p, Cdc27p, Apclp, the cullin-like protein Apc2p, the RING-H2 finger protein Apc11, Apc4, and Apc5 (Lamb et al., 1994; Zachariae et al., 1996; Kramer et al., 1998; Yu et al., 1998; Levenson et al., 2000). Yeast-two hybrid analysis and co-immunoprecipitation experiments identified Cdc16p, Cdc23p, and Cdc27p as core subunits of the APC. These proteins interact via multiple copies of a 34 residue repeat motif called the tetratricopeptide repeat (TPR), and disruptive mutations in the TPR of Cdc27p reduce or abolish
the association with Cdc23p and Cdc16p (Lamb et al., 1994). Temperature sensitive cdc23 (Irniger et al., 1995), apc1 (Zachariae et al., 1996), and apc11 mutants (Leverson et al., 2000) were defective in entering and exiting anaphase. This phenotype is due to the inability of these APC mutants to target for degradation inhibitors of anaphase and mitotic cyclins. CDC23, CDC16, CDC26, and APC1 were required for proteolysis of the B-type cyclin CLB2, as extracts of these mutants were defective in the ubiquitylation of mitotic cyclins (Irniger et al., 1995; Zachariae et al., 1996).

The RING-H2 finger of Apc11p was required to recruit the ubiquitin-conjugating enzyme Ubc4p, as mutations in the zinc chelating cysteinyl residues of Apc11p decreased or abolished this interaction. Apc11p co-immunoprecipitated with other components of the APC, namely Cdc16p and Cdc27p, and the RING finger of Apc11p was dispensable for these interactions. In vitro, Apc11p catalysed the multiubiquitylation of Clb2p in a Ubc4p- and RING finger-dependent manner (Leverson et al., 2000).

The conserved WD40-repeat proteins Cdc20p and Cdh1p were identified as substrate specific activators of the APC. Pds1, an inhibitor of the metaphase-anaphase transition and an APC substrate (Cohen-Fix et al., 1996), was stabilised in cdc20-1 mutants. Overexpression of CDC20 increased degradation of Pds1 in an APC-dependent manner. Similarly, Cdh1p directed the specificity of the APC towards the substrates Clb2p and Ase1p (Juang et al., 1997) and overexpression of CDH1 induced degradation of Clb2p and Ase1p (Visintin et al., 1997). Figure 5 presents structural models of the SCF, VCB, and APC ubiquitin-protein ligases.

6. The 26S Proteasome

The 26S proteasome is a 1700 kDa multisubunit ATP-dependent protease found in the nucleus and cytoplasm of eukaryotes that degrades multiubiquitylated proteins. The 26S proteasome resembles a cylinder consisting of a core 20S particle and two 19S regulatory caps at either end. The 20S particle is the proteolytically active core and is composed of four rings each containing either seven α or seven β subunits. The trypsin-like, chymotrypsin-like and peptidyl-glutamyl-peptide hydrolysing activities of the 20S proteasome are sequestered inside a channel that runs the length of the 26S proteasome. The 19S regulatory caps contain
Figure 5: Compositional Models of the Multisubunit Ubiquitin-Protein Ligases SCF, VCB, and APC

All three ubiquitin-protein ligase complexes recruit and perhaps activate ubiquitin-conjugating enzymes via a RING finger protein, Rbx1 (in the cases of the SCF and the VCB) or Apc11 (in the case of the APC). The E3 complexes recruit adapter proteins (such as F-box proteins, Cdc20, Cdh1, or VHL) which bind substrates via protein-protein interactions with WD40 or leucine rich repeats. Ubiquitin (Ub) is transferred from the ubiquitin-conjugating enzyme to the tethered substrate. The many subunits of the APC are represented by stippled ovals. This figure was adapted from Tyers and Willems, 1999.
multiubiquitin chain binding sites to tether a ubiquitylated substrate as well as several ATPases to unfold proteins targeted for degradation. Upon unfolding, the protein is fed into the 20S core where it is cleaved into small peptides. The ubiquitin chain is cleaved from the protein by a deubiquitylating enzyme and free ubiquitin is released by isopeptidase T (reviewed in Jentsch and Schlenker, 1995). A schematic representation of the 26S proteasome is presented in Figure 1.

Several reports have shown that ubiquitin-protein ligases associate with components of the 19S cap of the proteasome. GST pulldown assays and in vivo co-immunoprecipitation experiments showed that the N-end Rule E3 Ubr1p interacts with proteasomal subunits Rpn2, Rpt1, and Rpt6. The hect ubiquitin-protein ligase Ufd4p interacts with Rpt6 and Rpn1 (Xie and Varshavsky, 2000). Another hect E3, KIAA10 interacts with Rpn1, possibly delivering a substrate directly to the proteasome for degradation (You and Pickart, 2001). The U box E3 CHIP interacts with the S5a and HC8 subunits of the proteasome (Connell et al., 2001). The association between proteasomal subunits and ubiquitin-protein ligases may facilitate delivery of multiubiquitylated substrates to the proteasome for degradation.

7. Deubiquitylating Enzymes

Deubiquitylating enzymes (DUBs) are thiol proteases which hydrolyze ester, thiolester, and amide bonds to the carboxyl-terminal glycine 76 of ubiquitin. DUBs are required for the post-translational processing of polyubiquitin precursor proteins to produce free ubiquitin monomers and for trimming/editing multiubiquitin chains on target proteins. DUBs catalyze the removal and subsequent disassembly of multiubiquitin chains on a target protein. This activity serves to replenish pools of free ubiquitin for more conjugation reactions. The two families of deubiquitylating enzymes are the ubiquitin carboxyl-terminal hydrolases (UCH) and the ubiquitin-specific processing proteases (UBP) (reviewed in Wilkinson, 2000).

The ubiquitin carboxyl-terminal hydrolases process ubiquitin-fusion proteins and remove ubiquitin from small peptides. UCH family members contain a conserved 210 residue catalytic domain encompassing conserved sequence motifs called the Cys and His boxes. The UCH BAP1 binds to the RING finger domain of the tumour suppressor breast cancer susceptibility protein BRCA1 and promotes BRCA1-mediated inhibition of cell growth (Jensen et al., 1998).
The ubiquitin-specific processing proteases remove ubiquitin from large proteins or from the remnants of degraded proteins and disassemble multiubiquitin chains. UBP family members contain a conserved 450 residue catalytic core domain composed of Cys and His boxes. *S. cerevisiae* Ubp14p is required for the disassembly of unanchored multiubiquitin chains as deletion of *UBP14* results in the accumulation of unanchored multiubiquitin chains and a defect in ubiquitin-dependent protein degradation. *H. sapiens* isopeptidase T (IsoT) can rescue the defects of *ubp14* yeast, suggesting that Ubp14p and human IsoT are functional homologues (Amerik et al., 1997).

Deubiquitylating enzymes have important roles in cell fate determination and embryogenesis. The *D. melanogaster* fat facets (*faf*) gene is a ubiquitin-specific processing protease required for normal eye development, as mutants have ectopic photoreceptors in each eye unit (facet) and eggs from *faf* females do not undergo embryogenesis (Huang et al., 1995). Deubiquitylation by UBPs can stabilise a protein targeted for degradation. The herpesvirus-associated ubiquitin-specific protease (HAUSP) binds to the tumour suppressor protein p53 and stabilises it via direct deubiquitylation. Interestingly, expression of a catalytically inactive point mutant of HAUSP increases the levels of p53 ubiquitylation and destabilises p53. This suggests that HAUSP might function as a tumour suppressor through its ability to rescue p53 from ubiquitin-dependent proteolysis (Li et al., 2002).

Deubiquitylation activities are associated with the proteasome and are necessary for efficient degradation of multiubiquitylated substrates. *S. cerevisiae* Rpn11, a subunit of the 19S proteasome lid, is a metalloisopeptidase that is required to deubiquitylate substrates bound to the proteasome. Mutation of the predicted active-site histidines to alanine was lethal and stabilised ubiquitin pathway substrates (Verma et al., 2002).

8. Signals for Degradation

Given that specificity of ubiquitylation rests upon recognition of a target protein, what are the degradation signals in a target protein? The answer to this interesting question is largely unknown, although a few degradation or ubiquitylation signals have been identified. The APC ubiquitin-protein ligase recognises a destruction box sequence (RXALGXIXN) in substrates, including the mitotic cyclins (Clb2p and Clb3p) and Pds1p (an anaphase inhibitor). Pds1p and
Clb2p derivatives lacking destruction boxes are stable and resistant to APC-dependent ubiquitylation (Cohen-Fix et al., 1996; Irniger et al., 1995).

Phosphorylation of seryl, threonyl, or tyrosyl residues in a protein targeted for proteolysis may be a signal for ubiquitin-mediated degradation. Substrates of the SCF such as the G1 cyclins (Cln1, Cln2), the cyclin-dependent kinase inhibitor Sic1, IkBα, and β-catenin must be phosphorylated in order to be recognised by their respective F-box adaptors (Skowyra et al., 1997; Feldman et al., 1997; Winston et al., 1999; Orford et al., 1997; Conaway et al., 2002). Activated epidermal growth factor receptor (EGFR) autophosphorylates on tyrosyl residues and this phosphorylation is required for recognition and binding by the E3, c-Cbl (Joazeiro et al., 1999). Finally, substrates of the N-end Rule Pathway are recognised by their destabilising amino-terminal residue (Bachmair et al., 1986; Bachmair and Varshavsky, 1989).

9. DYSREGULATION OF UBIQUITIN-DEPENDENT PROTEOLYSIS AND DISEASE

9.1. Cancer

The rapid destruction of tumour suppressors is one mechanism by which oncogenic viruses can cause cellular transformation. The HPV E6 protein is a classic example of how a viral protein (E6) can form a complex with a cellular ubiquitin-protein ligase, E6-AP, and target the tumour suppressor protein p53 for rapid proteolysis (Scheffner et al., 1993). Other oncogenic viruses encode ubiquitin-protein ligases that act in a dominant negative fashion and fail to degrade cellular substrates such as activated receptors. The oncogenic CAS NS-1 retrovirus encodes mutant forms of c-cbl, a cellular E3 that binds to activated epidermal growth factor receptor (EGFR) and targets it for proteolysis. These oncogenic v-cbl and 70Z-cbl associate with pre-B cell lymphoma fail to ubiquitylate activated EGFR because both mutant c-cbls lack RING finger domains essential for recruiting E2s to target EGFR for degradation. Thus, the cell proliferating signal is not stopped/modulated and the result is cellular transformation (Joazeiro et al., 1999; Waterman et al., 1999).

Colorectal cancer has been linked to mutations in several genes of the Wnt signalling pathway that result in inappropriate activation of colonic epithelial cell proliferation (reviewed in Conaway et al., 2002). In the absence of Wnt signalling, the glycogen synthase kinase-3β (GSK3β) phosphorylates β-catenin, an oncogenic transcription factor. Phosphorylated β-
catenin is recognised by an F-box protein called β-TrCP (β-transducin repeat-containing protein) in complex with a Skp1/cullin-1/F-box protein (SCF) E3. SCFβTrCP ubiquitylates phosphorylated β-catenin, marking it for rapid proteolysis (Winston et al., 1999). The destruction of β-catenin prevents it from entering the nucleus and activating transcription of cell proliferation genes. Mutations that prevent phosphorylation of β-catenin stabilise it by preventing the recognition and ubiquitylation of β-catenin by SCFβTrCP (Aberle et al., 1997; Orford et al., 1997). The stabilised oncogenic β-catenin can enter the nucleus and activate gene expression for cell proliferation.

9.2. Liddle’s Syndrome

The epithelial Na⁺ channel (ENaC) regulates sodium absorption in the kidney. Liddle’s Syndrome is an autosomal dominant form of hypertension, in which mutations in the C-terminus of the β or γ subunits of ENaC result in increased renal sodium absorption. These mutations delete the PY motif in the C-terminus of ENaC and abolish the interaction with Nedd4, a hect domain ubiquitin-protein ligase (Goulet et al., 1998). Nedd4 ubiquitylates ENaC, perhaps targeting ENaC subunits for proteasomal degradation or fully assembled ENaC for internalization and subsequent lysosomal degradation (Staub et al., 1997; Abriel et al., 1999). Wild type Nedd4, but not a catalytically inactive Nedd4, significantly reduced the number of ENaC at the plasma membrane. Mutant ENaC derivatives, in which the PY motifs were deleted as in Liddle’s syndrome, were not down-regulated and were unresponsive to the presence of Nedd4 (Abriel et al., 1999). Thus, the Nedd4-dependent down-regulation of ENaC contributes to the regulation of renal sodium absorption by decreasing the number of ENaC at the plasma membrane. The hypertensive phenotype associated with Liddle’s Syndrome is due to the inability of mutant ENaC to interact with and be targeted for proteolysis by Nedd4.

9.3. Parkinson’s Disease

Parkinson’s Disease is a neurodegenerative disorder associated with the progressive loss of dopamine-containing neurons and the accumulation of Lewy bodies, cytoplasmic accumulations of proteins that stain for ubiquitin. Symptoms of Parkinson’s Disease include rigidity, slow movement, tremors, and loss of balance. Several mutations in the components of
the ubiquitin-proteasome system have been identified in various forms of familial Parkinson’s Disease. Zhang et al. (2000) showed that the RING finger protein Parkin was a ubiquitin-protein ligase that targeted a synaptic vesicle-associated protein, CDCrel-1, for degradation. Parkin underwent autoubiquitylation and this decreased the in vivo half-life of Parkin. Interestingly, mutations in the Parkin gene responsible for autosomal recessive Parkinson’s Disease effectively destroyed the RING finger of Parkin. These Parkin mutants failed to associate with UbcH8, undergo autoubiquitylation, or promote ubiquitylation and degradation of the substrate CDCrel-1. The aetiology of autosomal recessive Parkinson’s Disease may be due to the failure of Parkin mutants to degrade CDCrel-1. The resulting overexpressed CDCrel-1 inhibits secretion and may inhibit dopamine release, leading to a Parkinson’s phenotype.

In co-immunoprecipitation studies, Parkin associated with UbcH7 and a glycosylated form of α-synuclein called αSp22 in human brain. In vitro, wild type Parkin ubiquitylated αSp22, whereas Parkin with a mutation in the RING finger (a mutation associated with autosomal recessive Parkinson’s Disease) could neither bind to UbcH7, nor multiubiquitylate αSp22. Also, αSp22 accumulated in Parkin-deficient autosomal recessive Parkinson’s Disease brains. One hypothesis is that the loss of E3 activity of Parkin mutants in autosomal recessive Parkinson’s Disease results in the accumulation of αSp22 and this may lead to accelerated neuronal loss and early onset of disease (Shimura et al., 2001).

9.4. Angelman Syndrome

Angelman Syndrome is an inherited disease characterised by moderate to severe mental retardation, seizures, abnormal gait, absence of speech, and inappropriate laughter. The deleted chromosomal segment that gives rise to Angelman Syndrome is always maternal in origin and the paternal allele cannot rescue the mutation as it is silenced through imprinting. The gene mutated in Angelman Syndrome is UBE3A, which encodes the hect ubiquitin-protein ligase E6-AP (Matsuura et al., 1997). Mice bearing a null mutation of Ube3a (maternal deficient heterozygotes) have motor dysfunction, seizures, and decreased context-dependent learning - phenotypes in keeping with human Angelman Syndrome. Strikingly, maternal deficient ube3a mice have increased amounts of p53 in hippocampal neurons compared to either wild type or paternal deficient mice. That ube3a maternal deficient mice had increased levels of p53 suggests
that E6-AP has a role in degrading p53 in the absence of viral E6 (Jiang et al., 1998). Whether the increased p53 levels in E6-AP mutants causes Angelman Syndrome is unknown. Indeed, there may be several E6-AP targets in hippocampal neurons and perhaps the inability of ube3a mutants to target these unknown substrates for proteolysis may contribute to an Angelman Syndrome phenotype.

10. CAENORHABDITIS ELEGANS

10.1. Caenorhabditis elegans as a Model System

The free living soil nematode C. elegans is a model system for studying biological processes in higher organisms. C. elegans has a short life cycle (3 1/2 days at 20°C) and maintaining thousands of animals in liquid culture or on agar plates seeded with bacteria is straightforward. C. elegans embryos develop through four larval stages (L1 to L4) to become self-fertilising adult hermaphrodites. Males, arising due to non-disjunction of the X chromosome (1/500 animals), can be crossed with hermaphrodites (reviewed in Riddle et al., 1997).

The transparency of C. elegans facilitates immunohistochemical and cell biology studies of the animal’s sensory, muscular, digestive, reproductive, and neuronal systems. The completion of the C. elegans sequencing project (C. elegans Sequencing Consortium, 1998) and the mapping of the complete cell lineage (Sulston and Horvitz, 1977; Sulston et al., 1983) have provided researchers with powerful tools for genetic and developmental studies. The International C. elegans Gene Knockout Consortium will use reverse genetics to produce null alleles of all 19,000 predicted genes within the C. elegans genome. This will provide investigators studying genes involved in development or human disease with a biological system in which to study their gene of interest.

10.2. Vulval Development in Caenorhabditis elegans

The hermaphrodite gonad consists of two U-shaped tubular arms that extend ventrally to the anterior and posterior of the animal. Germ cells in the gonad undergo meiosis to produce oocytes and spermatocytes. The vulva is a passageway that connects the gonad to the external environment and is required for egg laying and copulation. Fertilised eggs and embryos held in the uterus are expelled through the vulva by contractions of the uterine and vulval (sex) muscles.
The development of the hermaphrodite vulva begins in the L1 stage when six vulval precursor cells (P3.p to P8.p) are born. In the L3 stage, signalling from the anchor cell induces P5.p, P6.p, and P7.p to adopt vulval fates while inhibitory signalling causes P3.p, P4.p, and P8.p to adopt the non-vulval fate. Once the vulval precursor cells become specified, P5.p, P6.p, and P7.p undergo several cell divisions to produce twenty-two cells. In the L4 stage, the twenty-two vulval cells migrate and invaginate to connect the uterus to the developing vulva. The vulval cells fuse with each other to form a stack of seven rings. At the L4 molt, this invagination everts slightly to form the mature vulva (Greenwald, 1997).

11. The Present Study

While studies in S. cerevisiae, humans, and mice implicate RAD6 in gene silencing and chromatin remodelling, DNA repair, and spermatogenesis, the cellular roles and protein targets of UBC-1, the Rad6p homologue in C. elegans, are unknown. Herein, we use C. elegans to study Rad6p in the context of a multicellular organism. Specifically, we were interested in identifying ubiquitin-protein ligases that interact with UBC-1 to target substrates for ubiquitylation and degradation. In an effort to elucidate the biochemical functions of UBC-1 in C. elegans, a yeast-two hybrid screen was performed to identify the protein partners of UBC-1. Upon confirmation of the interaction between UBC-1 and its partner RFP-1, RNA interference and immunohistochemical staining experiments were employed to learn more about this novel interaction.
MATERIALS AND METHODS

1. GENERAL MOLECULAR BIOLOGY TECHNIQUES

1.1. Bacterial Transformations

Purified plasmids or ligations were transformed into competent DH5α bacteria (GIBCO/BRL) according to the manufacturer’s instructions. BL21 (DE3) bacteria were made competent by CaCl₂ treatment and transformed as described (Sambrook et al., 1989). HB101 bacteria were made electrocompetent as described (Ausubel et al., 1998) and used to amplify pACT clones rescued from Y190. A BioRad Electroporator was used according to the manufacturer’s instructions to electroporate 50 µl of HB101 cells with 1 µl of pACT in a 0.1 cm BioRad electroporation cuvette at 1.8 Volts, 200 Ω, and a capacitance of 25 µF.

1.2. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed in 50 µl aliquots containing 50 ng of template, 0.2 mM of each deoxyribonucleoside triphosphate, 0.5 mM MgCl₂, 50 pmol of each oligonucleotide primer, and 1 U of Vent DNA polymerase (New England BioLabs) in 1X PCR Buffer provided by the manufacturer. PCR of long templates (>1000 nucleotides) was performed in 100 µl aliquots using 5 U of Pfu DNA polymerase (Stratagene). PCR reactions were overlaid with mineral oil and pre-incubated at 94°C for 10 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds; and finally one cycle at 72°C for 10 minutes. Deviations from this profile were typically in the annealing temperature (50°C or 55°C) or the duration of the cycles.

1.3. Plasmid DNA Purification from E. coli

Plasmid DNA was purified from saturated overnight E. coli cultures by a modified alkaline lysis method (Birnboim and Doly, 1979). Plasmid DNA for sequencing, yeast two-hybrid screens, or for transient transfection of insect cells was purified with Qiagen’s Mini or MaxiPrep Kit according to the manufacturer’s instructions.
1.4. Double Stranded DNA Sequence Analysis

The nucleotide sequences of double stranded DNA templates were determined by the University of British Columbia Nucleic Acids and Protein Service (NAPS) using the dideoxy chain termination method (Sanger et al., 1977).

1.5. Amplification of λ-ACT RB2 Phage Library

The cDNA library used in the yeast two-hybrid screen was a kind gift from R. Barstead at the Washington University School of Medicine. The C. elegans cDNA library, named λ-ACT RB2, consisted of randomly primed cDNAs (>600 bp) cloned into the Xho I site of the λ phage vector λ.ACT. This phage library would be used to produce plasmids encoding C. elegans cDNAs fused to GAL4 activation domain sequences for yeast two-hybrid screens (Elledge et al., 1991; Durfee et al., 1993). To amplify the λ-ACT RB2 phage library, a 0.5 ml saturated culture of RB3E bacteria in lambda broth (1% tryptone, 0.25% NaCl) containing 10 mM MgSO\textsubscript{4} and 0.2% maltose was incubated with 1 x 10\textsuperscript{7} plaque forming units (pfu) of λ-ACT RB2 for 15 minutes at 37°C. Next, 6.5 mL of molten (47°C) LB top agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.7% agar) was added to each tube and then spread on 150 mm LB plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.001 N NaOH, 1.5% agar) pre-warmed at 37°C. The plates were incubated at 37°C for 6 to 8 hours. A few drops of chloroform were added to the plates, followed by a 5 minute incubation. Next, 15 ml of lambda broth was added to the plates, and the plates were then rocked overnight at 4°C. The phage were pooled, extracted with chloroform, centrifuged, and dimethylsulfoxide (DMSO) was added to 7% to the supernatant. The λ-ACT RB2 phage was stored at -70°C.

1.6. Titering Amplified λ-ACT RB2 Phage by Serial Dilution

To determine the number of plaque forming units (pfu) or titre of amplified λ-ACT RB2 phage, 0.1 ml of several serial dilutions of λ-ACT RB2 in Suspension Media SM (50 mM Tris pH 7.5, 100 mM NaCl, 8 mM MgSO\textsubscript{4} • 7H\textsubscript{2}O, 0.01% Difco gelatine) were added to each 0.3 ml saturated culture of RB3E bacteria in lambda broth supplemented with 0.2% maltose and 10 mM MgSO\textsubscript{4}. The tubes were incubated at 37°C for 10 minutes, and 2.5 ml of molten (47°C) lambda top agar (1% tryptone, 0.25% NaCl, 0.7% agar) was added to each tube, mixed and poured onto
lambda plates (1% tryptone, 0.25% NaCl, 1% agar) pre-warmed to 37°C. The plates were incubated at 37°C for 8 to 12 hours and the number of plaques was scored. The titer of the amplified λ-ACT RB2 phage was 1.8 x 10^10 pfu/ml.

1.7. Conversion of a Randomly Primed *C. elegans* cDNA Library in λ-ACT RB2 Phage into a Plasmid Library in pACT

To convert the phage library into a plasmid library containing GAL4 activation domain sequences fused to randomly primed *C. elegans* cDNAs cloned into the *Xho* I site of plasmid pACT, ten tubes each containing 2 ml of logarithmically growing (3 x 10^8 cells/ml) RB4E bacteria with 10 mM MgCl2 were each incubated with 10^8 pfu of λ-ACT RB2 phage at 28°C for 30 minutes. Each tube received 2 ml of Luria Broth (LB: 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.001 N NaOH) and they were shaken at 30°C for 1 hour. The infected cells in each of the ten tubes were concentrated to 200 µl and spread on each of 10 fresh, 150 mm LB/ 50 ug/ml ampicillin/ 0.2% glucose plates and incubated at 37°C overnight. The ampicillin resistant colonies on each plate were resuspended in 10 ml of LB and pooled. The pooled bacteria were used to inoculate 3 litres of Terrific Broth (TB: 1.2% Bacto tryptone, 2.4% Bacto yeast extract, 0.4% glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄) containing 50 µg/ml ampicillin. The culture was grown overnight at 37°C and the library plasmids were purified. The resulting pACT plasmids excised from λACT contain the CoE1 origin of replication and *bla* gene for replication and selection in *E. coli*, and *LEU2*, 2µ origin, and the *ADC1* (Adh1) promoter sequences for selection, replication, and expression in *S. cerevisiae*. The *ADC1* promoter drives expression of the GAL4 activation domain (amino acids 768-881) fused to a randomly primed *C. elegans* cDNA (Elledge *et al.*, 1991; Durfee *et al.*, 1993).

1.8. Oligonucleotide Primers Used in This Study

The sequences and important restriction enzyme sites of the oligonucleotide primers (Gibco BRL) used in PCR or for sequencing double stranded DNA are shown in Table 3.
Table 3: Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA Sequence (5' → 3') *</th>
<th>Direction</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 1</td>
<td>ggaattccatATGACGACGCCCAGCCGTAGACG</td>
<td>Forward</td>
<td>Nde I</td>
</tr>
<tr>
<td>EC 2</td>
<td>aaggatccCAGCATTCGATCCACTGGCTCC</td>
<td>Reverse</td>
<td>BamHI</td>
</tr>
<tr>
<td>SEQEC 1</td>
<td>tcgatgatgaagatcccc</td>
<td>Forward</td>
<td>none</td>
</tr>
<tr>
<td>SEQEC 2</td>
<td>aggcaaaacagatgtataa</td>
<td>Reverse</td>
<td>none</td>
</tr>
<tr>
<td>R05D3.4 R</td>
<td>gtgagcccgatcgggc</td>
<td>Reverse</td>
<td>none</td>
</tr>
<tr>
<td>EC 11</td>
<td>cccttcacatttgtgATGATGAAAGAAGTAATGAAGGG</td>
<td>Forward</td>
<td>Nco I</td>
</tr>
<tr>
<td>EC 13</td>
<td>gctgccatgtgtATGTCTGAAGAGACATCA</td>
<td>Forward</td>
<td>Nco I</td>
</tr>
<tr>
<td>EC 14</td>
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<td>BamHI</td>
</tr>
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<td>Reverse</td>
<td>BamHI</td>
</tr>
<tr>
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<td>Reverse</td>
<td>EcoRI</td>
</tr>
<tr>
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<td>Xho I</td>
</tr>
<tr>
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</tr>
<tr>
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<td>BamHI</td>
</tr>
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<td>Nde I</td>
</tr>
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<td>Forward</td>
<td>none</td>
</tr>
<tr>
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<td>Reverse</td>
<td>none</td>
</tr>
<tr>
<td>UBC-1 F</td>
<td>aaaaatcgtcttaatcccc</td>
<td>Forward</td>
<td>none</td>
</tr>
<tr>
<td>UBC-1 R</td>
<td>gaaaaatcctgatgtgatcggatatcgc</td>
<td>Reverse</td>
<td>none</td>
</tr>
</tbody>
</table>

* Engineered restriction sites are underlined and capitalised bases correspond to coding regions of a particular cDNA.
2. CONSTRUCTION OF PLASMID CLONES USED IN THIS STUDY

2.1. UBC-1 Constructs in pAS1-CYH2

PCR was performed on plasmid pR1.7a (containing the *ubc-1* coding sequence in pBSIISK) with oligonucleotides EC 1 and EC 2. The *ubc-1* PCR product (600 bp) was subcloned into the *Nde* I (5') and *BamH* I (3') sites of pRSETA. This clone was digested with *Acc* I and *Bsm* I and the *ubc-1* PCR product was replaced with a *ubc-1* cDNA obtained from pR1.7a. The *ubc-1* cDNA obtained from pR1.7a had a correct DNA sequence. The *ubc-1* cDNA was cut out of pRSETA and cloned into the *Nde* I and *BamH* I sites of pAS1-CYH2. The resulting bait plasmid, named pAS1-CYH2::UBC-1, contained TRP1 and the 2μ origin for selection and replication in yeast, and the *ADC1* promoter for expression of the GAL4 DNA binding domain (amino acids 1-147) fused to the *ubc-1* cDNA.

A GAL4 DNA binding domain::UBC-1 Δ152 construct was created by performing PCR on pR1.7a with *Vent* DNA polymerase (New England Biolabs) and oligonucleotide primers EC 1 and EC 20. The *ubc-1Δ-152* PCR product (456 bp) was cloned into the *Nde* I (5') and *BamH* I (3') sites of pAS1-CYH2 and sequenced.

2.2. RFP-1 in pBSIIKS

Plasmid pACT 38 (which contained a full length RFP-1 cDNA lacking only the first two nucleotides of the open reading frame and containing approximately 100 base pairs of 3' untranslated sequence in pACT) was restriction digested with *EcoR* I and *Bgl* II and the fragments were separated by electrophoresis. The *EcoR* I/*Bgl* II fragment (1188bp) containing the 3' half of *rfp-1* was purified and ligated into the *EcoR* I and *BamH* I sites of pBSIIKS (Stratagene). This construct was transformed into DH5α, amplified, purified, and restriction digested with *EcoR* I. Plasmid pACT 38 was cut with *EcoR* I and the 1318 bp band containing the 5' half of *rfp-1* was ligated into the *EcoR* I site of pBSIIKS containing the 3' half of *rfp-1*. The construct was cut with *BamH* I and *Not* I in order to remove some extra pACT vector sequence upstream of *rfp-1* and cloned into pBSIIKS. The resulting full length *rfp-1* cDNA cloned into pBSIIKS, called pBSIIKS::RFP-1, was restriction digested with *Sea* I and *Sal* I to confirm the correct orientation of *rfp-1*.
2.3. GAL4 Activation Domain::RFP-1 Truncation Constructs in pACT2

The SMART program (Schultz et al., 1998; Letunic et al., 2002) was employed to identify domains in RFP-1 to facilitate the construction of four RFP-1 truncation proteins. The N-terminus of RFP-1 (amino acids 1 to 183) was produced by performing PCR on rfp-l in pACT (clone pACT 38) using oligonucleotide primers EC 11 and EC 23 and Pfu DNA polymerase. The rfp-l PCR product (573 bp) was cloned into the Nco I (5') and EcoR I (3') sites of pACT2. This RFP-1 (1-183) derivative included the first α-helical region of RFP-1 and terminated before the start of the second α-helical region.

PCR was performed on pACT 38 with Pfu DNA polymerase and oligonucleotide primers EC 25 and EC 24. The rfp-l PCR product (414 bp) was cloned into the Nco I (5') and BamH I (3') sites of pACT2. This RFP-1 (184-313) derivative contained only the second α-helical region and terminated before the start of the basic leucine zipper in RFP-1.

PCR was performed on pACT 38 with oligonucleotide primers EC 11 and EC 24 and the rfp-l PCR product (963 bp) was cloned into the Nco I (5') and BamH I (3') sites of pACT2. This RFP-1 truncation (1-313) included the first and second α-helical regions of RFP-1 and terminated before the beginning of the basic leucine zipper.

PCR with oligonucleotide primers EC 13 and EC 14 was performed on pACT 38 and the rfp-l PCR product (1322 bp) was cloned into the Nco I (5') and BamH I (3') sites of pACT2. This RFP-1 truncation (408-839) included the fifth and sixth α-helical regions and the C-terminal C3HC4 RING finger domain. The four constructs were sequenced with SEQEC 1 and SEQEC 2 primers.

2.4. Plasmid Construction for RFP-1 Expression

To produce the N-terminal region of RFP-1 with a C-terminal histidine tag (6x His tag), RFP-1 (1-313) in pACT2 was digested with Nco I and BamH I and the 950 bp fragment was cloned into pET28a (Novagen). This construct was then digested with Sac I and Xho I and the pET28a vector band (5508 bp) containing some 5' rfp-l sequence was separated by electrophoresis and purified. Plasmid pBSIJKS::RFP-1 was digested with Sac I and Xho I and the rfp-l restriction fragment (845 bp) was ligated into the pET28a vector containing the 5' end of rfp-l. The resulting construct was named aa 1-329 RFP-1::pET28a.
To produce the C-terminus of RFP-1 with a C-terminal histidine tag (6x His tag), RFP-1 (408-839) in pACT2 was digested with Nco I and Xho I and the 1106 bp fragment was cloned into pET28a. The construct was named aa 408-775 RFP-1::pET28a.

2.5. RNA Interference Constructs

To produce a ubc-1 template for bi-directional transcription, pR1.7 a was digested with Xba I and Hind III and the ubc-1 cDNA (600 bp) was cloned into pPD129.36 (Timmons et al., 2001). The resulting clone was called pPD129.36::UBC-1. To produce rfp-1 double stranded RNA, pACT 38 was digested with Xho I to yield nt 3-982 of the rfp-1 cDNA. This fragment was cloned into the Xho I site of pPD129.36 to yield plasmid pPD129.36::RFP-1 nt 3-982.

2.6. LIN-35 and LIN-53 Constructs

The lin-35 and lin-53 cDNAs were kind gifts of R. Horvitz and C. Ceol (Massachusetts Institute of Technology, Boston, Massachusetts, U. S. A.). PCR was performed on the lin-35 cDNA in pCITE-4a with Pfu DNA polymerase and oligonucleotide primers EC 38 and EC 33. The lin-35 PCR product (1500 bp) was cloned into the Nde I (5') and Sal I (3') sites of pAS2. The resulting construct, named pAS2::LIN-35 (aa 467-961), was sequenced with pAS2 F and pAS2 R sequencing primers.

PCR with Pfu DNA polymerase and oligonucleotide primers EC 36 and EC 37 was performed on the lin-53 cDNA in pGEX 4T-3. The lin-53 PCR product (1270 bp) was cloned into the Nco I (5') and BamH I (3') sites of pACT2. The resulting construct, pACT2::LIN-53, was sequenced with SEQEC1 and SEQEC2 primers.

3. CAENORHABDITIS ELEGANS TECHNIQUES

3.1. Maintenance of Caenorhabditis elegans

C. elegans N2, lin-15 A (n767) and lin-15 B (n744) strains were maintained at 15°C or 20°C on NG plates (0.3% NaCl, 0.25% tryptone, 5 mg/ml cholesterol, 1 mM MgSO4, 25 mM KH2PO4 pH 6.0, 1.7% agarose) spread with OP50 E. coli (Brenner, 1974). The lin-15 A (n767) and lin-15 B (n744) strains were kind gifts of R. Horvitz and C. Ceol (Massachusetts Institute of Technology, Boston, Massachusetts, U. S. A.).
Synchronous *C. elegans* populations were produced by inoculating liquid cultures (Sulston and Brenner, 1974) with 1x10^6 embryos/L at 15°C and allowing them to arrest in the L1 stage prior to the addition of *E. coli* food. Embryos were prepared by dissolving gravid adults in alkaline sodium hypochlorite (Emmons *et al.*, 1979). Long term storage of *C. elegans* strains involved washing worms off overgrown plates (containing a high percentage of L1 and L2 larvae) with M9 buffer and storing the worms in M9 buffer with 15% glycerol at -80°C overnight. The tubes were transferred to liquid nitrogen storage the next day.

### 3.2. Production of a *C. elegans* ubc-1 Deletion Mutant: ubc-1(gk14)

The *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, a member of the International *C. elegans* Gene Knockout Consortium, used formaldehyde to create a putative deletion mutation in *ubc-1* (C35B1.1) called *ubc-1*(gk14). Protein extracts of wild type and *ubc-1*(gk14) animals were Western blotted for the presence of UBC-1 with a monoclonal UBC-1 antibody.

### 3.3. PCR of Wild type and *ubc-1* (gk14) Animals

N2 (wild type) or *ubc-1* (gk14) animals were rinsed off a small plate with 1 ml of M9 buffer. The animals were collected by centrifugation (400 x g, 30 seconds), resuspended in 200 μl of Worm PCR Lysis Buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl₂, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatine) containing 200 μg/ml Proteinase K, and incubated overnight at 65°C. Proteinase K was inactivated by incubation at 95°C for 30 minutes and 10 μl from the N2 or *ubc-1* (gk14) samples were used as template DNA for PCR with 10 pmoles of primers UBC-1 F and UBC-1 R on Ready-to-Go-PCR Beads (Amersham Pharmacia). As negative controls, the PCR reactions were performed lacking either primer or lacking DNA template. The PCR profile was 1 cycle at 94°C for 30 seconds, followed by 35 cycles at 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1.5 minutes, and finally 1 cycle at 72°C for 10 minutes. The PCR reactions (20 μl) were separated by electrophoresis on 1.2% agarose gels.
3.4. Preparation of *C. elegans* Protein Extracts

Synchronous populations of N2 *C. elegans* animals were established at 15°C in liquid culture. Five developmental stages (embryos, L1 stage, L2 stage, L3/L4 stage, and young adults) were individually collected by centrifugation (100 x g, 2 minutes), rinsed twice with M9 buffer, and the worms were resuspended in approximately 2 ml of Worm Lysis Buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT, 1 protease inhibitor tablet per 10 ml buffer). Dounce homogenisation (100 to 200 strokes) was used to produce protein extracts of each of the developmental stages. The protein extracts were clarified by centrifugation (10,000 x g, 10 minutes, 4°C) and their concentrations were determined by Bradford Assays (BioRad).

3.5. Western Blot Analysis of *C. elegans* Extracts

The protein extracts of the five developmental stages were each standardised to 1 mg/ml in 1X LSB and 30 µg of total protein for each developmental stage was loaded onto two 12.5% SDS polyacrylamide gels, separated by SDS PAGE, and electroblotted to two PVDF membranes. One membrane was probed for the presence of RFP-1 (97.6 kDa) with polyclonal RFP-1 antibodies generated against the C-terminus of RFP-1 (Rabbit 1). As a control for protein loading, the second PVDF membrane containing immobilised protein extracts from the five developmental stages was probed with a monoclonal antibody against actin.

4. YEAST TECHNIQUES AND STRAINS

4.1. *Saccharomyces cerevisiae* Strains

Yeast strain Y190 (MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, -112 + URA3::GAL→lacZ, LYS2::GAL→HIS3 cyh') was used for the library transformation in the yeast two-hybrid screen. Yeast strain Y187 (MATα gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, -112 GAL→lacZ) was employed for false positive testing of clones recovered in the yeast two-hybrid screen. General yeast protocols were as described (Guthrie and Fink, 1991).

4.2. Yeast Transformation

A transformation mix consisting of 400 µl of TLP (10 mM Tris pH 7.5, 1 mM EDTA, 100 mM lithium acetate, 44% PEG 4000), 40 µl of DMSO, 5 µl sheared salmon sperm carrier
DNA and 5 μg of plasmid DNA was placed in a microcentrifuge tube. A large loopful (6 to 7 large colonies) of yeast (Y190) was added and the mixture was mixed and incubated at room temperature for 6 to 14 hours. The yeast were heat shocked (15 minutes, 42°C), plated on selective media, and incubated for 3 to 5 days at 30°C (Guthrie and Fink, 1991).

For high efficiency yeast transformation, a modified protocol of Gietz and Schiestl (1995) was employed. Yeast strain Y190 was grown to saturation in 20 ml of selective medium at 30°C. Then, 2.5 x 10^6 cells were used to inoculate 50 ml of YEPD medium (1% Bacto yeast extract, 2% Bacto tryptone, 2% glucose) and grown at 30°C until the concentration reached 2 x 10^7 cells/ml. The yeast were collected by centrifugation (3000 x g, 5 minutes), washed with distilled H_2O, resuspended in 1 ml of 100 mM lithium acetate, and collected in a microcentrifuge tube (10,000 x g for 15 seconds). The lithium acetate was removed and the cell pellet was resuspended in 500 μl of 100 mM lithium acetate. The cells were dispensed in 50 μl aliquots to sterile microcentrifuge tubes and collected by centrifugation (10,000 x g, 15 seconds). To each cell pellet, 240 μl of 50% polyethylene glycol, 36 μl of 1.0 M lithium acetate, 5 μl of sheared salmon sperm carrier DNA (10 mg/ml), 5 μg of purified pACT library, and distilled H_2O to 545 μl were added. The mixture was shaken, incubated at 30°C for 30 minutes, and heat shocked at 42°C for 25 minutes. The cells were collected by centrifugation (8000 x g, 15 seconds), resuspended in 1 ml of sterile distilled H_2O, and 10 μl was spread onto selection plates to determine transformation efficiency. Typical transformation efficiency was 2 x 10^4 colonies/ml.

4.3. Preparation of Yeast Protein Extracts

Two methods to make protein extracts from yeast were employed. In the first method, protein extracts of Y190 were prepared by growing a 10 ml yeast culture in the appropriate selective medium to O.D._{600}= 0.6-1.0. The cells were collected by centrifugation (300 x g, 10 minutes), washed with distilled water, and again collected by centrifugation (300 x g, 5 minutes). The cell pellet was resuspended in 100 μl SDS sample buffer (100 mM Tris pH 6.8, 4% SDS, 10% glycerol, 20% β-mercaptoethanol) and agitated with 425-500 μm and 150-212 μm glass beads (Sigma) three times (2 minutes each time) with cooling on ice in between each mixing. The sample was boiled for 5 minutes, then mixed for 10 seconds and clarified by centrifugation.
(10,000 x g, 10 minutes). Approximately 30 μg of protein was separated by SDS-polyacrylamide gel electrophoresis (Chang and Herskowitz, 1992).

In the second method, a 30 ml yeast culture was grown to O.D. 0.6-1.0 in the appropriate selective medium. Yeast cells were collected by centrifugation (4000 x g, 5 minutes), washed with distilled water, and again collected by centrifugation (4000 x g, 5 minutes). The yeast pellet was resuspended in 400 μl Yeast Lysis Buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 150 mM NaCl, 2 mM PMSF) and agitated with 425-500 μm and 150-212 μm glass beads (Sigma) for 20 minutes at 4°C. Next, 400 μl of 2X RIPA buffer (20 mM Tris pH 8.0, 200 mM NaCl, 2 mM EDTA, 2% Nonidet-P 40, 1% sodium deoxycholate, 0.2% SDS) was added and mixed (20 minutes, 4°C). The mixture was clarified by centrifugation (10,000 x g, 20 minutes, 4°C) and the protein supernatant was analysed by SDS PAGE.

4.4. X-gal Colony Filter Lift Assay

The X-gal Colony Filter Lift Assay detects β-galactosidase activity (lacZ reporter gene expression) of yeast colonies. Yeast colonies were lifted from a plate onto a High Bond-C 45 μm circular nitrocellulose filter (Amersham) and flash-frozen in liquid nitrogen to permeabilize the cells. The filter was placed cell side up in a Petri dish lined with 3 MM chromatography paper soaked with 0.30 ml/square inch of Z Buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 38 mM β-mercaptoethanol, pH 7.0) containing 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The Petri plate was incubated at 30°C for 4 hours to allow development of blue-coloured yeast colonies (Ausubel et al., 1998).

5. YEAST TWO-HYBRID SCREEN

5.1. A Yeast Two-Hybrid Screen for Proteins that Interact with UBC-1

A randomly primed C. elegans cDNA library was screened using ubc-1 as a bait in a yeast two-hybrid screen (Fields and Song, 1989). The yeast strains and vectors were kind gifts of S. Elledge (Baylor College of Medicine). The construction of the C. elegans cDNA library in plasmid pACT and the construction of the ubc-1 bait plasmid are described in Sections 1.5, 1.7, and 2.1. Yeast strain Y190 was transformed with pAS1-CYH2::UBC-1 and transformants were selected on - Trp Synthetic Complete (SC) plates (0.7% yeast nitrogen base without amino acids,
2% glucose, 0.074% -Trp dropout supplement powder, 2% agar). Expression of the GAL4 DNA binding domain: UBC-1 fusion protein in Y190 was confirmed by Western blotting yeast extracts with a monoclonal antibody against UBC-1. To confirm that the bait plasmid pAS1-CYH2::UBC-1 did not activate transcription of reporter genes, yeast transformants were tested for β-galactosidase activity (i.e. activation of the lacZ reporter gene) by the X-gal Colony Filter Lift Assay. Y190 transformed with pAS1-CYH2::UBC-1 was also plated on - His SC + 35 mM 3-amino-1, 2, 4 triazole (3-aminotriazole) to test for activation of the His3 reporter gene. The addition of 3-amino-1, 2, 4 triazole was required to prevent leaky expression of His3. These “auto-activation” tests were performed in parallel with a negative control consisting of Y190 transformed with pSE1112 (SNF4 fused to the DNA binding domain of GAL4 in pAS1). A positive control for activation of transcription of lacZ and His3 was Y190 transformed with pSE1112 and pSE1111 (SNF1 fused to the GAL4 activation domain in pAS1). SNF1 and SNF4 are known protein binding partners (Fields and Song, 1989).

Y190 transformed with pAS1-CYH2::UBC-1 was then transformed with a random primed C. elegans cDNA library in plasmid pACT using a high efficiency yeast transformation protocol. In order to screen the pACT library five fold for potential protein binding partners of UBC-1, a total of 2.1 x 10^6 colonies were screened on 150 mm - Trp - Leu - His SC + 35 mM 3-aminotriazole plates. Putative positive transformants were streaked for single colonies on - Trp - Leu - His SC + 35 mM 3-aminotriazole plates and tested for β-galactosidase activity. Putative positive transformants were grown overnight at 30°C in 10 ml of - Trp - Leu - His SC + 35 mM 3-aminotriazole. Glycerol was added to 15% final concentration and the putative positives were stored at -80°C.

5.2. Rescue and Amplification of pACT Clones from Y190 Transformants

To select for plasmid pACT, Y190 containing both pAS1-CYH2::UBC-1 and a particular pACT was serially cultured in - Leu SC liquid medium at 30°C for several generations. This saturated yeast culture was used to inoculate 10 ml of fresh - Leu SC liquid medium containing 2.5 µg/ml cycloheximide and grown at 30°C to saturation. The yeast from this culture were plated on - Trp SC plates (to select for loss of pAS1-CYH2::UBC-1) and - Leu SC plates
containing 2.5 μg/ml cycloheximide (to select for maintenance of pACT) and incubated at 30°C for 3 days.

A single yeast colony was resuspended in 200 μl Lysis Buffer (2% Triton x-100, 1% SDS, 10 mM Tris pH 8.0, 1mM EDTA) and 200 μl phenol/chloroform (1:1) and mixed for 2 minutes with 100 μl of 425-600 μm beads. The mixture was clarified by centrifugation (10,000 x g, 5 minutes) and the supernatant containing pACT was placed in a clean microcentrifuge tube. The DNA was precipitated with 20 μl of 3M sodium acetate and 500 μl of 95% ethanol and clarified by centrifugation (10,000 x g, 5 minutes). The DNA pellet was washed with 70% ethanol, centrifuged (10,000 x g, 5 minutes), and resuspended in 20 μl TE (Hoffman and Winston, 1987). HB101 bacteria were electroporated with 1 μl of rescued pACT and the HB101 transformants were grown overnight at 37°C in M9 liquid medium (42 mM Na2HPO4, 22 mM KH2PO4, 19 mM NH4Cl, 8.5 mM NaCl, 1 mM MgSO4•7H2O, 0.2% glucose) containing 200 μg/ml ampicillin and 25 μg/ml streptomycin. The pACT plasmids were purified using standard plasmid DNA purification techniques.

5.3. False Positive Testing of Putative Interactions with UBC-1

To test whether the protein encoded by the cDNA in pACT independently activated transcription of lacZ or His3, Y190 transformants lacking pAS1-CYH2::UBC-1 but maintaining pACT (i.e. Leu +, Trp -) were streaked on - Leu SC plates and tested for lacZ reporter gene activation. Y190 transformants lacking pAS1-CYH2::UBC-1 but maintaining pACT were also streaked on - His SC + 35 mM 3-aminotriazole plates to test for activation of His3 transcription.

Putative positive Y190 transformants containing only pACT were transformed with pAS1-CYH2::UBC-1 and plated on - Trp - Leu - His SC + 35 mM 3-aminotriazole plates to ensure that activation of His3 transcription occurred. Transformants (+ Trp + Leu + His) were tested for β-galactosidase activity.

To test whether the interaction with a particular C. elegans protein encoded by pACT was specific to UBC-1, Y190 transformed with pACT was mated to an opposite mating type strain Y187 transformed with either CDK2 or SNF1 in pAS1. Briefly, Y190 transformed with a particular pACT (Y190/pACT) was patched onto a YEPD plate (1% Bacto yeast extract, 2% Bacto tryptone, 2% glucose, 2% agar) and incubated overnight at 30°C. Y187 transformed with
CDK2 in pAS1 (Y187/CDK2-pAS1) was streaked for single colonies on a separate YEPD plate and incubated overnight at 30°C. A large loopful of Y187/CDK2-pAS1 was resuspended in 1 ml of YEPD liquid media and 300 µl was spread on a fresh YEPD plate and incubated at 30°C for approximately 2 hours until the lawn of yeast was dry. The patch of Y190/pACT was replica plated onto the lawn of Y187/CDK2-pAS1 and incubated overnight at 30°C. Diploids were selected by replica plating onto - Trp - Leu SC plates, incubated overnight at 30°C, and tested for β-galactosidase activity. A positive control for β-galactosidase activity was Y190 transformed with both pSE1111 and pSE1112. Also, β-galactosidase activity of the diploids was compared to the Y190 strain transformed with the relevant pACT and pAS1-CYH2::UBC-1.

The mating tests with Y190/pACT were repeated with Y187 transformed with another unrelated bait, SNF1 in pAS1.

5.4. Analysis of Positive Protein Interactions with UBC-1

Purified pACT clones were mapped by restriction enzyme digestion with Bgl II and the DNA fragments were separated by electrophoresis on 1% agarose gels. Clones that had similar DNA fragment patterns were grouped together and the nucleotide sequences of representative clones of each group were determined with SEQEC 1 primer (5') and SEQEC 2 primer (3'). DNA sequences were searched for homologies to other *C. elegans* genes as well as to genes from other organisms using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Altschul et al., 1997).

Since many of the sequenced pACT clones encoded regions of a previously uncharacterised *C. elegans* gene, R05D3.4, all the pACT clones were subjected to PCR with a primer to the pACT vector (SEQEC 1) and an internal primer to R05D3.4 (R05D3.4 R) to identify all those pACT clones encoding R05D3.4. Briefly, each pACT clone was used as a template for PCR using *Vent* DNA polymerase with 50 pmoles of SEQEC 1 and 50 pmoles of R05D3.4 R primers with 4 mM MgSO4. The PCR profile was 93°C for 5 minutes, followed by 30 cycles of 93°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute; and finally one cycle at 72°C for 10 minutes. A 500 bp or smaller PCR product indicated that a particular pACT clone encoded R05D3.4.
5.5. Yeast Interaction Trap Studies

Y190 was singly transformed with pAS1-CYH2::UBC-1Δ152, full length RFP-1 (pACT38 clone), RFP-1 (1-183) in pACT2, RFP-1 (184-313) in pACT2, RFP-1 (1-313) in pACT2, and RFP-1 (408-839) in pACT2. Protein extracts of the single transformants were prepared, fractionated by SDS PAGE at 140 V, and electro-blotted to PVDF membranes. The membranes were probed with monoclonal UBC-1 antibodies to detect the GAL4 DNA binding domain: UBC-1Δ152 fusion protein or with polyclonal RFP-1 antibodies to confirm expression of the GAL4 activation domain: RFP-1 fusion proteins.

As negative controls, Y190 was singly transformed with the empty pACT2 vector, full length RFP-1 in pACT (clone pACT 38), RFP-1 (1-183) in pACT2, RFP-1 (184-313) in pACT2, RFP-1 (1-313) in pACT2, and RFP-1 (408-839) in pACT2. Each of the six transformation reactions was plated on separate - Leu SC plates and the transformants were tested for lacZ expression. The transformants were also plated on - His SC + 35 mM 3-aminotriazole plates to test for His3 expression.

To map the regions of RFP-1 important for interaction with UBC-1, yeast strain Y190 was singly transformed with the empty pACT2 vector, full length RFP-1 in pACT (clone pACT 38), RFP-1 (1-183) in pACT2, RFP-1 (184-313) in pACT2, RFP-1 (1-313) in pACT2, and RFP-1 (408-839) in pACT2. Each of the six transformation reactions was plated on separate - Leu SC plates and incubated at 30°C for 3 days. The transformants were then transformed with either pAS1-CYH2, pAS1-CYH2::UBC-1, or pAS1-CYH2::UBC-1Δ152 and plated on - Leu - Trp SC plates. The transformants were tested for β-galactosidase activity upon exposure to X-gal using the X-gal colony filter lift assay. The transformants were also tested for His3 reporter gene expression by plating them on - Trp - Leu - His SC + 35 mM 3-aminotriazole plates.

6. PROTEIN AND IMMUNOLOGICAL METHODS

6.1. SDS-Polyacrylamide Gel Electrophoresis

Protein samples were separated by size using discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Protein samples were boiled (3 min.) in 1X loading sample buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.01% bromphenol blue, 100 mM DTT), loaded on a denaturing SDS-polyacrylamide gel, and fractionated at 140 V.
6.2. Western Blotting

Proteins separated by SDS-PAGE were transferred to PVDF membranes (Pall Gelman Laboratory) by electro-blotting. The PVDF membrane was briefly wetted in 100% methanol and then soaked in Transfer Buffer (25 mM Tris, 192 mM glycine, 10% methanol) for 5 minutes. The gel was equilibrated in Transfer Buffer for 5 minutes, and the gel and PVDF membrane were then sandwiched between sheets of filter paper and placed in the transfer apparatus (Trans-Blot Cell, BIORAD). Electro-blotting was performed at 225 mA for 45 minutes.

Following transfer of the proteins to the membrane, Western blots were equilibrated in TBS-Tween (20 mM Tris pH 7.5, 137 mM NaCl, 0.1% Tween-20) for 5 minutes and blocked with 10% skim milk powder in TBS-Tween either for 1 hour at room temperature or overnight at 4°C. The blots were incubated for 1 hour with primary antibody and then for 1 hour with horseradish peroxidase-conjugated secondary antibody at room temperature (refer to Table 4 for antibody dilutions in TBS-Tween). The blots were washed three times for 15 minutes with TBS-Tween after the blocking, primary, and secondary antibody incubations. The protein-antibody complexes were visualised with enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Table 4: Summary of primary and secondary antibody dilutions used for Western blot analyses

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Type</th>
<th>Dilution</th>
<th>Horseradish Peroxidase Conjugated Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-UBC-1</td>
<td>Monoclonal</td>
<td>1:4000</td>
<td>anti-mouse</td>
</tr>
<tr>
<td>anti-RFP-1</td>
<td>Polyclonal</td>
<td>1:2500</td>
<td>goat anti-rabbit</td>
</tr>
<tr>
<td>C-terminus (Rabbit 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-actin C4</td>
<td>Monoclonal</td>
<td>1:10,000</td>
<td>anti-mouse</td>
</tr>
<tr>
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<td>1:10,000</td>
<td>anti-mouse</td>
</tr>
<tr>
<td>anti-RFP-1</td>
<td>Polyclonal</td>
<td>1:2500</td>
<td>goat anti-rabbit</td>
</tr>
<tr>
<td>N-terminus (Rabbit 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.3. RFP-1 Protein Expression

Expression plasmid aa 1-329 RFP-1::pET28a or aa 408-775 RFP-1::pET28a was transformed into BL21 (DE3) bacteria, and transformants were grown at 37°C in LB containing 25 μg/ml kanamycin to O.D._600 = 0.6. Protein expression was induced with 1 mM IPTG for 2 to 4 hours. The induced bacteria were collected by centrifugation (3000 x g, 5 minutes) and sonicated (10 seconds on, 30 seconds off, power setting 3, for 4 minutes at 4°C) in 50 mM sodium phosphate pH 8, 300 mM NaCl, with one Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics) per 10 ml. The sonicated material was clarified by centrifugation (10,000 x g, 10 minutes) and soluble and insoluble fractions were separated by SDS PAGE and Coomassie blue stained.

To produce soluble RFP-1 (1-329), BL21 (DE3) bacteria transformed with aa 1-329 RFP-1::pET28a were grown at 30°C in 100 ml of LB containing 25 μg/ml kanamycin to O.D._600 = 0.8. Protein expression was induced with 0.1 mM IPTG overnight at 15°C. The induced bacteria were collected by centrifugation (3000 x g, 5 minutes), resuspended in Lysis Buffer (50 mM Tris pH 8, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) and sonicated. The sonicated material was clarified by centrifugation (10,000 x g, 10 minutes) and soluble and insoluble fractions were separated by SDS PAGE and Coomassie blue stained.

6.4. Purification of Insoluble RFP-1

Inclusion bodies containing either RFP-1 (1-329) or RFP-1 (aa 408-775) were resuspended in 15 ml of Denaturing Buffer (50 mM sodium phosphate pH 8.6, 300 mM NaCl, 6 M guanidine HCl) and applied to 2 ml of pre-equilibrated Talon Resin (Clontech) in a 50 ml conical centrifuge tube. The resin and proteins were rocked at room temperature for 20 minutes, centrifuged (300 x g, 1 minute), and washed twice for 10 minutes with 10 bed volumes of Denaturing Buffer containing 10 mM imidazole. The bound proteins were eluted with 5 bed volumes of Denaturing Buffer containing 150 mM imidazole.
6.5. RFP-1 Antibody Production

Polyclonal antibodies against RFP-1 were prepared as described (Harlow and Lane, 1988). Rabbits 1 and 2 were immunised with amino acids 408-775 RFP-1 with a C-terminal 6 x His Tag. Rabbits 3 and 4 were immunised with amino acids 1-329 RFP-1 with a C-terminal 6 x His Tag. After purification on Talon Resin, the eluted antigen was dialysed extensively at 4°C against PBS (136 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.7 mM KH₂PO₄ pH 7.4). The dialysed antigen was clarified by centrifugation (10,000 x g, 10 minutes) and the antigen was resuspended in 1X loading sample buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.01% bromphenol blue, 100 mM DTT), loaded on a denaturing SDS polyacrylamide gel, and fractionated at 140 V. The antigen band was excised from the gel, rinsed in PBS, and emulsified in a 1:1 ratio in PBS and Freund’s Complete Adjuvant. These preparations contained approximately 0.5 to 1 mg of antigen.

All four rabbits received boosts at approximately three week intervals using Incomplete Freund’s Adjuvant until the titres of the antibodies were sufficiently high as determined by Western blotting C. elegans extracts. After the full body bleeds, the sera were precipitated with ammonium sulphate and the antibodies were exhaustively dialysed against PBS at 4°C (Harlow and Lane, 1988).

6.6. RFP-1 and UBC-1 Co-Immunoprecipitation Studies

Protein extracts of adult C. elegans were prepared by Dounce homogenising animals in Co-immunoprecipitation Buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100, 2.5 mM PMSF). Each co-immunoprecipitation experiment contained 50 μg of worm protein extract, 2 mM ATP, 4 μg of ubiquitin (Sigma), and 4 μg of recombinant UBC-1 in a final reaction volume of 300 μl of Co-immunoprecipitation Buffer. The recombinant UBC-1 was expressed and purified as described (Leggett and Candido, 1997). To confirm the interaction between UBC-1 and RFP-1, 50 μg of soluble RFP-1 (amino acids 1-329 with a C-terminal 6 x His Tag) was added to each of two tubes along with either 2.5 μg of polyclonal RFP-1 antibody (Rabbit 4) or 2.5 μg of polyclonal Hsp43 antibody and the mixtures were incubated (30 min., 4°C). In order to capture the protein-antibody complexes, 50 μl of 10% formalin-fixed Staphylococcus aureus (Zymed) was added to the tubes and rocked (1 hour, 4°C).
The S. aureus was collected by centrifugation (10,000 x g, 10 seconds) and washed twice with Co-immunoprecipitation Buffer. Protein-antibody complexes on the S. aureus pellet were eluted with 50 µl of loading sample buffer, followed by incubation at 37°C for 10 minutes, and centrifugation (10,000 x g, 1 minute). The proteins in the supernatant were separated by SDS PAGE, transferred to a PVDF membrane and probed with a monoclonal antibody against UBC-1.

The co-immunoprecipitation experiment was repeated in the absence of worm protein extract, ATP, or ubiquitin to determine if any of these components were important for the UBC-1 and RFP-1 interaction.

7. INDIRECT IMMUNOHISTOCHEMICAL STAINING OF C. ELEGANS

7.1. RFP-1 and UBC-1 Indirect Immunohistochemical Staining of Larvae and Adults

Indirect immunohistochemical staining of C. elegans animals was performed as described (Loer and Kenyon, 1993). The primary antibodies used were RFP-1 polyclonal antibodies (1:500) or UBC-1 polyclonal antibodies (1:200). Alexa-488 anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, U.S.A.) was added at 1:200. The stained animals were mounted on microscope slides with coverslips, and viewed with epifluorescence on an Axioplan 2 microscope (Zeiss). As a negative control, animals were only incubated with Alexa-488 anti-rabbit secondary antibody (1:200).

In order to confirm that the observed staining pattern was specific to RFP-1 or UBC-1, competition experiments were performed by pre-incubating the RFP-1 or UBC-1 primary antibodies with 0.25 mg of purified RFP-1 or UBC-1.

7.2. RFP-1 and UBC-1 Indirect Immunohistochemical Staining of C. elegans Embryos

C. elegans embryos were extracted from gravid adults and subjected to indirect immunohistochemical staining as described (Goh and Bogaert, 1991). The primary antibodies employed were RFP-1 polyclonal antibody (1:400) or UBC-1 polyclonal antibody (1:300). Alexa-488 anti-rabbit secondary antibody was used at 1:200. After rinsing, the embryos were resuspended in SlowFade Light Antifade mounting medium (Molecular Probes, Eugene, Oregon, U.S.A.) and placed on slides for viewing by fluorescence microscopy (Axioplan 2 microscope; Zeiss). Competition experiments and negative controls were as described in Section 7.1.
8. DOUBLE STRANDED RNA INTERFERENCE TECHNIQUES

8.1. RNA Interference Against *ubc-1* or *rfp-l* by Injection of Double Stranded RNA in *C. elegans*

Double stranded RNA interference (RNAi) against *ubc-1* or *rfp-l* was performed as described (Fire *et al.*, 1998). Briefly, plasmid pR1.7 was cut with *Apa* I to put *ubc-1* transcription under the control of the T3 promoter. To put *ubc-1* transcription under the control of the T7 promoter, pR1.7 was restricted with *Sac* I. The restricted plasmids were extracted with 1:1 phenol:chloroform, precipitated with 95% ethanol, and the DNA pellets were resuspended in DEPC-treated water. Sense and antisense *ubc-1* RNA were made by incubating 1 μg of restricted pR1.7 DNA in transcription buffer (Promega) containing 1 μl of T3 or T7 RNA polymerase, 1 μl Rnasin, 10 mM DTT, and 0.5 mM rNTPs in a 40 μl final reaction volume. The T3 RNA polymerase reaction was incubated at 25°C and the T7 RNA polymerase reaction was incubated at 37°C for 1.5 hours. The sense and antisense *ubc-1* RNA strands were combined and the double stranded *ubc-1* RNA was purified and resuspended in DEPC-treated water. A 1 μl aliquot was separated by electrophoresis on a 1% agarose gel to confirm that *ubc-1* double stranded RNA at approximately 1.7 kb was produced.

Double stranded *rfp-l* RNA (approximately 2.6 kb) was made as described above with the following exceptions: 5 μg of pBSIIKS::RFP-1 was restriction digested with *Not* I to place *rfp-l* transcription under the control of the T3 promoter. To put *rfp-l* transcription under the control of the T7 promoter, pBSIIKS::RFP-1 was restricted with *BamH* I. After the transcription reactions were complete, a 2656 base pair RNA of full length *rfp-l* and some 3' untranslated region was produced.

The double stranded *ubc-1* or *rfp-l* RNA was microinjected into the syncytial gonads of young adult N2 *C. elegans* using a Zeiss microscope and Leitz micromanipulator. The injected animals were placed on NG plates spread with OP50 *E. coli* and allowed to recover for 6 hours at 15°C. Each injected animal was then moved to a separate NG plate spread with OP50 *E. coli* and allowed to lay embryos for 12 hours (Brood A). Each injected animal was then moved to a new separate NG plate spread with OP50 *E. coli* and allowed to lay embryos for 12 hours (Brood B). The number of hatched embryos was scored and the development of Broods A and B was followed.
8.2. RNA Interference Against *ubc-1* or *rfp-1* by the Feeding Method

Double stranded RNA interference (RNAi) against *ubc-1* or *rfp-1* was performed as described (Kamath *et al.*, 2000; Timmons *et al.*, 2001). Plasmid pPD129.36::UBC-1 or pPD129.36::RFP-1 nt 3-982 was transformed into HT115 (DE3) bacteria and a single colony was used to inoculate 5 ml of LB containing 50 μg/ml of ampicillin. The bacteria were grown at 37°C for 8 to 16 hours, concentrated by centrifugation (8000 x g, 1 minute), and spread on NGM plates (50 mM NaCl, 0.25% peptone, 0.005 mg/ml cholesterol, 1 mM CaCl₂, 1 mM MgSO₄, 25 mM potassium phosphate pH 6.0, 1.7% agar) containing 25 μg/ml carbenicillin and 1 mM IPTG. This resulted in lawns of HT115 (DE3) bacteria expressing *ubc-1* or *rfp-1* double stranded RNA.

Five to ten L4 hermaphrodite N2 animals were placed on one plate covered with HT115 (DE3) bacterial lawns expressing double stranded *ubc-1* or *rfp-1* RNA at 15°C. Each animal was moved to a separate plate after 72 hours, during which time the animals laid embryos. The adults were destroyed after 24 hours and the offspring were scored for phenotypes produced by double stranded RNA interference against *ubc-1* or *rfp-1* at 20°C. In the negative control, five to ten L4 hermaphrodite N2 animals were raised on plates with OP50 bacterial lawns (no exposure to dsRNA) and treated as above.

Double stranded RNA interference against *rfp-1* was also carried out in *lin-15A (n767)* and *lin-15B (n744)* mutant backgrounds as described above. The negative control consisted of placing five to ten L4 *lin-15A (n767)* hermaphrodites or LA *lin-15B (n744)* hermaphrodites on OP50 bacterial lawns.
RESULTS

1. THE YEAST TWO-HYBRID SCREEN

The yeast two-hybrid system is a genetic system to detect protein-protein interactions in vivo. The *S. cerevisiae* GAL4 protein is a transcriptional activator composed of two separable domains: a DNA binding domain (amino acids 1-147) and a transcriptional activation domain (amino acids 768-881). Two plasmids are constructed in which one plasmid (the bait) encodes a particular protein fused to the GAL4 DNA binding domain and the second plasmid (the prey) encodes a protein (or cDNA library) fused to the GAL4 activation domain. The plasmids, bearing selectable markers TRP and LEU, are transformed into yeast. If the two proteins interact, they reconstitute the GAL4 transcriptional activator and drive transcription of a reporter gene (*lacZ* or *His3*) under the control of the *GAL1* promoter. Thus, yeast colonies containing interacting fusion proteins grow in the absence of histidine and develop a blue colour when exposed to X-gal (Fields and Song, 1989; Chien *et al.*, 1991). Figure 6 is a schematic representation of the yeast two-hybrid system. The yeast two-hybrid screen was employed in this study to identify *C. elegans* proteins that interact with UBC-1.

1.1. A Yeast Two-Hybrid Screen for *C. elegans* Proteins that Interact with UBC-1

A randomly primed *C. elegans* cDNA library was screened using *ubc-1* as a bait in a yeast two-hybrid screen. Yeast strain Y190 was transformed with the bait plasmid pAS1-CYH2::UBC-1 and expression of the GAL4 DNA binding domain: UBC-1 fusion protein was confirmed. Figure 7 is a UBC-1 Western blot of protein extracts from Y190 transformed with pAS1-CYH2::UBC-1 (lane 2) or from Y190 that had not been transformed with the plasmid (lane 3). It shows that the 40 kDa GAL4 DNA binding domain: UBC-1 fusion protein was expressed only in Y190 cells carrying the pAS1-CYH2::UBC-1 plasmid. A positive control for Western blotting was the 20 kDa purified UBC-1 protein (lane 1).

Yeast strain Y190 was transformed with pAS1-CYH2::UBC-1 and transformants were selected on - Trp SC plates and tested for β-galactosidase activity (i.e. activation of the *lacZ* reporter gene) by the X-gal Colony Filter Lift Assay.
A: Native GAL4

B: Individual hybrids with GAL4 domains

C: Interaction between hybrids reconstitutes GAL4 activity

Figure 6: Schematic Representation of the Yeast Two-Hybrid System

Native GAL4 contains both the DNA binding domain (DBD) and activation domain (ACT) to induce transcription of lacZ under the control of the GAL1 promoter (A). Hybrid proteins containing either the DBD fused to protein X or the ACT domain fused to protein Y cannot induce transcription (B). An interaction between X and Y reconstitutes GAL4, resulting in transcriptional activity (C). This figure was adapted from Fields and Song, 1989.
Figure 7: The GAL4 DNA Binding Domain: UBC-1 Fusion Protein is Expressed in Y190

Cultures of yeast strain Y190 carrying the plasmid pAS1-CYH2::UBC-1 (lane 2) or lacking plasmid (lane 3) were grown and protein extracts were prepared as described in Materials and Methods. The proteins were separated by electrophoresis on a 15% SDS polyacrylamide gel, transferred to a PVDF membrane, and probed with a monoclonal antibody against UBC-1. Lane 1 contained purified UBC-1 (20 kDa) as a positive control for Western blotting. Lane 2 shows expression of the expected 40 kDa GAL4 DNA binding domain: UBC-1 fusion protein in Y190.
Y190 transformed with pAS1-CYH2::UBC-1 was also plated on - His SC + 35 mM 3-aminotriazole to test for activation of the His3 reporter gene. The transformants lacked β-galactosidase activity and hence failed to turn blue when exposed to X-gal (data not shown). These Trp + transformants failed to grow on plates lacking histidine, indicating that the His3 reporter gene was not expressed (data not shown). Thus, the UBC-1 bait did not independently activate transcription of the His3 or lacZ reporter genes.

Y190 bearing pAS1-CYH2::UBC-1 was then transformed with a randomly primed C. elegans cDNA library in plasmid pACT. In order to screen the pACT library five-fold for potential protein binding partners of UBC-1, a total of $2.1 \times 10^6$ colonies were screened on 150 mm - Trp - Leu - His SC + 35 mM 3-aminotriazole plates. Of $2.1 \times 10^6$ colonies screened, 62 contained C. elegans cDNAs that upon expression reconstituted the GAL4 transcription factor, allowing the transformants to grow in the absence of histidine. These transformants also had β-galactosidase activity and turned blue when exposed to X-gal.

1.2. Identification of pACT Clones Isolated in the UBC-1 Yeast Two-Hybrid Screen

The results of the yeast two-hybrid screen are summarised in Table 5. Seventy-seven percent of the recovered cDNAs encoded the uncharacterised protein R05D3.4. Fourteen percent of the cDNAs (pACT 2, pACT 10, pACT 13, pACT 16, pACT 26, pACT Q, pACT Y, pACT Z, pACT T) encoded C32E8.11. A single clone, pACT E, encoded K11D9.2, a calcium-dependent ATPase of the sarcoplasmic reticulum. In Table 5 two asterisks (**) indicate that a particular pACT clone was sequenced as well as subjected to PCR with a primer to the pACT vector (SEQEC 1) and R05D3.4 R (an internal primer to R05D3.4) to ascertain if the particular pACT clone encoded R05D3.4. One asterisk (*) indicates that a particular pACT clone was only sequenced and was not subjected to PCR. No asterisk indicates that a particular pACT clone was only subjected to PCR and was not directly sequenced. Plasmids pACT 9 and pACT X were not analysed because they could not be rescued from yeast and amplified in bacteria. Plasmids pACT F and pACT 35 were not analysed because they encoded cDNAs that gave rise to false positive interactions.
Table 5: Summary of the identity of pACT plasmids isolated from Y190 in a yeast two-hybrid screen for proteins that interact with UBC-1.

<table>
<thead>
<tr>
<th>pACT Clone</th>
<th>Clone Identification</th>
<th>pACT Clone</th>
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<th>Clone Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>A **</td>
<td>R05D3.4</td>
<td>3 *</td>
<td>R05D3.4</td>
<td>25</td>
<td>R05D3.4</td>
</tr>
<tr>
<td>B</td>
<td>R05D3.4</td>
<td>4 **</td>
<td>R05D3.4</td>
<td>26 **</td>
<td>C32E8.11</td>
</tr>
<tr>
<td>D</td>
<td>R05D3.4</td>
<td>5 **</td>
<td>R05D3.4</td>
<td>27 **</td>
<td>R05D3.4</td>
</tr>
<tr>
<td>E **</td>
<td>K11D9.2</td>
<td>6 *</td>
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<td>7</td>
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<td>29 **</td>
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</tr>
<tr>
<td>H</td>
<td>R05D3.4</td>
<td>8</td>
<td>R05D3.4</td>
<td>30 **</td>
<td>R05D3.4</td>
</tr>
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<td>K *</td>
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<tr>
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<td>10 **</td>
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<td>C32E8.11</td>
<td>35</td>
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</tr>
<tr>
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<td>14 **</td>
<td>R05D3.4</td>
<td>36</td>
<td>R05D3.4</td>
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<tr>
<td>Q *</td>
<td>C32E8.11</td>
<td>15 **</td>
<td>R05D3.4</td>
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<td>R05D3.4</td>
<td>16 **</td>
<td>C32E8.11</td>
<td>38 *</td>
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</tr>
<tr>
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<td>R05D3.4</td>
<td>17</td>
<td>R05D3.4</td>
<td>39</td>
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</tr>
<tr>
<td>T **</td>
<td>C32E8.11</td>
<td>18</td>
<td>R05D3.4</td>
<td>41 *</td>
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<td>C32E8.11</td>
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<td>44</td>
<td>R05D3.4</td>
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<td>Y **</td>
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<td>2 **</td>
<td>C32E8.11</td>
<td>24</td>
<td>R05D3.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Clone was directly sequenced and subjected to PCR with primers SEQEC1 and R05D3.4 R.
* Clone was subjected to sequencing only.
No asterisk: Clone was subjected to PCR with primers SEQEC1 and R05D3.4 R only.
1.3. False Positive Testing of Putative Interactions with UBC-1

A drawback to yeast two-hybrid screens is the propensity to uncover false positive interactions with a particular bait. Four false positive tests were performed on each putative positive pACT clone and the results are summarised in Tables 6, 7, and 8. All pACT clones encoding R05D3.4 gave identical results in the four false positive tests and are presented as one R05D3.4 group. Similarly, all of the pACT clones identified as C32E8.11 behaved identically when tested for false positive interactions and are denoted as one C32E8.11 group.

False positive interactions can occur when the prey protein independently activates transcription of the reporter genes. The first false positive test examined whether the protein encoded by the cDNA in pACT independently activated transcription of lacZ or His3. Y190 transformants lacking pAS1-CYH2::UBC-1 but maintaining a particular pACT (i.e. Leu +, Trp -) were streaked on - Leu SC plates and tested for β-galactosidase activity upon exposure to X-gal using the X-gal Colony Filter Lift Assay. Leu + Trp - transformants that turned blue were false positives, i.e. they activated lacZ transcription in the absence of UBC-1. Y190 transformants lacking pAS1-CYH2::UBC-1 but maintaining a pACT clone were also streaked on - His SC + 35 mM 3-aminotriazole plates to test for activation of His3 transcription. Transformants that grew on these plates were false positives as they did not require UBC-1 to activate transcription of His3 and were not pursued further. Finally, Y190 transformants lacking pAS1-CYH2::UBC-1 but maintaining a pACT clone were streaked on - Leu - Trp SC plates to ensure that the bait plasmid was absent. Table 6 shows that pACT clones encoding R05D3.4, C32E8.11, and K11D9.2 failed to activate reporter gene expression on their own in Y190. Therefore, none of these interactions were classified as false positive by this test.
Table 6: Summary of the first false positive test to determine if the pACT clones independently activate reporter gene transcription.

<table>
<thead>
<tr>
<th>Prey (cDNA in pACT)</th>
<th>Bait</th>
<th>Growth on - Leu Plates</th>
<th>Growth on - Leu - Trp Plates</th>
<th>β-galactosidase Activity</th>
<th>Growth on - His Plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>empty pACT2 vector</td>
<td>none</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>R05D3.4</td>
<td>none</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>C32E8.11</td>
<td>none</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>K11D9.2</td>
<td>none</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

The second false positive test, summarised in Table 7, simply examined whether reintroduction of pAS1-CYH2::UBC-1 into Y190 containing only a putative positive pACT clone would activate transcription of the reporter genes. Putative positive transformants containing a pACT clone were transformed with pAS1-CYH2::UBC-1 and plated on - Trp - Leu - His SC + 35 mM 3-aminotriazole plates to ensure that activation of His3 transcription occurred. Transformants (+ Trp + Leu) were tested for β-galactosidase activity to ensure that they could activate lacZ transcription.

Table 7: Summary of the second false positive test to determine if the pACT clones activate reporter gene transcription in the presence of UBC-1.

<table>
<thead>
<tr>
<th>Prey (cDNA in pACT)</th>
<th>Bait</th>
<th>Growth on - Trp - Leu Plates</th>
<th>β-galactosidase Activity</th>
<th>Growth on - Trp - Leu - His Plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACT 35</td>
<td>UBC-1</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>pACT F</td>
<td>UBC-1</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>R05D3.4</td>
<td>UBC-1</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>C32E8.11</td>
<td>UBC-1</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>K11D9.2</td>
<td>UBC-1</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>
Table 7 shows that addition of plasmid pAS1-CYH2::UBC-1 to Y190 bearing pACT clones encoding R05D3.4, or C32E8.11, or K11D9.2 restores β-galactosidase activity (lacZ transcription) and histidine prototrophy (His3 transcription). Two clones, pACT F and pACT 35, did not re-activate transcription of the reporter genes when pAS1-CYH2::UBC-1 was transformed into the yeast. These two clones were classified as false positive interactors and were not pursued further.

Another false positive interaction occurs when the prey protein interacts non-specifically with the bait protein, thereby reconstituting the GAL4 transcription factor and the reporter genes are expressed. Third and fourth false positive tests, summarised in Table 8, were performed in which all positive interacting pACT clones were tested to see if they interacted with CDK2 or SNF1, baits unrelated in sequence to UBC-1. Any interaction between CDK2 or SNF1 and a particular pACT clone would be a false positive interaction, and the apparent interaction with UBC-1 would be classified as non-specific. Briefly, Y190 transformed with each of the isolated pACT clones were mated to an opposite mating type strain Y187 transformed with CDK2 or SNF1 in pAS1. The diploids were tested for β-galactosidase activity. False positives were those diploids that had β-galactosidase activity as they did not specifically require UBC-1 to activate transcription of lacZ. A positive control for β-galactosidase activity was Y190 transformed with both pSE1111 and pSE1112. Also, β-galactosidase activity of the diploids was compared to that of the Y190 strain transformed with the relevant pACT and pAS1-CYH2::UBC-1.
Table 8: Summary of the third and fourth false positive tests to determine if a pACT clone interacts specifically with UBC-1.

<table>
<thead>
<tr>
<th>Prey (cDNA in pACT) in Y190; Mat a</th>
<th>Bait (cDNA in pAS1) in Y187; Mat α</th>
<th>Growth of Diploids on - Trp - Leu Plates</th>
<th>β-galactosidase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>R05D3.4</td>
<td>CDK2</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>C32E8.11</td>
<td>CDK2</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>K11D9.2</td>
<td>CDK2</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>R05D3.4</td>
<td>SNF1</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>C32E8.11</td>
<td>SNF1</td>
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<td>no</td>
</tr>
<tr>
<td>K11D9.2</td>
<td>SNF1</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

Table 8 shows that when Y190 cells containing pACT clones encoding R05D3.4 or C32E8.11 or K11D9.2 were mated to Y187 cells containing CDK2 in pAS1, the diploids did not express β-galactosidase activity. Similarly, when Y190 cells containing pACT clones encoding R05D3.4 or C32E8.11 or K11D9.2 were mated to Y187 containing SNF1 in pAS1, the diploids lacked β-galactosidase activity. This confirmed that the apparent interactions between UBC-1 and R056D3.4, C32E8.11 or K11D9.2 were specific.

1.4. **C32E8.11 Encodes a Putative Ubiquitin-Protein Ligase (E3)**

Fourteen percent of pACT clones that interacted with UBC-1 encoded a putative ubiquitin-protein ligase or E3 called C32E8.11. Ubiquitin-protein ligases catalyse the transfer of ubiquitin to target proteins and hence form complexes with ubiquitin-conjugating enzymes and their substrates. *C. elegans* C32E8.11 is located on chromosome I and encodes a 1927 amino acid protein with a predicted molecular weight of 218 kDa and a pKi of 6.38. That UBC-1 interacted with the C32E8.11 protein was a particularly reassuring result as it was expected that this ubiquitin-protein ligase (E3) should interact with UBC-1. BLAST analysis (Altschul et al., 1997) of the C32E8.11 protein shows that it is 47% similar to *S. cerevisiae* Ubr1p, the N-end Rule ubiquitin protein-ligase that interacts with Rad6p. As RAD6 is the yeast homologue of *ubc-
I (Leggett et al., 1995; Jones et al., 2001), it was gratifying to find an E3 in *C. elegans* that was similar to Ubr1p. C32E8.11 shared 32% identity and 49% similarity to the 1757 residue *M. musculus* ubiquitin-protein ligase E3α, the mouse homologue of Ubr1p (Kwon et al., 1998). The *H. sapiens* KIAA0349 protein was 32% identical and 51% similar to C32E8.11.

SMART (Schultz et al., 1998; Letunic et al., 2002) analysis of the predicted protein sequence of C32E8.11 identified an N-terminal zinc finger domain that comprises the N-recognin, or the recognition component for substrates that are degraded by the N-end Rule Pathway (Sung et al., 1991). There is also a C3H2C3 RING finger domain spanning amino acids 1140 to 1254.

1.5. R05D3.4 Encodes a Novel RING Finger Protein

Sequence analysis of various interacting pACT clones showed that 77% encoded a novel RING finger protein located on chromosome III, called R05D3.4. R05D3.4 was renamed RFP-1 for RING Finger Protein 1 and RFP-1 will be the main focus of this thesis. Interestingly, RING finger domains reside in both of the “hits” (RFP-1 and C32E8.11) resulting from this yeast two-hybrid screen. Plasmid pACT 38 contained an almost full length *rfp-l* cDNA consisting of bp 3 to 2520 of the open reading frame as well as 103 bp of 3′ untranslated region. The predicted *rfp-l* coding region consists of 2520 bp and upon expression produces an 839 amino acid protein with a predicted molecular weight of 97.6 kDa and a pKi of 6.87. The DNA sequence of the *rfp-l* cDNA isolated from the yeast two-hybrid screen revealed a six bp insertion (two extra amino acids) and a nine bp insertion (three extra amino acids). This suggests that the GenBank coding sequence as described contains incorrectly assigned exons, specifically exons six and seven. The SMART program (Letunic et al., 2002; Schultz et al., 1998) predicted six α-helical regions, a “hidden” (poor match to the consensus sequence) basic region leucine zipper, and a C3HC4 RING finger domain.

While several predicted RING finger proteins are encoded in the *C. elegans* genome, BLAST analysis (Altschul et al., 1997) yielded no clear RFP-1 relatives in *C. elegans*, i.e. RFP-1 did not appear to belong to a protein family. BLAST analysis of the RFP-1 sequence showed that it was 48% similar to a 1002 residue *R. norvegicus* RING finger protein called Staring or Rnf40. RFP-1 shared 48% similarity and 23% identity to the human ortholog of Staring, a 1001 residue *Homo sapiens* protein K1AA0661. *S. cerevisiae* Bre1p was 40% similar and 21%
identical to RFP-1. RFP-1 also had considerable similarity (43%) to an *Anopheles gambiae* 861 amino acid protein called ebiP9515. All of these proteins contain RING finger domains important for protein-protein interactions. Figure 8 is an alignment of the predicted amino acid sequence of RFP-1 with its putative homologues.

2. ANALYSIS OF THE UBC-1 AND RFP-1 INTERACTION

2.1. Mapping the UBC-1 and RFP-1 Interaction with Yeast Interaction Trap Studies

In order to map the region of interaction between UBC-1 and RFP-1, studies were performed with either full length UBC-1 or a mutated UBC-1 lacking the acidic C-terminal tail, called UBC-1Δ152. These two UBC-1 baits were assayed individually for their ability to interact with the full length GAL4 activation domain: RFP-1 fusion protein or with each of four GAL4 activation domain: truncated RFP-1 fusion proteins.

Expression of the UBC-1Δ152 bait construct was confirmed by Western blotting extracts of Y190 transformed with UBC-1Δ152::pAS1-CYH2. Figure 9 (lane 1) is a UBC-1 Western blot showing expression of the 36.4 kDa GAL4 DNA binding domain: UBC-1Δ152 fusion protein in Y190.

For the yeast interaction trap experiments, four RFP-1 truncation proteins fused to the GAL4 activation domain in pACT2 were cloned. The RFP-1 truncations were chosen based on domains identified by the SMART program (Schultz *et al.*, 1998; Letunic *et al.*, 2002). The C-terminal half of RFP-1 (amino acids 408 to 839) contained two helical regions (Helices 5 and 6) and a C3HC4 RING finger domain. As RING finger domains often recruit ubiquitin-conjugating enzymes (Lorick *et al.*, 1999), this RFP-1 truncation construct was of great interest. RFP-1 (408-839) was cloned into pACT2 and the resulting construct was transformed into Y190. Expression of the GAL4 activation domain: RFP-1 (408-839) fusion protein in Y190 was confirmed by Western blotting protein extracts with polyclonal antibodies against the C-terminus of RFP-1 (RFP-1 antibody preparation is described in Materials and Methods). Figure 9 (lane 2) shows expression of the 68.2 kDa fusion protein in Y190. Either proteolysis of the fusion protein or antibody cross reactivity could account for the detection of polypeptides smaller than 68 kDa.
Figure 8. Conservation of *C. elegans* RING Finger Protein 1 (RFP-1) in Eukaryotes

*C. elegans* RFP-1 is an 839 amino acid protein with a predicted molecular weight of 97.6 kDa and a pKi of 6.87. Shown is a Clustal W alignment of RFP-1 sequence homologues in *R. norvegicus* (Staring), *H. sapiens* (KIAAO661), and *S. cerevisiae* (Bre1p). Asterisks indicate the positions of the cysteinyl and histidyl residues that likely co-ordinate two zinc atoms in the conserved C-terminal C3HC4 RING fingers. Black shading, identical residues. Grey shading, conserved residues.
The second RFP-1 truncation protein fused to the GAL4 activation domain in pACT2 consisted of the first 183 amino acids of RFP-1. This region included the first helix of RFP-1 and terminated before the start of the second helix. Expression of this truncation protein was confirmed by probing Western blots of Y190 protein extracts with monoclonal antibodies against the GAL4 activation domain (Figure 9, lane 3). While the fusion protein runs about 3 kDa smaller than the predicted 38 kDa, the strong signal at 35 kDa is indicative of expression of the fusion protein.

RFP-1 (184-313) contained only the second helix and terminated before the start of the basic leucine zipper. Expression of this GAL4 activation domain: RFP-1 (184-313) fusion protein was confirmed by probing Western blots with polyclonal antibodies against the N-terminus of RFP-1 (Figure 9, lane 4). While the fusion protein runs approximately 4 kDa larger than the predicted 32 kDa, the strong banding at 35 kDa is indicative of expression of this protein.

The N-terminal half of RFP-1 (1-313), containing the first two helical regions of RFP-1 and terminating before the beginning of the basic leucine zipper, was also analysed. The expression of the GAL4 activation domain: RFP-1 (1-313) fusion protein was confirmed by probing Western blots of Y190 protein extracts with polyclonal antibodies against the N-terminus of RFP-1 (refer to Materials and Methods, Section 6.5. for RFP-1 antibody production). Figure 9 (lane 5) shows expression of the 52.6 kDa fusion protein in Y190. Finally, expression of full length RFP-1 was confirmed using polyclonal antibodies against the N-terminus of RFP-1. Figure 9 (lane 6) shows expression of the expected 113 kDa GAL4 activation domain: RFP-1 fusion protein.
Figure 9: The Constructs Used in UBC-1 and RFP-1 Yeast Interaction Traps are Expressed in Y190.

Yeast strain Y190 was individually transformed with one of six constructs and protein extracts of the transformants were separated by electrophoresis on SDS polyacrylamide gels, transferred to PVDF membranes, and probed with the indicated antibodies (Ab) to detect expression of the GAL4 DNA binding domain (DBD) or GAL4 activation domain (ACT) fusion proteins. An asterisk (*) indicates the position of the fusion protein of interest. RFP-1 antibodies generated against the N- and C-termini of RFP-1 are referred to as RFP-1 Ab (N-term.) and RFP-1 Ab (C-term.), respectively.
Having established that the two UBC-1 baits and the five RFP-1 preys were all expressed in yeast, the yeast interaction trap experiments were executed. Yeast strain Y190 was singly transformed with the empty pACT2 vector, full length RFP-1 in pACT (clone pACT 38), RFP-1 (1-183) in pACT2, RFP-1 (184-313) in pACT2, RFP-1 (1-313) in pACT2, and RFP-1 (408-839) in pACT2. Each of the six transformation reactions was plated on separate - Leu SC plates and incubated at 30°C for 3 days. The transformants were then transformed with either no construct, or empty pAS1-CYH2 vector, or pAS1-CYH2::UBC-1, or pAS1-CYH2::UBC-1Δ152, and the twenty-four transformation experiments were plated on separate - Leu - Trp SC plates. The Leu + Trp + transformants were tested for β-galactosidase activity upon exposure to X-gal using the X-gal Colony Filter Lift Assay. The transformants were also tested for their ability to activate transcription of the His3 reporter gene by plating them on - Trp - Leu - His SC + 35 mM 3-aminotriazole plates.

As summarised in Figure 10, none of the GAL4 activation domain: RFP-1 fusion proteins independently activated His3 or lacZ transcription. None of the GAL4 activation domain: RFP-1 fusion proteins interacted with empty pAS1-CYH2, indicating that the RFP-1 interaction was specific to UBC-1. Similarly, neither the UBC-1 nor the UBC-1Δ152 baits could interact with empty pACT2 vector, indicating that the interaction was RFP-1-dependent.

Full length RFP-1 interacted with both UBC-1 and UBC-1Δ152. Thus, the acidic C-terminal tail of UBC-1 was dispensable for the interaction with RFP-1. Neither the first 183 amino acids, nor amino acids 184 to 313 of RFP-1 were sufficient for the interaction with UBC-1. The N-terminus of RFP-1 (1 to 313), containing the first two helical regions, was required for the interaction with UBC-1. The N-terminus of RFP-1 (1-313) interacted with UBC-1Δ152, thereby demonstrating that the acidic tail of UBC-1 is dispensable for this interaction. Interestingly, the C-terminal half of RFP-1 (containing the RING finger domain) was dispensable for the interaction with UBC-1.
Figure 10: Summary of the UBC-1 and RFP-1 Yeast Interaction Trap Experiments

Yeast strain Y190 was individually transformed with the indicated GAL4 activation domain constructs. Each construct was assayed for the ability to interact with each of three baits: empty pAS1-CYH2 vector, pAS1-CYH2::UBC-1, or pAS1-CYH2::UBC-1Δ152. Trp + Leu + transformants were assayed for lacZ transcription, resulting in β-galactosidase activity using the X-gal Colony Filter Lift Assay. Transformants were also tested for His3 transcription by assaying for growth on - Leu - Trp - His SC plates containing 35 mM 3-amino-1, 2, 4 triazole. Lack of transcriptional activation is denoted with a blank ( - ) and activation of lacZ or His3 is denoted with a plus sign ( + ). Grey boxes represent helical regions in REP-1. The basic leucine zipper (residues 313-375) together with helix 3 (amino acids 303-353) and helix 4 (amino acids 376-399) are represented by a divided box. Hatched boxes represent the C3HC4 RING finger domain in RFP-1.
<table>
<thead>
<tr>
<th>GAL4 Activation Domain Construct</th>
<th>None</th>
<th>pAS1-CYH2</th>
<th>pAS1-CYH2::UBC-1</th>
<th>pAS1-CYH2::UBC-1Δ152</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-gal*</td>
<td>β-gal</td>
<td>β-gal</td>
<td>β-gal</td>
</tr>
<tr>
<td></td>
<td>His**</td>
<td>His</td>
<td>His</td>
<td>His</td>
</tr>
<tr>
<td>Empty pACT2 Vector</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RFP-1</td>
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<td>+</td>
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<tr>
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</tr>
<tr>
<td>aa 408-839</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*β-galactosidase activity

**Growth on histidine dropout medium containing 35 mM 3-amino-1, 2, 4 triazole
3. Expression of Soluble RFP-1 (amino acids 1-329) in E.coli

It was paramount to express RFP-1 in order to independently confirm the interaction between RFP-1 and UBC-1 and to study the function of RFP-1. Unfortunately, expressing RFP-1 proved to be difficult and the inability to produce full length RFP-1 limited functional studies. Several attempts were made to express soluble, full length RFP-1 (amino acids 1 to 839) in E. coli, insect cell lines Kcl, Sf9, and High Five, and also in rabbit reticulocyte lysates. Full length RFP-1 was not expressed in any of the insect cell lines studied, nor was it expressed to any appreciable level in rabbit reticulocyte lysates. While E. coli could express small quantities of recombinant full length RFP-1, the protein was entirely insoluble (data not shown).

A partially soluble truncation of RFP-1, the 38 kDa amino terminal fragment, was expressed in bacteria as described in Materials and Methods. Figure 11 (lane 1) contained extract from un-induced BL21 (DE3) bacteria, while lane 2 clearly shows strong expression of RFP-1 (amino acids 1-329 with a C-terminal 6 x His Tag). Lane 3 contained the insoluble fraction and lane 4 contained the soluble fraction after sonicating the induced bacteria. The results show that the 38 kDa RFP-1 truncation protein is partially soluble. This soluble fraction was used for subsequent co-immunoprecipitation studies.

4. RFP-1 AND UBC-1 CO-IMMUNOPRECIPITATION EXPERIMENTS

4.1. Confirmation of the Interaction Between UBC-1 and RFP-1: Co-immunoprecipitation Experiments in C. elegans Extract

As an independent approach to study the interaction between UBC-1 and RFP-1, co-immunoprecipitation experiments were performed. Each experiment contained C. elegans protein extract, ATP, ubiquitin, and recombinant UBC-1 in a final reaction volume of 300 μl. Worm protein extract was included to provide any co-factors necessary for the interaction. ATP and ubiquitin were present in case UBC-1 required modification with ubiquitin in order for it to interact with RFP-1. To examine the interaction between UBC-1 and RFP-1, soluble RFP-1 (amino acids 1-329 with a C-terminal 6 x His Tag) was added to each of two tubes containing the above components, along with either polyclonal RFP-1 antibodies or polyclonal Hsp43.
Figure 11: Expression of a Partially Soluble RFP-1 Truncation Protein (amino acids 1-329 with a C-terminal histidine tag)

The expression of RFP-1 (amino acids 1-329 with a C-terminal histidine tag) in BL21 (DE3) bacteria was induced as described in Materials and Methods. After sonication and centrifugation, the soluble and insoluble protein fractions were separated by electrophoresis on a 15% SDS polyacrylamide gel and Coomassie blue stained. Lane 1 is a protein sample of the bacterial culture before induction with 0.1 mM IPTG. Lane 2 shows strong expression of the 38 kDa RFP-1 (amino acids 1-329 with a C-terminal 6 x His Tag) after 16 hours of IPTG induction at 15°C. Lane 3 contains the insoluble protein fraction. Lane 4 contains the soluble protein fraction. The 38 kDa RFP-1 derivative was partially soluble.
antibodies (as a negative control). The protein-antibody complexes were captured on 10% 
*Staphylococcus aureus*, washed twice with Co-immunoprecipitation Buffer, and analysed by 
Western blotting with a monoclonal antibody against UBC-1 (Figure 12).

In the absence of any antibody or *S. aureus*, UBC-1 remains soluble (Figure 12, lane 2). UBC-1 did not bind to the *S. aureus* in the absence of any antibody (lane 3), nor did it bind to *S. aureus* with the addition of RFP-1 antibody in the absence of recombinant RFP-1 protein (lane 4). UBC-1 was not immunoprecipitated with Hsp43 antibody either with (lane 7) or without (lane 5) RFP-1. RFP-1 antibodies immunoprecipitated recombinant UBC-1 only when RFP-1 protein (1-329) was added to the extract (lane 6).

### 4.2. UBC-1 and RFP-1 Interact in the Absence of ATP, Ubiquitin, or *C. elegans* Extract

The co-immunoprecipitation experiments were repeated in the absence of *C. elegans* 
protein extract, ATP, or ubiquitin to determine if any of these three components were important 
for the UBC-1 and RFP-1 interaction. RFP-1 antibodies co-immunoprecipitate UBC-1 and RFP-1 
in buffer that was not supplemented with either ubiquitin or ATP (Figure 13, lane 2). Similarly, RFP-1 antibodies co-immunoprecipitated UBC-1 and RFP-1 in extract that was not 
supplemented with ubiquitin or ATP (lane 3). In the negative controls, Hsp43 antibodies did not 
co-immunoprecipitate UBC-1 and RFP-1 (lanes 4 and 5).

### 5. EXPRESSION STUDIES OF RFP-1 AND UBC-1 IN *C. elegans*

Having detected and mapped the interaction between UBC-1 and RFP-1 *in vitro*, 
experiments were performed in *C. elegans* to examine the tissue specificity of RFP-1 and UBC-1. A similar pattern of tissue expression of RFP-1 and UBC-1 would strengthen the hypothesis 
that these two proteins interact *in vivo*.

#### 5.1. RFP-1 is Expressed in All Life Stages of *C. elegans*

Five developmental stages (embryos, L1 larvae, L2 larvae, L3/L4 larvae, and young adults) from a synchronous population of N2 *C. elegans* animals were collected and protein 
extracts of each stage were prepared as described in Materials and Methods. The protein extracts
<table>
<thead>
<tr>
<th></th>
<th>N2 extract</th>
<th>ATP + Ubiquitin</th>
<th>UBC-1</th>
<th>1-329 RFP-1</th>
<th>Hsp 43 Ab</th>
<th>RFP-1 Ab</th>
<th>10% S. aureus</th>
<th>Lane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>ATP + Ubiquitin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>1-329 RFP-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Hsp 43 Ab</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>RFP-1 Ab</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>6</td>
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<tr>
<td>10% S. aureus</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
</tbody>
</table>

**Figure 12: RFP-1 and UBC-1 Co-Immunoprecipitate in C. elegans Extract**

Protein extracts of adult *C. elegans* were prepared as described in Materials and Methods. Each co-immunoprecipitation experiment contained 50 μg of N2 worm protein extract, 2 mM ATP, 4 μg of ubiquitin, and 4 μg of recombinant UBC-1 in a final reaction volume of 300 μl of Co-immunoprecipitation Buffer. 50 μg of soluble RFP-1 (amino acids 1-329 with a C-terminal 6 x His Tag) was added along with either 2.5 μg polyclonal RFP-1 antibodies (Ab) or 2.5 μg polyclonal Hsp43 antibodies as indicated. The protein-antibody complexes were captured on a 10% suspension of *Staphylococcus aureus* cells, washed twice with Co-immunoprecipitation Buffer, eluted, and separated by SDS PAGE. The gel was electro-blotted and probed with a monoclonal UBC-1 antibody to detect the 20 kDa UBC-1 in the immunoprecipitates. The positions of IgH, heavy chains of precipitating antibodies, and UBC-1 are indicated.
<table>
<thead>
<tr>
<th>Buffer</th>
<th>N2 extract</th>
<th>UBC-1</th>
<th>1-329 RFP-1</th>
<th>RFP-1 Ab</th>
<th>Hsp 43 Ab</th>
<th>Lane</th>
</tr>
</thead>
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<td></td>
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<td>-</td>
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</tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 13: RFP-1 and UBC-1 Co-Immunoprecipitate in Buffer Lacking ATP and Ubiquitin

Protein extracts of adult *C. elegans* were prepared as described in Materials and Methods. Recombinant UBC-1 (4 µg) was added to either 50 µg worm protein extract or Co-immunoprecipitation Buffer as indicated. The immunoprecipitation experiments were not supplemented with ATP or ubiquitin. 50 µg of soluble RFP-1 (amino acids 1-329 with a C-terminal 6 x His Tag) was added, followed by either 2.5 µg polyclonal RFP-1 antibodies (Ab) or 2.5 µg polyclonal Hsp43 antibodies, as indicated. The protein-antibody complexes were captured on a 10% suspension of *Staphylococcus aureus* cells, washed twice with Co-immunoprecipitation Buffer, eluted, and separated by SDS PAGE. The gel was electro-blotted and probed with a monoclonal antibody to UBC-1. The positions of UBC-1 and IgH, heavy chains of the precipitating antibodies, are indicated.
were analysed by Western blotting for the presence of RFP-1 (97.6 kDa) using polyclonal antibodies generated against the C-terminus of RFP-1. Figure 14, A shows that RFP-1 is expressed in all life stages of *C. elegans*. RFP-1 expression is high in the embryonic, L1 and L2 stages (lanes 1 through 3) and appears to decrease slightly in the L4 (lane 4) and young adult (lane 5) stages. Extracts of the L2 larval stage appear to have enhanced proteolytic activity. As a control for protein loading, the extracts were also probed with a monoclonal antibody against actin. Except for a slightly lower intensity in embryos, the actin concentration was similar in all stages (Figure 14, B).

5.2. RFP-1 Indirect Immunohistochemical Staining of *C. elegans* Embryos

RFP-1 indirect immunohistochemical staining of N2 *C. elegans* embryos was performed as described in Materials and Methods. Briefly, embryos were extracted from gravid adults, formaldehyde-fixed, rinsed, blocked, and incubated with RFP-1 polyclonal antibodies. The embryos were rinsed, incubated with Alexa-488 anti-rabbit secondary antibody, resuspended in mounting medium, and placed on slides for viewing by fluorescence microscopy. In a negative control, embryos incubated only with the secondary anti-rabbit antibody show very weak background staining (Figure 15, A). In a competition experiment, the RFP-1 antibodies were pre-incubated with purified RFP-1 protein to "titrate" away the RFP-1 antibody from the RFP-1 in the embryo and demonstrate the antibody specificity (Figure 15, B). RFP-1 is a nuclear protein in 40 cell stage embryos (Figure 15, C), late stage embryos (Figure 15, D), and three-fold stage embryos (Figure 15, E). RFP-1 is excluded from nucleoli (Figure 15, D and E).
Figure 14: RFP-1 is Expressed in All Life Stages of *C. elegans*

Protein extracts of *C. elegans* embryos (E), larval stage 1 (L1), larval stage 2 (L2), larval stages 3 and 4 (L3/L4), and young adults (YA) were prepared as described in Materials and Methods. The proteins were separated by electrophoresis on 12.5% SDS polyacrylamide gels, transferred to a PVDF membrane, and probed with polyclonal RFP-1 antibodies generated against the RFP-1 C-terminal region (A). The 97.6 kDa RFP-1 is expressed in all life stages of *C. elegans*. As a control for protein loading, the protein extracts from the five developmental stages were probed with a monoclonal antibody against actin (48 kDa) (B).
Figure 15. RFP-1 is Localised to the Nucleus in *C. elegans* Embryos

RFP-1 indirect immunohistochemical staining of N2 *C. elegans* embryos was performed as described in Materials and Methods. A, negative control in which the embryos were incubated with only the secondary anti-rabbit antibody (1:200). B, competition experiment in which the RFP-1 antibodies (1:400) were pre-incubated with 0.25 mg of purified RFP-1 protein. C, a 40 cell stage embryo (approximate stage) that has been treated with RFP-1 (1:400) antibodies followed by Alexa-488 anti-rabbit secondary antibodies (1:200). D, a late stage embryo that has been treated with RFP-1 antibodies followed by Alexa-488 anti-rabbit secondary antibodies. RFP-1 is excluded from the nucleolus. E, a three-fold stage embryo stained for RFP-1. The scale bar represents 30 μm.
5.3. RFP-1 Indirect Immunohistochemical Staining of C. elegans Larvae and Adults

To examine the tissue specificity of RFP-1 in larvae and adults, RFP-1 indirect immunohistochemical staining of a mixed population of N2 C. elegans animals was performed as described in Materials and Methods. The results are presented in Figure 16 (A to F). A very weak overall staining was observed with the secondary antibody alone on all animals, represented by the LI shown (A). In a competition experiment, there was a general RFP-1-independent staining pattern observed in the L2 animal. The buccal opening and the posterior bulb of the pharynx (grinder) stained particularly strongly in an RFP-1 independent manner, suggesting that the antibody may have cross-reacted with bacteria in the pharynx (B).

In L1 larvae, specific RFP-1 staining was observed in the nuclei of pharyngeal, intestinal, and nerve ring cells (C). RFP-1 was not located in the nucleoli, as these subcellular compartments remained dark. The nuclear staining of RFP-1 in most tissues was observed in L2, L3, and L4 animals (data not shown). In adult animals, RFP-1 was localised to the nuclei of oocytes and germ cells in the syncytial gonad. Again, the nucleoli did not stain for RFP-1 (E). Competition with RFP-1 abolished this signal (D). RFP-1 nuclear staining was observed in pharyngeal and intestinal cell nuclei in the head region of the adult animal (F).

5.4. UBC-1 Indirect Immunohistochemical Staining of C. elegans Larvae and Adults

As RFP-1 was a nuclear protein in embryos, larvae, and adults, it was hypothesised that UBC-1 should be localised to the same regions if indeed UBC-1 and RFP-1 interacted in vivo.

To determine the tissue specificity of UBC-1 in N2 animals, UBC-1 indirect immunohistochemical staining was performed as described in Materials and Methods. Unfortunately, both the polyclonal and monoclonal UBC-1 antibodies worked poorly for this purpose, giving high non-specific background staining (data not shown). This was particularly true for embryos. Attempts to affinity purify the UBC-1 antibodies to increase their specificity were unsuccessful. Despite these specificity problems, some limited staining data were obtained on adults.
Figure 16. RFP-1 is Localised to the Nucleus in *C. elegans* Larvae and Adults

RFP-1 indirect immunohistochemical staining of a mixed population of larvae and adults was performed as described in Materials and Methods. A, negative control in which the animals, represented by the L1 shown, were incubated only with Alexa-488 anti-rabbit secondary antibodies (1:200). B, competition experiment in which the RFP-1 antibodies were pre-incubated with 0.25 mg of purified RFP-1 protein, followed by incubation with the Alexa-488 anti-rabbit secondary antibodies (1:200). The L2 shown was representative of the general RFP-1-independent staining pattern. C, L1 animal from a mixed population of N2 animals incubated with RFP-1 antibodies (1:500), followed by Alexa-488 anti-rabbit secondary antibodies (1:200). D, the gonad of an adult from a competition experiment in which RFP-1 antibodies were pre-incubated with 0.25 mg of purified RFP-1 protein. E, gonad of an adult animal incubated with RFP-1 antibodies (1:500), followed by incubation with Alexa-488 anti-rabbit secondary antibodies (1:200). F, RFP-1 nuclear staining in pharyngeal and intestinal cell nuclei in the head region of an adult animal. The anterior of the animal is to the left. The scale bar represents 50 μm.
A mixed population of N2 C. elegans animals was fixed with paraformaldehyde and immunostained with UBC-1 polyclonal antibodies as described in Materials and Methods. As a negative control, the anti-UBC-1 primary antibodies were omitted from the procedure (data not shown). The specificity of the observed staining pattern was tested in competition experiments, in which UBC-1 primary antibodies were pre-incubated with 0.25 mg of purified UBC-1. The adult gonad shows an overall staining pattern that is UBC-1-independent. Note, however, that the oocyte nuclei do not stain for UBC-1 (Figure 17, A). In contrast, oocyte nuclei stain specifically for UBC-1 (Figure 17, B).

6. RNA INTERFERENCE STUDIES

Double stranded RNA-mediated interference (RNAi) is a reverse-genetics tool used to study gene function in C. elegans. The introduction of double stranded RNA (dsRNA) results in strong and specific inactivation of the corresponding gene due to degradation of endogenous mRNA. RNA interference can cross cell boundaries and cause potent gene-specific interference in the progeny of the RNAi-treated animals (Fire et al., 1998; Montgomery et al., 1998).

The two methods of introducing dsRNA into C. elegans are the injection method, whereby dsRNA is synthesised and injected into the gonad of a young adult, or the feeding method, in which animals are fed E.coli expressing dsRNA under the control of IPTG-inducible T7 promoters (Fire et al., 1998; Timmons and Fire, 1998; Timmons et al., 2001). The ingested dsRNA is absorbed through the gut and distributed to the germ line and somatic tissues. The optimised feeding method is less labour-intensive than the injection method, and is conducive to performing RNAi on a large number of genes (Kamath et al., 2000). Regardless of the RNAi method employed, the development of the progeny of treated hermaphrodites is examined for a phenotype produced by the inactivation of the corresponding gene.

Several recent studies in C. elegans and D. melanogaster suggest that RNAi acts at the posttranscriptional level to degrade mRNAs complementary to the dsRNA (Matzke et al., 2001). The mechanism of RNAi involves cleavage of the initiating dsRNA into sense and antisense RNAs 21 to 23 nucleotides long by a dsRNA endonuclease called DCR-1 (C. elegans)
Figure 17. UBC-1 is Localised to the Nucleus in *C. elegans* Oocytes

UBC-1 indirect immunohistochemical staining of a mixed population of larvae and adults was performed as described in Materials and Methods.  

A, competition experiment in which the UBC-1 antibodies (1:200) were pre-incubated with 0.25 mg of purified recombinant UBC-1 protein, followed by incubation with Alexa-488 anti-rabbit secondary antibodies (1:200).  

B, UBC-1 staining of an adult gonad incubated with UBC-1 antibodies (1:200), followed by Alexa-488 anti-rabbit secondary antibodies (1:200). Note the nuclear staining of UBC-1 in the oocytes. The scale bar represents 30 μm.
(Knight and Bass, 2001). These small interfering RNAs (siRNA) combine with a 500 kDa nuclease called the RNA-Induced Silencing Complex (RISC), a component of which is *C. elegans* RDE-1 or the *D. melanogaster* homologue Argonaute2 (Tabara *et al.*, 1999; Hammond *et al.*, 2001). The siRNAs act as guides that, in conjunction with RISC, bind to endogenous homologous mRNAs and degrade them. Both *dcr-1* and *rde-1* are required for RNA interference, as animals carrying null mutations in either gene do not respond to injected or ingested dsRNA against several genes tested (Tabara *et al.*, 1999; Knight and Bass, 2001).

### 6.1. RNA Interference Against *ubc-1* or *rfp-1* in *C. elegans*

To investigate the *in vivo* function of *rfp-1* and *ubc-1*, double stranded *ubc-1* RNA (approximately 1.7 kb) or double stranded *rfp-1* RNA (approximately 2.6 kb) was produced and RNA interference experiments were performed as described in Materials and Methods. Each dsRNA was microinjected into the syncytial gonads of young adult N2 *C. elegans*. The number of hatched embryos (F1 generation) was scored and their development at 20°C was followed. A GFP/UNC-54 *C. elegans* strain was used as a positive control for RNA interference by microinjecting these animals with double stranded *gfp* RNA and examining the hermaphrodite and its F1 progeny for loss of GFP fluorescence. The results of injecting hermaphrodites with double stranded RNA against *ubc-1* or *rfp-1* are summarised in Table 9.

Double stranded RNA interference (RNAi) against *ubc-1* or *rfp-1* was also performed by the feeding method as described in Materials and Methods. Briefly, HT115 (DE3) bacteria were transformed with either plasmid pPD129.36::UBC-1 or plasmid pPD129.36::RFP-1 nt 3-982 and induced with IPTG to express double stranded *ubc-1* RNA or double stranded *rfp-1* (nt 3-982) RNA. The induced bacteria were spread on plates to produce bacterial lawns as a food source for N2 hermaphrodites. The number of hatched embryos (F1 generation) from the treated hermaphrodites was scored and their development at 20°C was followed. As a negative control, L4 hermaphrodite N2 animals were fed HT115 (DE3) bacterial lawns containing the empty pPD129.36 vector (no double stranded RNA produced) and the development of the F1 generation was followed. Table 9 summarises the effect of *ubc-1* or *rfp-1* RNA interference on the embryonic viability and development of the progeny of treated hermaphrodites.
Table 9: Summary of the effect of double stranded RNA interference against rfp-1 or ubc-1 on embryonic viability and development

<table>
<thead>
<tr>
<th>C. elegans Strain</th>
<th>Double Stranded RNA</th>
<th>Method ofRNA Interference</th>
<th>Total Number of Embryos</th>
<th>Percent Viability of Embryos</th>
<th>Normal Development to Adult Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP/UNC-54</td>
<td>ubc-1</td>
<td>Injection</td>
<td>2410</td>
<td>93</td>
<td>yes</td>
</tr>
<tr>
<td>GFP/UNC-54</td>
<td>ubc-1 + gfp</td>
<td>Injection</td>
<td>1855</td>
<td>96</td>
<td>yes</td>
</tr>
<tr>
<td>N2</td>
<td>ubc-1</td>
<td>Injection</td>
<td>3226</td>
<td>100</td>
<td>yes</td>
</tr>
<tr>
<td>GFP/UNC-54</td>
<td>rfp-1</td>
<td>Injection</td>
<td>1161</td>
<td>94</td>
<td>no</td>
</tr>
<tr>
<td>N2</td>
<td>rfp-1</td>
<td>Injection</td>
<td>1422</td>
<td>97</td>
<td>no</td>
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<tr>
<td>N2</td>
<td>empty pPD129.36</td>
<td>Feeding</td>
<td>543</td>
<td>100</td>
<td>yes</td>
</tr>
<tr>
<td>N2</td>
<td>ubc-1</td>
<td>Feeding</td>
<td>1218</td>
<td>96</td>
<td>yes</td>
</tr>
<tr>
<td>N2</td>
<td>rfp-1 (nt.3-982)</td>
<td>Feeding</td>
<td>1663</td>
<td>100</td>
<td>no</td>
</tr>
</tbody>
</table>

Table 9 shows that no obvious phenotype was induced by RNA interference against ubc-1, either by injection or feeding double stranded ubc-1 RNA. RNA interference against ubc-1 in either N2 or GFP/UNC-54 animals had no deleterious effects on embryonic viability, i.e. embryos hatched as L1 animals with normal developmental timing. Moreover, these F1 animals progressed through the four larval stages to become egg-laying adults, with no obvious abnormalities. RNA interference against rfp-1 in either N2 or GFP/UNC-54 animals had no visible effect on embryonic viability.

6.2. RNA Interference Against rfp-1 in C. elegans Results in Abnormal Vulval Eversion and Egg-laying Defects

The development of the F1 progeny of N2 or GFP/UBC-54 hermaphrodites exposed to double stranded rfp-1 RNA was followed. After hatching, the F1 animals progressed through the
four larval stages to adulthood with varying degrees of success. The \textit{rfp-1} RNA interference-induced phenotypes of the F1 progeny are summarised in Table 10.

Table 10: Summary of the phenotypes of the F1 progeny of hermaphrodites exposed to double stranded RNA interference against \textit{rfp-1}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{C. elegans Strain} & \textbf{GFP/UNC-54} & \textbf{N2} & \textbf{N2} \\
\hline
\textbf{\textit{rfp-1} RNA Interference Method} & Injection & Injection & Feeding \\
\hline
\textbf{Total Number of Embryos} & 1161 & 1422 & 1663 \\
\hline
\textbf{Percent Dead Larvae L1 + L2} & 1 & 1 & ND * \\
\textit{(actual number)} & (10) & (15) & \\
\hline
\textbf{Percent L3 Arrested Larvae} & 3 & 5 & 2 \\
\textit{(actual number)} & (38) & (77) & (32) \\
\hline
\textbf{Percent Adults with Everted Vulvas} & 4 & 2 & 10 \\
\textit{(actual number)} & (45) & (28) & (168) \\
\hline
\textbf{Percent Egg-laying Defective} & 7 & 6 & 5 \\
\textbf{Adults} & (78) & (80) & (79) \\
\textit{(actual number)} & & & \\
\hline
\end{tabular}
\end{table}

* No Data: The number of dead larvae were not assessed in this sample of 1663 F1 animals.

Although only 1% of the larvae died, 2% to 5% of the larvae arrested at the L3 stage (Table 10). Overall, the F1 generation was developmentally delayed and this slow growth phenotype was later confirmed by Kamath \textit{et al.} (2003). While many of the larvae became egg-laying adults, approximately 2% to 10% of the young adults had everted vulvas that subsequently burst. Intestinal and gonadal tissue extruded from the body cavity. Approximately 7% of the adults had egg-laying defects characterised by a bloated appearance (a bag of eggs) and these animals laid few eggs. Figure 18 shows the abnormally everted vulva of an F1 adult from a hermaphrodite injected with double stranded RNA against \textit{rfp-1}.
Figure 18. RNA Interference Against rfp-1 Produces an Everted Vulva Phenotype

Nomarski photomicrograph of an F1 adult *C. elegans* exposed to *rfp-1* RNA. The most striking phenotype was the development of an abnormally everted vulva. A, an animal which has already begun to burst. The anterior is towards the top. After vulval eversion, the animals exposed to *rfp-1* RNA burst and died. These animals were typically bloated with embryos (B). The anterior of the animal is to the left. The scale bars represent 30 μm.
7. RFP-1 AND THE SYNTHETIC MULTIVULVA PATHWAY

Retinoblastoma protein (pRB) is a tumour suppressor protein found mutated in several cancers (Taya, 1997). In their yeast two-hybrid screen for proteins that interact with \textit{H. sapiens} pRb, Wen and Ao (2000) identified a novel leucine zipper-containing protein called Retinoblastoma Binding Protein 95 (RBP95) that interacted with pRb. RBP95 had a conserved pRb-binding motif Leu-X-Cys-X-Glu (LXCXE) which was required for the interaction with pRb. RBP95 was localised to the nucleus in HeLa cells and RBP95 and pRb co-immunoprecipitated \textit{in vitro}. These data were of interest because the isolated RBP95 cDNA was an alternatively spliced product of \textit{H. sapiens} KIAA0661, such that RBP95 and K1AA0661 contained different C-termini upon translation. Sequence analysis of RFP-1 showed 49% similarity to KIAA0661 and 33% similarity to RBP95. Moreover, RFP-1 contains the LXCXE motif, suggesting it could interact with pRb. Since RFP-1 binds to UBC-1, it was of great interest to determine if RFP-1 also interacted with pRb, potentially directing the ubiquitin-proteasome pathway towards pRb.

The \textit{C. elegans} pRb is encoded by \textit{lin-35}, a member of the Synthetic Multivulva Pathway (SynMuv Pathway) that inhibits differentiation of vulval precursor cells. The vulva is derived from six vulval precursor cells (VPC): P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p. All six VPCs have the ability to take on the vulval cell fate, but in wild type animals P6.p has the primary vulval cell fate, P5.p and P7.p take on the secondary vulval cell fate, and P3.p, P4.p, and P8.p receive the tertiary non-vulval cell fate. Vulval formation is regulated through inductive signalling from the gonadal anchor cell, lateral signalling between adjacent VPCs, and inhibitory signalling from hyp7, a syncytial hypodermal cell (Kornfeld, 1997).

The SynMuv genes define two functionally redundant pathways that negatively regulate expression of vulval cell fates. As their name suggests, SynMuv genes are loss of function mutations that result in a multivulva phenotype due to all VPCs taking the vulval cell fate. SynMuv genes are divided into two classes A and B, and animals must carry a mutation in a gene from both A and B classes to have a multivulva phenotype (Ferguson and Horvitz, 1989). It is thought that \textit{lin-35}, a Class B SynMuv gene, inhibits transcription of genes required for vulval differentiation by binding to transcription factors (Lu and Horvitz, 1998) and recruiting a
nucleosome remodelling and histone deacetylase complex to repress vulval development genes (Solari and Ahringer, 2000).

To determine if \textit{rfp-1} belongs to the SynMuv pathway and binds to \textit{LIN-35}, double stranded RNA interference against \textit{rfp-1} in both a Class A mutant background (\textit{lin-15A}) and a Class B mutant background (\textit{lin-15B}) were performed. A multivulva phenotype in the progeny of treated animals would be good evidence to place \textit{rfp-1} in this genetic pathway. If RFP-1 and LIN-35 do indeed interact, this might link the ubiquitin-proteasome pathway to vulval differentiation and transcriptional repression. Interestingly, RFP-1 and LIN-35 are both nuclear proteins in embryos and L1 animals. LIN-35 expression becomes limited to nuclei of certain cells in the adult's head and tail regions and in the nuclei of vulval precursor cells (Lu and Horvitz, 1998).

7.1. Double Stranded RNA Interference Against \textit{rfp-1} in \textit{lin-15A (n767)} and \textit{lin-15B (n744)} Mutant Backgrounds

Double stranded RNA interference experiments against \textit{rfp-1} were performed in \textit{lin-15A (n767)} and \textit{lin-15B (n744)} mutants as described in Materials and Methods. The clone pPD129.36::RFP-1 nt 3-982 was used as the template for bi-directional transcription of \textit{rfp-1} in HT115 (DE3) bacteria. The number of hatched embryos from RNAi-treated \textit{lin-15A (n767)} or \textit{lin-15B (n744)} hermaphrodites was scored at 15°C or 20°C and the development of the progeny was followed. As a negative control, L4 \textit{lin-15A (n767)} or L4 \textit{lin-15B (n744)} hermaphrodites were placed on OP50 bacterial lawns (no exposure to double stranded RNA).

While RNA interference against \textit{rfp-1} in a \textit{lin-15A (n767)} or a \textit{lin-15B (n744)} background had no effect on embryonic viability (hatching), the progeny displayed similar phenotypes to N2 animals exposed to RNA interference against \textit{rfp-1}. Table 11 summarises these results. The data at 15°C and 20°C were combined for each RNA interference experiment. There is no significant difference between RNA interference at 15°C versus 20°C (Kamath et al., 2000).
Table 11: Summary of *rfp-l* RNA interference-induced phenotypes in *lin-15A (n767)* and *lin-15B (n744)* animals

<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Double Stranded RNA</td>
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<td><em>rfp-l</em></td>
<td>none *</td>
<td><em>rfp-l</em></td>
</tr>
<tr>
<td>Total Number of Embryos</td>
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<td>1587</td>
<td>773</td>
<td>2800</td>
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<tr>
<td>Percent Dead Larvae</td>
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<td>0.4</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td>(actual number)</td>
<td>(0)</td>
<td>(7)</td>
<td>(0)</td>
<td>(20)</td>
</tr>
<tr>
<td>Percent Delayed Larvae</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>(actual number)</td>
<td>(0)</td>
<td>(111)</td>
<td>(0)</td>
<td>(60)</td>
</tr>
<tr>
<td>Percent Adults with Everted Vulvas</td>
<td>0</td>
<td>11</td>
<td>2</td>
<td>57</td>
</tr>
<tr>
<td>(actual number)</td>
<td>(0)</td>
<td>(181)</td>
<td>(12)</td>
<td>(1606)</td>
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<tr>
<td>Percent Egg-laying Defective Adults</td>
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<td>8</td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td>(actual number)</td>
<td>(0)</td>
<td>(134)</td>
<td>(1)</td>
<td>(2800)</td>
</tr>
<tr>
<td>Percent Adults with Multivulvas</td>
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<td>0.1</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>(actual number)</td>
<td>(0)</td>
<td>(2)</td>
<td>(0)</td>
<td>(14)</td>
</tr>
</tbody>
</table>

* Control experiments in which animals were raised on OP50 bacteria and were not exposed to *rfp-l* dsRNA.

Table 11 shows that the F1 progeny of *lin-15A (n767)* hermaphrodites raised on OP50 bacterial lawns did not display phenotypes such as delayed larval development, multiple vulvas, vulval eversion and rupture, or egg-laying defects. Interestingly, 2% of the F1 progeny of *lin-15B (n744)* hermaphrodites raised on OP50 plates displayed an everted vulva phenotype.

When raised on HT115 (DE3) bacterial lawns expressing double stranded *rfp-l* RNA, the progeny of *lin-15A (n767)* hermaphrodites displayed numerous phenotypes that were not more severe than the *rfp-l* RNAi-induced phenotypes in N2 animals (Table 11 and Table 10). Approximately 7% of the F1 *lin-15A (n767)* generation were delayed in the L2 and L3 larval
stages, but subsequently developed to adulthood. Eleven percent (11%) of the F1 generation developed abnormal vulval eversion (evl), followed by rupture, much like N2 animals exposed to rfp-1 double stranded RNA interference. While most of the F1 animals developed into egg-laying adults, 8% had egg-laying defects (egl) and these animals looked like bags of hatching embryos. Finally, there were virtually no F1 lin-15A (n767) animals (0.1%) with multiple vulvas (muv) in these rfp-1 RNA interference experiments. The absence of a multivulva phenotype genetically excludes rfp-1 from being a Class B synthetic multivulva gene.

Table 11 also presents the results of raising the progeny of lin-15B (n744) hermaphrodites on HT115 (DE3) bacterial lawns expressing double stranded rfp-1 RNA. While only 2% of the F1 generation experienced delays in the L2 and L3 stages, 57% of the lin-15B (n744) F1 generation consisted of adults with abnormally everted vulvas, which subsequently ruptured. Moreover, all the F1 adults appeared slow and sickly and failed to lay eggs. Despite this dramatic phenotype, only 0.5% of the F1 generation had the multivulva phenotype (muv) when exposed to rfp-1 double stranded RNA interference. The absence of a multivulva phenotype genetically excludes rfp-1 from being a Class A synthetic multivulva gene.

The enhanced evl and egl phenotypes in the Class B mutant background suggest that rfp-1 interacts in a genetic pathway that has a more general role in vulval structure. The lin-15B (n744) strain itself does not appear wild type as 2% of the adults had everted vulvas in the control experiments (Table 11). When the lin-15B (n744) animals were exposed to rfp-1 RNAi, the inherent vulval “weakness” in the lin-15B (n744) strain was clearly exacerbated.

7.2. RFP-1 and LIN-35 Yeast Interaction Trap

To determine if RFP-1 directly interacts with LIN-35, a Class B SynMuv protein, a yeast interaction trap was performed. Yeast strain Y190 was transformed with pAS2::LIN-35 (aa 467-961) or pACT2::LIN-53. The pAS2::LIN-35 (467-961) construct encompasses the A and B pockets of pRb that bind to E2F and viral oncoproteins, and presumably other unidentified partners (Taya, 1997). The pACT2::LIN-53 construct encodes full length LIN-53, which is a known binding partner of LIN-35 in vitro and is 72% identical to Rb-associated protein 48 (Lu and Horvitz, 1998).
Protein extracts of cultured transformants were prepared, separated by SDS PAGE, and transferred to a PVDF membrane. The membrane was probed with a monoclonal antibody against the hemagglutinin tag to detect expression of the 77 kDa GAL4 DNA binding domain: LIN-35 fusion protein or a monoclonal antibody against the GAL4 activation domain to detect the 64 kDa GAL4 activation domain: LIN-53 fusion protein (Figure 19).

Figure 20 summarises the results of the LIN-35 and RFP-1 interaction trap experiments. Y190 carrying pAS2::LIN-35 (467-961) was transformed with either no construct, or empty pACT2 vector, or full length RFP-1 in pACT, or each of the four RFP-1 derivatives in pACT2. As a positive control, yeast were transformed with pAS1-CYH2::UBC-1 and pACT::RFP-1. For a specificity test, Y190 bearing pAS2::LIN-35 (467-961) was transformed with SNF4 in pACT. Y190 bearing pAS2::LIN-35 (467-961) was transformed with pACT2::LIN-53, a known binding partner of LIN-35 (Lu and Horvitz, 1998). The Leu + Trp + transformants were tested for β-galactosidase activity upon exposure to X-gal using the X-gal Colony Filter Lift Assay. The transformants were also tested for their ability to activate transcription of the His3 reporter gene by plating them on - Trp - Leu - His SC + 35 mM 3-aminotriazole plates.

As shown in Figure 20, the pAS2::LIN-35 (aa 467-961) construct does not activate transcription of lacZ or His3 independently. The LIN-35 (467-961) bait neither interacts with full length RFP-1 nor with the four RFP-1 truncations. Surprisingly, the LIN-35 (467-961) bait also failed to interact with LIN-53, a binding partner of LIN-35 in vitro.

8. THE UBC-1 MUTANT

To study the function of ubc-1 in vivo, the C. elegans Reverse Genetics Core Facility at the University of British Columbia used formaldehyde to create a putative deletion mutation in ubc-1 (C35B1.1) called ubc-1(gk14).
Figure 19: The LIN-53 and LIN-35 (467-961) Constructs Used in Yeast Interaction Traps are Expressed in Y190.

Yeast strain Y190 was transformed with no construct, pAS2::LIN-35 (467-961), or pACT2::LIN-53 and protein extracts of the transformants were separated by electrophoresis on SDS polyacrylamide gels, transferred to PVDF membranes, and probed with the indicated antibodies (Ab) to detect expression of the GAL4 DNA binding domain (DBD) or GAL4 activation domain (ACT) fusion proteins. An asterisk (*) indicates the position of the fusion protein of interest. HA-tag antibodies recognize the hemagglutinin tag in the GAL4 DNA binding domain: LIN-35 (467-961) fusion protein.
Yeast strain Y190 was transformed with the bait plasmid pAS2::LIN-35 (amino acids 467-961) and tested for β-galactosidase activity and the ability to grow in the absence of histidine. Y190 carrying the pAS2::LIN-35 (amino acids 467-961) bait was then singly transformed with the indicated GAL4 activation domain constructs. As a positive control, Y190 was transformed with pAS1-CYH2::UBC-1 and pACT::RFP-1. Trp + Leu + transformants were assayed for lacZ transcription, resulting in β-galactosidase activity, using the X-gal Colony Filter Lift Assay. Transformants were also tested for His3 transcription by assaying for growth on -Leu - Trp - His SC plates containing 35 mM 3-amino-1, 2, 4 triazole. Lack of transcriptional activation is denoted with a blank (-) and activation of lacZ or His3 is denoted with a plus sign (+). Grey boxes represent helical regions in RFP-1. The basic leucine zipper (residues 313-375) together with helix 3 (amino acids 303-353) and helix 4 (amino acids 376-399) are represented by a divided box. Hatched boxes represent the C3HC4 RING finger domain in RFP-1.
<table>
<thead>
<tr>
<th>GAL4 DNA Binding Domain Construct</th>
<th>GAL4 Activation Domain Construct</th>
<th>β-galactosidase Activity</th>
<th>Growth on - Histidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAS2::LIN-35 (aa 467-961)</td>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pAS2::LIN-35 (aa 467-961)</td>
<td>Empty pACT2 Vector</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pAS2::LIN-35 (aa 467-961)</td>
<td>pACT::SNF4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pAS2::LIN-35 (aa 467-961)</td>
<td>RFP-1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pAS2::LIN-35 (aa 467-961)</td>
<td>aa 1-313</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pAS2::LIN-35 (aa 467-961)</td>
<td>aa 1-183</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pAS2::LIN-35 (aa 467-961)</td>
<td>aa 184-313</td>
<td>-</td>
<td>-</td>
</tr>
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<td>pAS2::LIN-35 (aa 467-961)</td>
<td>aa 408-839</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pAS1-CYH2::UBC-1</td>
<td>RFP-1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pAS2::LIN-35 (aa 467-961)</td>
<td>pACT2::LIN-53</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
8.1. The *ubc-1*(gk14) Strain is Not Deleted for *ubc-1*

Protein extracts of wild type and *ubc-1*(gk14) animals were prepared, fractionated by SDS PAGE, transferred to a PVDF membrane, and probed with a monoclonal UBC-1 antibody. Figure 21, A shows that both wild type (lane 3) and *ubc-1*(gk14) (lane 4) animals had a UBC-1-reactive band at 20 kDa. Lane 1 contained 50 ng of purified UBC-1 (20 kDa) and lane 2 contained 50 ng of purified UBC-2 (16 kDa) for positive and negative controls, respectively. This result suggested that *ubc-1*(gk14) was not a *bona fide* *ubc-1* knockout as it contained UBC-1.

To confirm that the observed bands on the Western blot were specific to UBC-1, the membrane was probed with UBC-1 antibodies that had been pre-adsorbed to purified UBC-1 protein (Figure 21, B). The band at 20 kDa was now significantly diminished in the UBC-1 positive control (lane 1) and absent from the wild type (lane 3) and *ubc-1*(gk14) (lane 4) animals, suggesting that the band detected by the UBC-1 antibodies (A, lane 4) was in fact UBC-1, and that these *ubc-1*(gk14) animals were therefore not deleted for *ubc-1*.

Finally, the presence or absence of *ubc-1* in wild type (N2) and *ubc-1*(gk14) animals was examined by PCR with primers (UBC-1 F and UBC-1 R) specific for the *ubc-1* coding region (Figure 21, C). PCR on DNA templates isolated from N2 (lane 4) or *ubc-1*(gk14) (lane 5) animals generated a 1223 base pair band specific for *ubc-1*. This 1223 base pair product was sequenced and encoded *ubc-1* (Dr. Ding Xue, Dept. of Molecular and Cellular and Developmental Biology, University of Colorado, personal communication). Lanes 1 to 3 were negative controls in which either primer or N2 DNA template was omitted from the PCR reaction. This result supports the Western blot data and confirms that *ubc-1*(gk14) animals are not *ubc-1* null mutants.
Figure 21: The *ubc-1*(gk14) Animals are Not *ubc-1* Null Mutants.
Protein extracts of wild type and *ubc-1*(gk14) animals were prepared, fractionated by SDS PAGE, transferred to a PVDF membrane, and probed with a monoclonal antibody against UBC-1. A, both wild type (lane 3) and *ubc-1*(gk14) (lane 4) animals had a UBC-1-reactive band at 20 kDa. Lane 1 contained 50 ng of purified UBC-1 (20 kDa) and lane 2 contained 50 ng of purified UBC-2 (16 kDa) for positive and negative controls, respectively. B, the membrane was probed with UBC-1 antibodies that had been pre-adsorbed to purified UBC-1 protein. C, the presence or absence of *ubc-1* in wild type (N2) and *ubc-1*(gk14) animals was detected by PCR with primers (UBC-1 F and UBC-1 R) specific for the *ubc-1* coding region. PCR on the DNA templates isolated from N2 (lane 4) or *ubc-1*(gk14) (lane 5) animals generated a 1223 base pair band specific for *ubc-1*. Lanes 1 to 3 were negative controls in which either primer or N2 DNA template was omitted from the PCR reaction.
A

Lane

kDa

18

32

43

73

1.0

0.8

0.6

1.2

1.4

Lane

B

Lane

kDa

18

32

43

73

1.0

0.8

0.6

1.2

1.4

Lane

C

UBC1 F primer + - + + + +
UBC1 R primer - + + + + +
N2 DNA + + - + -
ubc1 gk14 DNA - - - - +
DISCUSSION

1. Analysis of a Yeast Two-Hybrid Screen for Proteins that Interact with UBC-1

The yeast two-hybrid system (Fields and Song, 1989) is a useful tool for discovering new protein binding partners of a protein of interest. In this study, UBC-1 fused to the GAL4 DNA binding domain was a suitable bait as it was expressed in yeast (Figure 7) and did not independently activate transcription of the reporter genes *His3* or *lacZ* (data not shown). UBC-1 contains an acidic C-terminal extension and this was maintained in the construction of the UBC-1 bait for this screen. It was hypothesised that this acidic tail might be required for UBC-1 to interact with unidentified protein partners in *C. elegans*.

After screening $2.1 \times 10^6$ transformants, 62 *C. elegans* cDNAs were recovered that upon expression reconstituted the GAL4 transcription factor, allowing the transformants to grow on media lacking histidine and to produce $\beta$-galactosidase. This yeast two-hybrid screen was considered thorough as the randomly primed cDNA library was effectively screened 18 times through, assuming that *C. elegans* contains about 19,000 predicted genes (*C. elegans* Sequencing Consortium, 1998) and allowing for the cDNAs to be in one of three possible reading frames in either orientation.

The cDNAs that encoded potential binding partners of UBC-1 were isolated, sequenced, and determined to encode C32E8.11, R05D3.4, and K11D9.2 (Table 5). Since such a large percentage (77%) encoded R05D3.4, this strongly suggests that R05D3.4 is probably a *bona fide* binding partner of UBC-1. Similarly, C32E8.11 was repeatedly uncovered in this screen, indicating that it too is probably an *in vivo* partner of UBC-1.

Only a single plasmid encoded K11D9.2 (*sca-1*), a calcium transport ATPase that pumps cytosolic Ca$^{2+}$ into the sarco-endoplasmic reticulum. The *sca-1* transcript is alternatively spliced to yield two SCA-1 isoforms, a and b, which are expressed predominantly in body wall, vulval, uterine, pharyngeal, and anal depressor muscles. Animals mutated for *sca-1* arrested in the first larval stage with decreased motility and pharyngeal pumping and defecation problems. Darier-White disease is caused by null mutations in the human homologue of *C. elegans* *sca-1* (Zwaal et al., 2001).
Unfortunately, yeast two-hybrid screens may also uncover false positive interactions with a bait. These arise due to non-specific interactions between prey and bait, and subsequent reconstitution of the GAL4 transcription factor. Another type of false positive result occurs if a particular prey protein is itself a transcription factor, and thus independently activates transcription of the reporter genes.

Neither R05D3.4, C32E8.11, nor K11D9.2 independently activated transcription of the reporter genes His3 or lacZ (Table 6). Re-introducing the pAS1-CYH2::UBC-1 bait into yeast carrying prey plasmids encoding R05D3.4, C32E8.11, or K11D9.2 reconstituted GAL4 and these transformants expressed lacZ and His3 (Table 7). Neither R05D3.4, C32E8.11, nor K11D9.2 interacted with CDK2 or SNF1, baits unrelated functionally to UBC-1 (Table 8). Hence, the observed interactions uncovered in this yeast two-hybrid screen were specific to UBC-1.

2. UBC-1 Interacts with C32E8.11, a Homologue of S. cerevisiae Ubr1p

One of the protein partners of UBC-1 identified in this study was C32E8.11, a putative ubiquitin-protein ligase of the N-end Rule Pathway. Ubiquitin-protein ligases provide the important specificity factor in the ubiquitin-proteasome proteolytic pathway by recognising degradation signals in target proteins and facilitating the transfer of ubiquitin from ubiquitin-conjugating enzymes to target proteins. C32E8.11 encodes a 1927 residue protein with a predicted molecular weight of 218 kDa. BLAST analysis (Altschul et al., 1997) of the predicted protein sequence encoded by C32E8.11 shows that it is similar to S. cerevisiae Ubr1p and M. musculus Ubr1 (E3α), ubiquitin-protein ligases of the N-end Rule proteolytic pathway. C32E8.11 will be referred to as C. elegans UBR1 for simplicity, although this designation is based only on sequence similarity, not experimental evidence.

The interaction between Rad6p and its cognate E3 Ubr1p in S. cerevisiae has been studied in detail, hence the interaction between UBC-1 and C. elegans UBR1 discovered herein was not examined further. In S. cerevisiae, the N-terminal nine residues and the acidic residue C-terminal extension of Rad6p were required for interaction with Ubr1p (Watkins et al., 1993; Xie and Varshavsky, 1999). Truncation and mutation studies showed that the basic residue-rich region (BRR) of Ubr1p (residues 1081 to 1220) was required for the interaction with Rad6p (Xie
and Varshavsky, 1999). Thus, electrostatic interactions between the positively charged BRR region of Ubr1p and the negatively charged extension of Rad6p mediate this E2-E3 interaction.

Although the RING-H2 finger of Ubr1p was dispensable for the interaction with Rad6p, it was required for ubiquitylation and degradation of artificial N-end rule substrates. Moreover, a RING-H2 mutation in Ubr1p had a dominant-negative effect as its overexpression in UBRI yeast strongly inhibited the degradation of artificial N-end rule substrates. The RING-H2 finger may recruit other factors required for N-end Rule degradation and/or provide a favourable environment for ubiquitin transfer from Rad6p to the substrate protein (Xie and Varshavsky, 1999).

Some of the in vivo functions of Ubr1p have recently been elucidated. S. cerevisiae Ubr1p was shown to multiubiquitylate and target for degradation Cup9, a transcriptional repressor of the di- and tripeptide transporter Ptr2p. This was an interesting finding as imported dipeptides bearing destabilising N-terminal residues bound to Ubr1p, allosterically activated it, and increased ubiquitylation of Cup9p. The resulting positive feedback regulatory loop relieved the repression of Ptr2 and increased the amount of imported peptides (Turner et al., 2000).

Until recently, the only N-end Rule substrates were artificial ubiquitin fusion proteins such as Leu-βgal. Scclp, a component of the cohesin complex, is the first physiological substrate of the Ubr1p-mediated N-end rule proteolytic pathway. Briefly, duplicated chromosomes (sister chromatids) are held together by a group of proteins called cohesins, including Smc1p, Smc3p, Scclp, and Scc3p in S. cerevisiae (Michaelis et al., 1997). Prior to chromosome segregation at the metaphase-anaphase transition, the Esp1p protease cleaves Scclp, generating a Scclp fragment bearing a destabilising amino-terminal arginyl residue (Arg-Sccl\textsuperscript{269-566}). Ubr1p was required to target Arg-Sccl\textsuperscript{269-566} for degradation, as the Sccl\textsuperscript{269-566} cleavage fragment was stabilised in ubr1Δ yeast. Pulse-chase experiments confirmed that Arg-Sccl\textsuperscript{269-566} was significantly stabilised in ubr1Δ yeast (Rao et al., 2001).

The inability to degrade cleaved Sccl fragments was lethal. It was hypothesised that the deleterious effects of metabolically stable Sccl fragments were caused by chromosome instability. Indeed, ubr1Δ yeast suffer chromosome loss about 100 times more frequently than wild type yeast. In a strain producing the wild type Sccl cleavage fragment, Arg-Sccl\textsuperscript{269-566}, the frequency of chromosome loss was indistinguishable from the congenic wild-type strain. However, a strain producing a metabolically stable Sccl fragment, Gly- Sccl\textsuperscript{269-566}, had an
increased frequency of chromosome loss, much like the chromosome instability of \textit{ubr1}Δ yeast. It was concluded that the chromosome instability in \textit{ubr1}Δ yeast was due to the inability to degrade the Esp-1-generated Scc1 fragment (Rao \textit{et al.}, 2001).

In \textit{C. elegans}, RNA interference against \textit{ubr1} has no obvious phenotype, i.e. the progeny of the treated adults appear wild type (Fraser \textit{et al.}, 2000). Studies in mice provide the most information about \textit{Ubr1} function in multicellular organisms. Whole-mount \textit{in situ} hybridization studies showed that \textit{Ubr1} was expressed in the branchial arches, forelimb and hindlimb buds, and tails of mouse embryos (Kwon \textit{et al.}, 1998). \textit{UBR1}\textsuperscript{−/−} mice were viable, fertile, and overtly healthy, although they weighed about 20\% less than \textit{UBR1}\textsuperscript{+/+} mice, presumably due to their decreased skeletal muscle and adipose tissue. Extracts of \textit{UBR1}\textsuperscript{−/−} skeletal muscle were defective in the N-end Rule Pathway, but \textit{UBR1}\textsuperscript{−/−} embryonic fibroblast cell lines had an active N-end Rule Pathway. This mosaicism suggests that other N-end Rule ubiquitin-protein ligases in the mouse, such as \textit{UBR2} and \textit{UBR3}, can compensate for the loss of \textit{UBR1} in certain tissues. Indeed \textit{UBR1}\textsuperscript{−/−} \textit{UBR2}\textsuperscript{−/−} mice die as embryos, underscoring the importance of the N-end Rule Pathway in multicellular organisms (Kwon \textit{et al.}, 2001).

3. Structure of RFP-1 and its Putative Orthologs

The most abundant clones (77\%) identified in this UBC-1 yeast two-hybrid screen encoded R05D3.4. R05D3.4 encodes a 97.6 kDa protein (839 amino acids) with a C-terminal C3HC4 RING finger, and was renamed RFP-1 for \textit{RING Finger Protein 1}. The interaction between UBC-1 and RFP-1 was later detected in proteome-wide yeast two-hybrid screens for proteins involved in the DNA damage response in \textit{C. elegans}. Other UBC-1-interacting proteins included UBC-13, MMS-2, MDF-1, ASP-6, GPD-2, HUM-6, and CRT-1. MDF-1 encodes a protein involved in the mitotic spindle assembly checkpoint. ASP-6 is very similar to an aspartyl protease and GPD-2 is a glyceraldehyde-3-phosphate dehydrogenase. HUM-6 resembles myosin and CRT-1 is a member of the calreticulin family of calcium-binding molecular chaperones. These putative interactions were not subjected to false positive tests and are considered “biological hypotheses until validated \textit{in vivo}” (Boulton \textit{et al.}, 2002).

SMART (Schultz \textit{et al.}, 1998; Letunic \textit{et al.}, 2002) analysis of the RFP-1 protein sequence identified six helical regions, a hidden (below threshold) basic region leucine zipper, and
a C-terminal C3HC4 RING finger domain. The putative orthologs of RFP-1 are Staring (*R. norvegicus*), K1AA0661/RNF40 (*H. sapiens*), Bre1p (*S. cerevisiae*), ebiP9515 (*Anopheles gambiae*), and CG10542 (*D. melanogaster*). All of these proteins contain helical regions and C-terminal RING finger domains important for protein-protein interactions.

### 4. Interaction of UBC-1 and RFP-1

The N-terminus of RFP-1 (1 to 313), containing the first two helical regions, was required for the interaction with UBC-1. Neither the first 183 amino acids, nor amino acids 184 to 313 of RFP-1 were sufficient for the interaction with UBC-1. The first two helical regions may be required for stability and folding of RFP-1, such that the interaction with UBC-1 is obliterated if either helix is missing. The C-terminal half (residues 408-839) of RFP-1 was dispensable for this interaction (Figure 10). While RING finger domains have been reported to recruit ubiquitin-conjugating enzymes (Lorick *et al.*, 1999), the C3HC4 RING finger of RFP-1 did not function in this capacity.

In *S. cerevisiae*, Rad6p binds to the basic rich region (BRR) of its cognate E3 Ubr1p, and Ubr1p’s RING finger domain was dispensable for this interaction (Xie and Varshavsky, 1999). The C3HC4 RING finger of Rad18p was also dispensable for the interaction with Rad6p (Bailly *et al.*, 1997). Similarly, the interaction between *C. elegans* UBC-1 and RFP-1 did not require the RING finger domain of RFP-1. Instead, this interaction was mediated by the basic N-terminus of RFP-1 (the N-terminus of RFP-1 has a calculated pKi of 9.2). Although RFP-1 and Ubr1p share no sequence similarity outside of the RING finger domain, it is interesting that both of these proteins contain basic residue rich regions that recruit the same UBC homologue (UBC-1 or Rad6p, respectively). It is somewhat surprising that RFP-1, Ubr1p, and Rad18p all interact with Rad6p (or its homologue UBC-1), and yet none of these interactions are RING finger-dependent. This suggests that the RING finger of RFP-1 recruits other proteins.

The N- or C-terminal extensions of UBCs can confer substrate specificity, modulate ubiquitylation activity, promote self-association, or direct subcellular localisation of a given UBC. In *C. elegans*, the C-terminal extension of UBC-1 (residues 153 to 192) contributes to homodimerization. UBC-1 autoubiquitylates on lysine-162, indicating that the acidic C-terminal extension has important roles in the post-translational modification of UBC-1 (Leggett and
Candido, 1997). In *S. cerevisiae*, the acidic C-terminal tail of Rad6p was required for the multiubiquitylation of histones H2A and H2B, as a Rad6p mutant lacking the tail could only monoubiquitylate H2A and H2B *in vitro* (Sung *et al*., 1988).

The C-terminal extension of a mammalian UBC, E2-25K, stimulated ubiquitin-conjugating activity and contributed to E1 selectivity (Haldeman *et al*., 1997). Human CDC34, a homologue of the *S. cerevisiae* ubiquitin-conjugating enzyme required for the G1 to S phase transition, has an acidic C-terminal extension that is phosphorylated *in vivo* by casein kinase 2. Interestingly, phosphorylation of specific residues in the tail localises CDC34 to the nucleus, as point mutations of the phosphorylation sites result in cytoplasmic localisation of CDC34 (Block *et al*., 2001).

The N-terminus of RFP-1 contained several basic residues that could potentially interact electrostatically with the acidic C-terminal extension of UBC-1. RFP-1 interacted with UBC-1Δ152, indicating that the acidic C-terminal tail of UBC-1 was dispensable for the interaction with RFP-1 (Figure 10). This argues against a simple electrostatic attraction as the basis of the interaction between UBC-1 and RFP-1 and implies that residues within the core domain of UBC-1 are required.

An apparent interaction between two proteins detected in a yeast two-hybrid screen is a biological hypothesis until the interaction is substantiated *in vitro* or *in vivo*. As an independent approach to study the interaction between UBC-1 and RFP-1, co-immunoprecipitation experiments were performed in *C. elegans* extract (Figure 12). RFP-1 antibodies immunoprecipitated recombinant UBC-1 only when recombinant RFP-1 protein (1-329) was present in the extract. The observation that RFP-1 antibodies could co-immunoprecipitate UBC-1 with the N-terminus of RFP-1 (1-329) was in agreement with the yeast interaction trap studies (Figure 10), in which RFP-1 (1 to 313) was sufficient to interact with UBC-1. RFP-1 antibodies co-immunoprecipitated recombinant UBC-1 and RFP-1 (1-329) in buffer that was not supplemented with either ubiquitin or ATP (Figure 13, lane 2). Similarly, RFP-1 antibodies co-immunoprecipitated UBC-1 and RFP-1 in *C. elegans* extract that was not supplemented with ubiquitin or ATP (lane 3).

The interaction between UBC-1 and RFP-1 appeared to be stronger in *C. elegans* extract than in buffer perhaps due to helpful chaperone activity in the extract which promoted proper
folding of the added recombinant UBC-1 and RFP-1 proteins. Native UBC-1 in the extract may have been co-immunoprecipitated with RFP-1. Another interpretation that cannot be excluded is that the protein concentration of the *C. elegans* extract (0.2 mg/ml) was high enough to contribute to "molecular crowding" that increased the association between UBC-1 and RFP-1 in these experiments (Ellis, 2001).

Finally, enhanced interaction between UBC-1 and RFP-1 in extract could be promoted by native ATP and/or ubiquitin in the *C. elegans* extract. These results show that ATP and ubiquitin are not required for the interaction between UBC-1 and RFP-1 (Figure 13, lane 2). However, ATP and/or ubiquitin may increase the interaction by post-translationally modifying either protein. UBC-1 itself is stably monoubiquitylated on Lys 162 (Leggett and Candido, 1997) and it’s possible that this modification is important for interactions with other proteins such as RFP-1. However, this seems less likely given that there is no difference in the mobility of UBC-1 incubated with extract (Figure 13, lane 2 versus 3), i.e. no evidence of monoubiquitylation of UBC-1 being correlated with RFP-1 binding in this experiment. Alternatively, RFP-1 may be ubiquitylated and this may strengthen the interaction with UBC-1. Attempts to co-immunoprecipitate endogenous RFP-1 and UBC-1 from *C. elegans* extract were unsuccessful, presumably due to low protein concentrations making detection difficult.

5. *In vivo* Expression Studies of RFP-1 and UBC-1 in *C. elegans*

Western blot analyses of *C. elegans* protein extracts show that the 98 kDa RFP-1 protein is expressed in all life stages of *C. elegans*. The RFP-1 expression level appears high in embryos, L1, and L2 animals, and then decreases slightly in the L4 and young adult stages (Figure 14).

The expression profile of RFP-1 was similar to the expression profile of *ubc-1* mRNA. Northern analysis of total *C. elegans* mRNA from embryos, larvae, and adults showed that *ubc-1* mRNA was expressed in all life stages (Leggett, 1996). These data indicate that UBC-1 and RFP-1 are expressed *in vivo* and may function together throughout development.

In order to examine the tissue specificity and subcellular localisation of RFP-1, N2 embryos, larvae, and adults were subjected to RFP-1 indirect immunohistochemical staining (Figures 15 and 16). RFP-1 is localised to the nucleus in embryos. RFP-1 is absent from nucleoli in late stage and three-fold stage embryos. RFP-1 is a nuclear protein in most tissues in larvae.
In L1 larvae, specific RFP-1 staining was observed in pharyngeal, intestinal, and nerve ring cell nuclei. RFP-1 was not detected in nucleoli. In adult animals, RFP-1 was localised to nuclei (but not nucleoli) of oocytes and germ cells in the syncytial gonad as well as in nuclei of pharyngeal and intestinal cells.

The nuclear localisation signal of the p50 subunit of NF-κB is QRKRQK (Kieren et al., 1990). RFP-1 contains two regions that are similar to this nuclear localisation signal: QKRRKI (residues 24 to 30) and RSKR (residues 77 to 82). The basic residues KKRR within the N-terminus of the human ubiquitin-activating enzyme Ela were necessary and sufficient for the nuclear localisation of Ela (Stephen et al., 1997). RFP-1 contains two similar sequences in the N-terminus: KRRK (residues 25 to 28) and RKKR (residues 218 to 221). A bipartite nuclear localisation signal KRIAELEKERSKRR (residues 66 to 82) was detected by the PSORT program (Nakai and Kanehisa, 1992). Some or all of these putative nuclear localisation sequences may be sufficient to direct RFP-1 to the nucleus and could account for the observed nuclear staining pattern in embryos and larvae.

As RFP-1 is a nuclear protein, it was hypothesised that UBC-1 should be localised to the same regions if indeed these proteins interact in vivo. UBC-1 indirect immunohistochemical staining was performed to determine the tissue specificity of UBC-1 in N2 animals. Due to specificity problems with the UBC-1 antibodies, only limited staining data were obtained on adults (Figure 17). The UBC-1 antibodies stained UBC-1 in oocyte nuclei.

UBC-1 was expected to be a nuclear protein since Rad6p is predominantly a nuclear protein (Watkins et al., 1993). Two human homologues of RAD6, called HHR6A and HHR6B, are constitutively expressed in all mammalian tissues, with a particular enrichment in testes. The HHR6 proteins are found in euchromatin regions within the nucleus, whereas the cytoplasm and nucleolus are devoid of the HHR6 proteins. Neither heat shock nor UV irradiation produces any changes in the mRNA or protein levels of the HHR6 proteins, suggesting that these proteins are not required for DNA repair or the stress response (Koken et al., 1996).

In summary, UBC-1 and RFP-1 localise to oocyte nuclei in the adult gonad, and this staining is obliterated by competition with purified antigen (compare Figure 16, D and E to Figure 17, A and B). Unlike RFP-1, UBC-1 did not appear to be localised to nuclei in the syncytial gonad, intestinal cells, or pharyngeal cells. However, it was very difficult to make
meaningful conclusions about the tissue specificity of UBC-1 due to the poor quality of the antibodies as immunostaining reagents. Laser scanning confocal microscopy studies of RFP-1 and UBC-1 would have been useful to determine if these proteins are co-localised in vivo. Unfortunately, it was not possible to double-label animals for RFP-1 and UBC-1 because it was necessary to use the same secondary antibody for both RFP-1 and UBC-1 immunostaining.

6. FUNCTION OF THE UBC-1 - RFP-1 PATHWAY

6.1. RNA Interference Against ubc-1 has No Obvious Phenotype

As yet there exist no mutants of ubc-1 in C. elegans. To circumvent this problem, RNA interference experiments against ubc-1 were performed as described in Materials and Methods. No obvious phenotype was induced by RNA interference against ubc-1, either by injection or feeding double stranded ubc-1 RNA. RNAi against ubc-1 had no deleterious effects on embryonic viability, i.e. embryos hatched as L1 animals with normal developmental timing and the animals appeared wild type (Table 9 and data not shown).

Animals exposed to ubc-1 RNA produced fertilised embryos suggesting that ubc-1 is not required for spermatogenesis in C. elegans. This differs from the role of RAD6 homologues in spermatogenesis in other multicellular organisms. Elimination of mHR6B in mice causes male sterility and more than 90% of the spermatozoa that mature are abnormal and immotile (Roest et al., 1996). Interestingly, histone H2A is ubiquitylated in elongating spermatids in both wild type and mHR6B ~m~ animals. This indicates that HR6B is not required for H2A ubiquitylation in elongating spermatids. One hypothesis is that HR6A (another RAD6 homologue) or other ubiquitin-conjugating enzymes are able to rescue the loss of HR6B and maintain H2A ubiquitylation. The male infertility of mHR6B ~m~ mutant animals is presumably caused by other unidentified interactions with HR6B targets (Baarends et al., 1999).

The apparent lack of a ubc-1 RNA interference-induced phenotype cannot be explained simply by assuming that the injection of the double stranded ubc-1 RNA was inefficient. Clearly, GFP/UNC-54 animals injected with a combination of double stranded ubc-1 RNA and double stranded gfp RNA lost their GFP fluorescence. This internal control (loss of GFP fluorescence in GFP/UNC-54 animals injected with double stranded gfp RNA) indicated that the
methodology was effective. Moreover, the feeding method of RNA interference also yielded no
ubc-1 RNA interference-induced abnormalities.

The absence of a ubc-1 RNA interference-induced phenotype may be due to
compensation by other ubiquitin-conjugating enzymes. There are twenty ubiquitin-conjugating
enzymes in C. elegans and it is possible that one or more of them is capable of substituting for
UBC-1. Perhaps UBC-3 and UBC-8, which also have acidic carboxy-terminal extensions, could
functionally substitute for UBC-1. Surprisingly, only four of the twenty predicted UBCs, ubc-
2, ubc-9, ubc-12, and ubc-14, had RNAi-induced phenotypes. These phenotypes included
embryonic lethality and secondary phenotypes such as larval arrest, abnormal body morphology,
vulval eversions, and egg-laying deficiencies (Jones et al., 2001). Alternatively, ubc-1 may be
required under certain circumstances such as when the animals suffer DNA damage. Clearly, a
ture ubc-1 null mutant would be helpful in addressing these questions.

6.2. RNA Interference Against rfp-1 Results in Vulval Eversion and Egg-laying Defects

RNA interference against rfp-1 in either N2 or GFP/UNC-54 animals had no visible effect
on embryonic viability, suggesting that rfp-1 is not required for embryogenesis (Table 9). The
lack of an embryonic phenotype was confirmed by Gonczy et al. (2000) in their RNA
interference experiments on approximately 96% of the 2315 predicted open reading frames on
chromosome III. Using an in vivo time-lapse differential interference contrast microscopy assay,
the authors monitored cell divisions of embryos from RNAi treated adults and identified several
RNAi-induced embryonic phenotypes. This was a thorough examination of embryos and yet the
authors also did not detect an rfp-1 RNAi-induced embryonic phenotype.

Although embryogenesis was not affected by rfp-1 RNAi, the F1 animals progressed with
difficulties through the four larval stages to adulthood. While many of the larvae became egg-
laying adults, approximately 2% to 10% of the young adults had everted vulvas (evl) that
subsequently resulted in bursting of the animals (Table 10 and Figure 18). The feeding method of
RNAi exposes the F1 animals constantly to rfp-1 RNA and this is reflected in the higher
frequency of vulval eversion produced by the feeding method (10%) versus the injection method
(2%). Approximately 7% of the adults had egg-laying defects (egl), such that the embryos
hatched inside the F1 adults. These results contradict the findings of Gonczy et al. (2000), who
failed to detect an *rfp-1* RNAi-inducible phenotype in F1 larvae or adults. Kamath et al. (2003) also failed to detect the *evl* and *egl* *rfp-1* RNAi-induced phenotypes, although they confirmed our observation that *rfp-1* RNAi is associated with slow growth. These *evl* and *egl* phenotypes may have been overlooked due to the high-throughput approach of their experiments.

Interestingly, the *rfp-1* RNAi-inducible phenotype is similar to some of the secondary phenotypes produced by RNAi against *ubc-2*, *ubc-9*, or *ubc-12* (Jones *et al.*, 2001). Specifically, RNAi against *ubc-2* or *rfp-1* results in L3 arrest. RNAi against *ubc-9*, *ubc-12*, or *rfp-1* induces vulval eversion and subsequent rupture of the animals. Egg laying defects arose in F1 animals exposed to *rfp-1* or *ubc-9* double stranded RNA. These similar phenotypes may indicate that *rfp-1* has similar targets as those of *ubc-2*, *ubc-9*, and *ubc-12*. In addition to UBC-1, perhaps RFP-1 can interact with UBC-2, UBC-9, or UBC-12. In this view, lack of RFP-1 would remove the specificity factor that controls ubiquitylation of substrates leading to phenotypes such as vulval eversion. By contrast, lack of UBC-1 could be rescued by other UBCs (perhaps UBC-3 or UBC-8) and no obvious null phenotype would be discernible.

The most striking *rfp-1* RNA interference-inducible phenotypes were abnormal vulval eversion and egg-laying defects. In their genetic screens for abnormal vulval eversion mutants, Seydoux *et al.* (1993) identified 30 *evl* (*everted vulva*) mutants in which the somatic gonadal tissues extruded out of the body cavity. Many of these mutants had abnormal or mispositioned germ cells and/or abnormal or absent gametes, giving rise to sterile phenotypes. These 30 mutations defined 24 complementation groups, suggesting that this screen was not exhaustive and that many genes in the *C. elegans* genome will have *evl* phenotypes. The positions of germ cells were not rigorously examined in the *evl* animals produced by *rfp-1* RNAi. These animals produced oocytes, which upon fertilisation sometimes developed and hatched inside the hermaphrodite. This indicates that spermatogenesis and fertilisation were not affected by *rfp-1* RNAi. The *evl* phenotype indicates that *rfp-1* has a role in maintaining proper vulval structure or development.

The egg-laying defects in animals exposed to *rfp-1* RNA interference may have stemmed from defects in the sex (vulval and uterine) muscles and/or defects in neural connections to the vulva. There are sixteen neurons which directly or indirectly control egg-laying in *C. elegans*. Two hermaphrodite-specific neurons (HSN) and six ventral C neurons (VCs) directly input to
the sex muscles. There are also five mechanosensory neurons, two interneurons, and one neuron with mechanosensory endings in the vulval region (Chalfie and White, 1988).

In their screen for egg-laying mutants, Trent et al. (1983) conducted pharmacological experiments on wild type and several egl (egg-laying defective) mutants to determine whether these defects were due to defects in sex muscle structure or to defects in HSN. Wild type hermaphrodites are stimulated to release their eggs in response to treatment with either serotonin or imipramine. By contrast, egl mutants with defects in vulval or uterine muscles failed to lay eggs in response to either drug. These animals tended to have severe egl phenotypes and appeared as bags of larvae. Egl mutants that had defects in the HSN neurons responded to serotonin, but did not respond to imipramine. These animals had a less severe phenotype, often releasing embryos later than wild type animals. Many of these animals lacked HSN cell bodies. Thus, this simple pharmacological test could be applied to egl animals exposed to rfp-1 dsRNA to discriminate between muscular or neuronal defects.

6.3. RFP-1 does Not Function in the Synthetic Multivulva Pathway

RNA interference experiments against rfp-1 in both a Class A mutant background (lin-15A) and a Class B mutant background (lin-15B) were performed to determine if rfp-1 was a Class A or Class B Synthetic Multivulva gene. The rationale for these experiments and a brief description of the Synthetic Multivulva Pathway are described in Results Section 7. A multivulva phenotype (muv) in the progeny of treated animals would suggest that rfp-1 functioned in this genetic pathway.

RNA interference against rfp-1 in a lin-15A (n767) background was similar to that seen in a wild type (N2) background (Tables 10 and 11). RNA interference against rfp-1 in a lin-15A (n767) background had no effect on embryonic viability (hatching), but adults developed abnormally everted vulvas and had egg-laying defects. In the negative control, F1 progeny of lin-15A (n767) hermaphrodites raised on OP50 bacterial lawns appeared wild type. Importantly, there were virtually no F1 lin-15A (n767) animals (0.1%) with multiple vulvas (muv) in these rfp-1 RNA interference experiments. In their studies of genes involved in the SynMuv Pathway, Lu and Horvitz (1998) classified mutants as symmuv if at least 18% of the animals had multiple
vulvas. The absence of a multivulva phenotype genetically excludes *rfp-1* from being a Class B synthetic multivulva gene.

RNA interference against *rfp-1* in a *lin-15B (n744)* background resulted in a phenotype which was significantly more penetrant than *rfp-1* RNA interference in the N2 background (Table 11). Indeed, 57% of the *lin-15B (n744)* F1 generation (versus 10% of N2 animals exposed to *rfp-1* RNAi) consisted of adults with everted vulvas, which subsequently ruptured. Moreover, all the F1 adults appeared slow and sickly and failed to lay eggs. Despite this dramatic phenotype, only 0.5% of the F1 generation had a multivulva phenotype (*muv*) when exposed to *rfp-1* double stranded RNA interference. Thus, *rfp-1* is not a Class A synthetic multivulva gene.

Clearly, *rfp-1* RNA interference in a *lin-15B (n744)* background results in dramatic everted vulva (*evl*) and egg-laying defective (*egl*) phenotypes. These data suggest that *rfp-1* has a more general role in vulval development and/or structure that is exacerbated in a *lin-15B* background. Although the function of *rfp-1* in vulval development is unknown, one possibility is that RFP-1 could be an E3 that targets for ubiquitylation specific regulators of vulval development.

### 6.4. RFP-1 and LIN-35 Do Not Interact

To determine if RFP-1 and LIN-35 directly interact, a yeast interaction trap was performed (Figure 20). The C-terminal half of LIN-35 (amino acids 467-961), encompassing the A and B pocket domains, was unable to interact with RFP-1. This result supports the *rfp-1* RNA interference studies in the Class A SynMuv mutant background and strongly suggests that *rfp-1* is not a Class B Synthetic Multivulva gene. The LIN-35 bait was expressed in yeast, thus the inability to interact with RFP-1 cannot be explained by lack of bait expression (Figure 19). The LIN-35 (467-961) bait and LIN-53 prey were used as a positive control for binding in these experiments. Unfortunately, the LIN-35 bait also failed to interact with LIN-53. This was surprising as *in vitro*, GST::LIN-53 pulled down a LIN-35 truncation that contained the A and B pocket domain (Lu and Horvitz, 1998). Significantly, the LIN-35 and LIN-53 interaction was not detected in large-scale two-hybrid analyses of proteins involved in vulval development in *C. elegans* (Walhout *et al.*, 2000). While LIN-35 and LIN-53 interact genetically and *in vitro*, evidently this interaction is difficult to detect with the yeast two-hybrid system. In retrospect,
LIN-53 was a poor choice as a positive control for binding to LIN-35. A better positive control for binding to LIN-35 would have been a histone deacetylase, HDA-1.

Interestingly, Boulton et al. (2002) detected an interaction between lin-35 (C32F10.2) and mms-2 in their screens for proteins involved in the DNA damage response in *C. elegans*. They also showed that MMS-2 interacts with UBC-13 and that UBC-13 interacts with UBC-1. Therefore, it is possible that UBC-1 may still be recruited to LIN-35, not via RFP-1, but via an interaction with MMS-2 and UBC-13. This is reminiscent of the cooperativity of Rad6p, Ubc13p and Mms-2p in the ubiquitylation of PCNA in *S. cerevisiae*. In response to DNA damage, PCNA became multiubiquitylated in a Rad6 and Rad18-dependent manner. Rad6p was thought to monoubiquitylate PCNA and Ubc13p and Mms2p (in a complex with Rad5p) were proposed to extend the ubiquitin chain via lys-63 linkages (Hoege et al., 2002). By analogy to PCNA, UBC-1 could function in concert with UBC-13 and MMS-2 to possibly ubiquitylate LIN-35.

6.5. Is RFP-1 Required for Silencing in *C. elegans*?

The function of RFP-1 is presumably required in the nucleus, where it may act as a RING finger ubiquitin-protein ligase that works in concert with UBC-1 to target specific nuclear proteins for ubiquitylation. Based on its interaction with the Rad6p homologue UBC-1, RFP-1 may be involved in DNA repair or chromatin remodelling and gene silencing in *C. elegans*. In *S. cerevisiae*, Rad6p-dependent ubiquitylation of histone H2B (Robzyk et al., 2000) is required for methylation of histone H3 and subsequent gene silencing (Dover et al., 2002; Sun and Allis, 2002). Significantly, Bre1p, the *S. cerevisiae* homologue of RFP-1 (Figure 8), was recently shown to be a RING finger E3 required for the Rad6p-dependent monoubiquitylation of histone H2B and subsequent methylation of histone H3 (Hwang et al., 2003).

Gene expression can be repressed/silenced by protein complexes that stabilise chromatin conformations that render DNA transcriptionally inactive. Gene silencing in *C. elegans* has been observed in the germline, where repetitive transgenic DNA is efficiently silenced. By contrast, transgenic DNA is not silenced in the soma. Mutations in the maternal effect sterile genes *mes-2, mes-3, mes-4, and mes-6* caused desilencing in germ cells in the gonad, resulting in abnormal germ cells, decreased numbers of germ cells, and sterility. This suggested that the products of *mes-2, mes-3, mes-4, and mes-6* maintained germline chromatin in a transcriptionally repressed state.
(Kelly and Fire, 1998). Histone H1.1 (there are eight predicted histone H1 proteins in *C. elegans*) was also required for germline-specific silencing, as RNA interference against *H1.1* caused desilencing of a *let-858::GFP* reporter gene in germ cells, spermatocytes, and oocytes. RNA interference against *H1.1* resulted in *mes*-like phenotypes including disordered gonad structures (5 to 11%), and decreased numbers of germ cells that appear swollen. Thus, histone H1.1 is also a component of the silencing machinery in the germline (Jedrusik and Schulze, 2001). It would be interesting to ascertain if *ubc-1* and *rfp-1*, the homologues of *RAD6* and *BRE1*, are required for histone ubiquitylation and germline-specific gene silencing in *C. elegans*.

6.6. Is RFP-1 a RING Finger E3?

The function of RFP-1 in *C. elegans* is unknown. RFP-1 could be a ubiquitylation substrate of UBC-1, a positive/negative regulator of UBC-1, or a RING finger ubiquitin-protein ligase that works in concert with UBC-1 to target specific proteins for ubiquitylation. Proteins containing RING finger domains were later shown to possess ubiquitin-protein ligase activity (Lorick *et al.*, 1999), with the RING finger domain usually recruiting and activating the ubiquitin-conjugating enzyme (Joazeiro *et al.*, 1999). The RING finger family of E3s is diverse and includes single subunit E3s that recognise and bind to motifs in their target proteins, and multisubunit E3s such as the SCF or APC that have a RING finger protein as an important component. Mutations in RING finger domains of E3s are associated with many diseases such as autosomal recessive Parkinson’s Disease (Shimura *et al.*, 2001; Zhang *et al.*, 2000) and cancer (Joazeiro *et al.*, 1999; Waterman *et al.*, 1999).

In addition to the cysteinyl residues in the RING finger domain, RFP-1 contains ten cysteinyl residues that could potentially form thiolesters with ubiquitin, similar to the hect E3s. E3s often autoubiquitylate in vitro, presumably due to the absence of a substrate in reconstitution experiments. Unfortunately, E3 assays with RFP-1 were impeded by the inability to express soluble full length RFP-1. In assays with recombinant UBC-1 and the RFP-1 (1-329) truncation protein, no ubiquitylation of RFP-1 was observed (data not shown). However, since the recombinant RFP-1 (1-329) protein was truncated, these experiments do not exclude the possibility that intact RFP-1 is a functional E3 in vivo.
Although the function of RFP-1 in *C. elegans* remains elusive, a recent study demonstrated that the rat homologue of RFP-1 was a ubiquitin-protein ligase for the neuronal membrane protein Syntaxin 1. In their yeast two-hybrid screen for proteins that interact with *R. norvegicus* Syntaxin 1B, Chin *et al.* (2002) identified a novel RING finger protein called Syntaxin 1-interacting RING finger protein (Staring). The 1002 residue Staring protein (114 kDa) is 26% identical and 38% similar to *C. elegans* RFP-1 (Figure 8) and both proteins share a similar domain structure composed of six helical regions and a C-terminal C3HC4 RING finger. GST pull down experiments *in vitro* and co-immunoprecipitation experiments *in vivo* confirmed the Staring-Syntaxin 1 interaction. Staring, UbcH8 and Syntaxin 1 immunoprecipitated as a complex from HeLa cells, suggesting that Staring may function as a ubiquitin-protein ligase to target Syntaxin 1 for proteolysis. Indeed, Syntaxin 1 was multiubiquitylated in a Staring-dependent manner, and this ubiquitylation was abolished when the RING finger of Staring was deleted. Finally, Syntaxin 1 proteolysis was inhibited by treatment with the proteasome inhibitor MG132, indicating that ubiquitylated Syntaxin 1 was targeted for proteasomal degradation by Staring.

This study was intriguing because it definitively established Staring as an E3. This suggests that RFP-1 could have a similar function in *C. elegans*. Thus, we propose a model in which RFP-1 is a single subunit RING finger ubiquitin-protein ligase that interacts with UBC-1 to perhaps target Syntaxin 1 as well as other unidentified substrates for ubiquitylation in *C. elegans*.

7. Conclusions

This yeast two-hybrid screen identified RFP-1 (R05D3.4), UBR1 (C32E8.11), and SCA-1 (K11D9.2) as potential binding partners of UBC-1. The RFP-1 and UBC-1 interaction was confirmed by immunoprecipitation experiments. This interaction was not dependent on the presence of ubiquitin or ATP, suggesting that ubiquitylation of UBC-1 or RFP-1 was not required for binding. Yeast interaction trap experiments mapped the region of interaction to the basic N-terminal 313 residues of RFP-1 and the UBC core domain of UBC-1. Both the RING finger domain of RFP-1 and the acidic carboxy extension of UBC-1 were dispensable for this interaction.
The RFP-1 and UBC-1 interaction was also detected in the proteome-wide yeast two-hybrid studies of Boulton et al. (2002), suggesting that this is a *bona fide* interaction. Moreover, Hwang et al. (2003) showed that *S. cerevisiae* Bre1p, the putative ortholog of RFP-1, was a conserved RING finger protein required for the Rad6p-dependent monoubiquitylation of histone H2B. Chin et al. (2002) showed that Staring, the rat ortholog of RFP-1, was a RING finger E3 that interacted with UbcH8 to target syntaxin for degradation. These data strongly suggest that the UBC-1 and RFP-1 interaction has been evolutionarily conserved and that RFP-1 is probably an E3.

Western blot analyses of *C. elegans* protein extracts showed that RFP-1 is expressed in all life stages of *C. elegans*. Indirect immunohistochemical staining experiments showed that RFP-1 is a nuclear protein in embryos, larvae, and adults, where it is found in nerve ring, intestinal, pharyngeal, gonadal, and oocyte cell nuclei. Limited UBC-1 indirect immunohistochemical staining experiments showed that UBC-1 is localised to oocyte nuclei. The nuclear localisation of RFP-1 and UBC-1 suggest that these proteins may function together to target nuclear proteins such as histones for ubiquitylation.

Double stranded RNA interference experiments against *rfp-l* indicate that *rfp-l* is not essential, but is required for proper vulval development and for egg-laying. RNA interference experiments against *ubc-1* gave no obvious phenotype, suggesting that *ubc-1* is non-essential or is functionally redundant.

Although an alternatively spliced human ortholog of RFP-1 interacts with pRb, RFP-1 failed to directly interact with LIN-35 (467-961), a Class B Synthetic Multivulva protein and the putative retinoblastoma protein in *C. elegans*. RNA interference experiments against *rfp-l* in *lin-15A* (n767) animals suggest that *rfp-l* is not a Class B Synthetic Multivulva gene. Similarly, RNA interference experiments against *rfp-l* in *lin-15B* (n744) animals suggest that *rfp-l* is not a Class A Synthetic Multivulva gene. Thus, *rfp-l* does not appear to function in pathways that negatively regulate vulval development. Interestingly, the *rfp-l* RNAi-inducible *evl* and *egl* phenotypes were exacerbated in *lin-15B* (n744) animals. This may suggest that *rfp-l* has a more general role in maintaining vulval structure.
8. Future Work

1. A yeast two-hybrid screen with RFP-1 as a bait would be worthwhile, especially in light of RFP-1 possibly being an E3. As RFP-1 is a 100 kDa protein and difficult to express, it may be reasonable to construct two RFP-1 baits: one consisting of the N-terminal half, and the second consisting of the C-terminal half. The C-terminal RFP-1 bait would be of particular interest, as this region encompasses the RING finger, an important protein-protein interaction domain, which may recruit substrates for ubiquitylation.

2. As the yeast homologue of ubc-1 is required for gene silencing, experiments to address the role of ubc-1 and rfp-1 in gene silencing in C. elegans could be fruitful. The germ cells of animals exposed to rfp-1 or ubc-1 RNA interference should be examined closely for any cytological defects or decrease in proliferation. Such abnormalities could suggest that rfp-1 or ubc-1 were required for germline-specific silencing. Also, performing ubc-1 or rfp-1 RNA interference in let-858::gfp animals could ascertain if these genes are required for silencing in the germline. Desilencing of the gfp transgene in the germline of animals exposed to ubc-1 or rfp-1 RNA interference would suggest that these genes are required for germline-specific silencing.

3. Pharmacological tests could be performed on animals exposed to rfp-1 RNA interference to determine the cause of the egg-laying deficiencies. Specifically, treating egl animals with either serotonin or imipramine could discriminate between defects in sex muscles and defects in neuronal connections that affect egg-laying. Egl animals with defective sex muscles fail to lay eggs in response to either drug. Egl animals with neuronal defects lay eggs in response to serotonin, but do not respond to imipramine.

4. It will be important to express full length RFP-1 for functional studies such as ubiquitin-protein ligase assays. Yeast (Pichia pastoris, S. pombe, or S. cerevisiae) may be a suitable expression host as RFP-1 was expressed in the yeast two-hybrid screen. Alternatively, the baculovirus expression system could be used to express RFP-1 in Sf9 cells. Whereas protein expression is constitutive with the Insect Select system previously tried, RFP-1 expression
would be induced upon infection with recombinant baculovirus. Using a late promoter in the recombinant virus may improve solubility of RFP-1.
REFERENCES


