Caenorhabditis elegans UBC-2 functions with the anaphase-promoting complex but also has other activities

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Summary

The anaphase-promoting complex or cyclosome (APC/C) is a multi-subunit ubiquitin ligase that regulates the eukaryotic cell cycle. APC/C belongs to the RING finger class of ubiquitin ligases that function by interacting with a ubiquitin-conjugating enzyme (Ubc), thus inciting the Ubc to transfer ubiquitin onto a target protein. Extensive studies with APC/C in other organisms have identified several possible Ubc s that might function as partners for APC/C. This report presents phenotypic and biochemical evidence showing that, in Caenorhabditis elegans, UBC-2 interacts specifically with the APC/C. This conclusion is based on three lines of evidence: first, the RNAi phenotype of ubc-2 is indistinguishable from RNAi phenotypes of APC/C subunits; second, RNAi of ubc-2 but not other Ubc s enhances the phenotype of hypomorphic APC/C mutants; third, purified UBC-2 and APC-11, the RING finger subunit of the APC/C, show robust ubiquitination activity in vitro assays. APC-11 interaction is specific for UBC-2 as ubiquitination is not seen when APC-11 is combined with other C. elegans Ubc s. As expected from the Ubc that functions with the APC/C, ubc-2(RNAi) produces metaphase blocks in both mitotic germ cells and in meiotic divisions of post-fertilization oocytes. In addition, ubc-2(RNAi) results in two germline phenotypes that appear to be unrelated to the APC/C: an expanded transition zone indicative of a pre-pachytene meiotic arrest and endoreplicated oocytes indicative of a problem in ovulation or oocyte-soma interactions.

Key words: APC/C, E2 ubiquitin-conjugating enzyme, RING finger, E3 ubiquitin ligase, Ubiquitination, Metaphase-to-anaphase transition

Introduction

The ubiquitin-protein conjugation system serves to regulate and/or modulate a wide range of cellular processes including cell cycle transitions, chromatin modifications, intracellular signal transduction, protein trafficking and the cellular stress response (Aguilar and Wendland, 2003; Hershko and Ciechanover, 1998; Pickart, 2001). During ubiquitination, the small 76 amino acid protein, ubiquitin, is covalently attached to a particular target protein through a multi-enzyme cascade. Key steps in this enzymatic cascade include the covalent linkage of ubiquitin to an E1 ubiquitin-activating enzyme and the subsequent transfer of the activated ubiquitin to any one of several E2 ubiquitin-conjugating enzymes (Ubc s). In the final step, E3 ubiquitin ligases bind to specific target substrates and mediate the covalent linkage of ubiquitin either directly to a lysine residue of the substrate protein or to a lysine on a growing polyubiquitin chain.

Specific trios of E1, E2 and E3 enzymes can mediate the addition of either ubiquitin or ubiquitin-like proteins such as NEDD8 or SUMO (Schwartz and Hochstrasser, 2003). In some cases, a specific lysine residue on a target protein has been shown to be an acceptor for both ubiquitin and a ubiquitin-like protein. The mechanisms that determine which residues become ubiquitin acceptors are not well understood. Presumably, specific combinations of Ubc s and ubiquitin ligases are critical in this determination.

The ubiquitination pathway is characterized by a hierarchy of complexity within the enzymatic cascade. Organisms typically contain only a few E1 ubiquitin-activating enzymes, several Ubc s and large classes of E3 ubiquitin ligases. For example, the Caenorhabditis elegans genome encodes a single E1, 17 different Ubc s, and 103 potential E3 s belonging to just the RING finger class (Clarke and Berg, 1998; Jones et al., 2001; Moore and Boyd, 2004). The formation of distinct Ubc-ligase pairs is thought to generate diversity within the ubiquitination pathway. One aspect of that diversity is that some ubiquitination events result in monoubiquitination whereas other events lead to polyubiquitination. Polyubiquitination can lead to recognition and degradation of the marked protein by the 26S proteasome. Monoubiquitination regulates a variety of cellular activities including transcription and protein localization (Schnell and Hicke, 2003). Yet another level of diversity arises from differences in the linkages in the polyubiquitin chain. Polyubiquitin chains formed in reactions with the ubiquitin ligase, BRCA1, are linked via lys6 of ubiquitin rather than the
Materials and Methods

Nematode strains and maintenance

The wild-type Bristol *C. elegans* strain, N2, and the OP50 and HT115 bacterial strains were obtained from the Caenorhabditis Genetics Center. Nematodes were cultured on agar plates seeded with OP50 or HT115 *Escherichia coli* according to standard methods (Brenner, 1974). Temperature-sensitive strains were maintained at 16°C. The temperature-sensitive APC/C mutants were isolated from two separate maternal-effect lethal screens (Cassada et al., 1981; Golden et al., 2000) and identified as meiotic metaphase I-arrest mutants (Golden et al., 2000).

DAPI and antibody staining

In order to examine the germline phenotypes of L1 fed worms, the adults were cut open in egg salts (Edgar, 1995), frozen in liquid nitrogen, fixed in methanol and mounted in Vectashield plus DAPI (Vectorlabs). In some DAPI staining experiments, the isolated gonads were fixed in Carnoy II fixative (6:1 ethanol/acetic acid/chloroform). Anti-phosphohistone H3 polyclonal antibody (Upstate Biotechnology) (Hendzel et al., 1997) was used as previously described (Golden et al., 2000).

Plasmid constructions

RNAi plasmids were generated with the GATEWAY recombinational cloning system (Invitrogen) and the vector pL4440GTWWY, a modified form of the L4440 plasmid (Timmons et al., 2001). pL4440GTWWY was constructed by inserting GATEWAY cassette B (Invitrogen) into the L4440 plasmid. RNAi clones for *ubc-2* and *apc-11* were made using recombinational cloning with the pL4440GTWWY and the corresponding entry clones (*ubc-2*M7.1 and *apc-11*/F35G12.9) obtained from M. Vidal (Reboul et al., 2003).

Expression constructs of the Ubcs, APC-11 and RBX-1 were generated using the GATEWAY recombinational cloning system. pDEST15 and pDEST17.1 vectors were used for GST and HIS tagged proteins respectively. Entry clones were obtained from M. Vidal and Open Biosystems (Huntsville, AL). The ubiquitin-HA expression plasmid was a gift from Ze’ev Ronai and the GST-UbcH5c expression plasmid was a gift from Yue Xiong.

To generate the GST-3APC-11 mutant protein, a cysteine in position 100 of the RING finger of APC-11 was mutated to a leucine using site-directed mutagenesis. The wild-type GST-APC-11 plasmid was amplified by PCR using primers (5’-GGA ATT CTT CGC CAT GAT TTT CAT CGT CAT-3’) and (5’-ATG AGT ATG AAA AGC ATG GCG AAC AAT TCC-3’). The product was digested with DpnI for 1 hour at 37°C to degrade template plasmid and then transformed into DH5α bacterial cells. The sequence of GST-3APC11 was confirmed using automated sequencing.

RNAi feeding method

RNAi was performed using a modified version of a described protocol (Timmons et al., 2001). Nematodes were fed bacteria expressing double stranded RNA from constructs as described above. Plasmids were freshly transformed into HT115 *E. coli*. 125 μl of saturated culture were seeded onto 35 mm NGM plates supplemented with 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and 100 mg/ml ampicillin. L4 worms were placed on plates 2-4 hours after seeding. Adults were transferred to a second plate after having laid 30-50 embryos (24-48 hours depending on temperature) and transferred to a third plate after 8-16 hours.

Protein expression and purification

To express fusion proteins for use in ubiquitination assays, 5 ml of a saturated culture of BL21(DE3)pLysS bacteria carrying the individual expression plasmid were introduced into 500 ml growth media and grown to an OD of 0.7-0.9. Induction occurred for 3 hours at 37°C using IPTG. The concentration of IPTG for GST-APC-11 and GST-C3APC-11 was 0.1 mM. For all other inductions, the final concentration was 1.0 mM of IPTG. The 6XHis-tagged enzymes (His-Ubcs and RBX-1) were purified with denaturing conditions (8 M urea) using recombinational cloning with the pL4440GTWWY and the corresponding entry clones (*ubc-2*M7.1 and *apc-11*/F35G12.9) obtained from M. Vidal (Reboul et al., 2003).

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Eluted proteins were diluted tenfold in buffer lacking urea and dialyzed against this buffer for 3 hours at 4°C. The GST-fusion proteins (GST-APC-11 and GST-C3APC-11) were purified using non-denaturing conditions (50 mM Tris pH 8.0, 150 mM KCl, 2 mM DTT, and 1 mM PMSF) with glutathione Sepharose affinity chromatography (Sigma).

normally accepted lys48 linkages (Wu-Baer et al., 2003). Additionally, Ubc13, a Ubc first identified in yeast, catalyses lys63-linked chains that play a role in DNA repair (Hofmann and Pickart, 1999).

In most cases, individual Ubcs have yet to be matched with specific ubiquitin ligases. Although numbers alone would suggest that each E1 interacts with more than one Ubc, and that each Ubc interacts with more than one ubiquitin ligase, individual ligases might also function with multiple Ubcs. For instance, one of the best-known RING finger ubiquitin ligases, the anaphase-promoting complex or cyclosome (APC/C) has been shown to interact biochemically with at least two different human Ubcs, UbcH10 and Ubc4 (Tang et al., 2001). It is not clear whether both of these are relevant Ubcs in vivo.

The APC/C is an important cell cycle regulator that controls both the metaphase to anaphase transition and exit from mitosis (Harper et al., 2002; Peters, 2002). The best-characterized substrates of APC/C are securin and cyclin B. The APC/C is a multisubunit ubiquitin ligase that contains at least 11 different subunits (Harper et al., 2002; Peters, 2002). Studies utilizing in vitro ubiquitination assays have shed light on the role of the different subunits and their interactions with Ubcs. The entire APC/C complex is required for substrate-specific ubiquitination in vitro. However, non-specific ubiquitination occurs in reactions containing certain Ubcs together with APC11, the RING finger subunit of the APC/C, and APC2, the cullin homolog (Gmachl et al., 2000; Levery et al., 2000; Tang et al., 2001). Thus, the APC11 and APC2 subunits are thought to facilitate interactions with the Ubc. Specific roles for the other APC/C subunits remain poorly defined, but recent evidence suggests that subunits containing tetratricopeptide repeats (TPRs) recruit the APC/C activators (Cdh1 or Cdc20) whereas the APC1/4/5 complex links these TPR-containing subunits with the APC2/11 complex (Vodermair et al., 2003).

In the current study, we extend earlier work on *C. elegans* Ubcs (Crowe and Candido, 2004; DeRenzo et al., 2003; Jones et al., 2001; Zhen et al., 1993; Zhen et al., 1996) and identify Ubcs (Crowe and Candido, 2004; DeRenzo et al., 2003; Jones et al., 2001; Zhen et al., 1993; Zhen et al., 1996) and identify Ubcs (Crowe and Candido, 2004; DeRenzo et al., 2003; Jones et al., 2001; Zhen et al., 1993; Zhen et al., 1996) and identify Ubcs (Crowe and Candido, 2004; DeRenzo et al., 2003; Jones et al., 2001; Zhen et al., 1993; Zhen et al., 1996) and identify Ubcs (Crowe and Candido, 2004; DeRenzo et al., 2003; Jones et al., 2001; Zhen et al., 1993; Zhen et al., 1996). The wild-type Bristol *C. elegans* strain, N2, and the OP50 and HT115 *Escherichia coli* according to standard methods (Brenner, 1974). Temperature-sensitive strains were maintained at 16°C. The GST-fusion proteins were diluted tenfold in buffer lacking urea and dialyzed against this buffer for 3 hours at 4°C. The GST-fusion proteins (GST-APC-11 and GST-C3APC-11) were purified using non-denaturing conditions (50 mM Tris pH 8.0, 150 mM KCl, 2 mM DTT, and 1 mM PMSF) with glutathione Sepharose affinity chromatography (Sigma).
In vitro ubiquitination assays

Ubiquitination assays were performed in a reaction volume of 30 μl for 1 hour at 37°C (buffer: 50 mM Tris pH 8.0, 5 mM MgCl₂, 2 mM NaF and 0.5 mM DTT). Yeast E1 was obtained from Affiniti Research Products Limited. The following were added to the assay: 40 ng E1, 0.5 μg Ubc, 0.5 μg ubiquitin ligase, 2 mM ATP and 1 μg of hemagglutinin-tagged ubiquitin (UB-HA). Sample buffer with SDS was used to stop the reaction. After SDS-PAGE and blotting onto PVDF membranes, ubiquitination was assessed by probing with anti-HA antibodies (Covance) and detection with chemiluminescent reagents (Pierce Biotechnology).

Results

**ubc-2** and **apc-11** share a common one-cell arrest embryonic phenotype

To investigate the role of ubiquitination in *C. elegans* development, we performed an RNAi screen of ubiquitin-conjugating (Ubc) enzymes. A similar RNAi screen of *C. elegans* Ubcs was previously reported (Jones et al., 2001). In our analysis of 12 different Ubcs, only **ubc-2** yielded an embryonic lethal phenotype by RNAi (Tables 1 and 2; data not shown). The essential role of **ubc-2** during larval development has been analyzed previously in mutants (Clark et al., 1988; Zhen et al., 1996). RNAi provides a powerful method for analyzing the function of this gene within the germline and early embryo.

Wild-type mothers fed on bacteria containing an **ubc-2 (RNAi)** construct produced fertilized, one-cell embryos that lacked meiotic polar bodies. Subsequent studies of fixed **ubc-2 (RNAi)** embryos co-stained with DAPI and anti-tubulin antibodies (FITC-labeled DM1A; Sigma-Aldrich) revealed that the affected embryos arrested development with their oocyte-derived chromosomes locked in meiotic metaphase I configuration and their sperm chromatin locked in a single, tightly condensed mass (Fig. 1 and data not shown).

**Table 1. ubc-2 (RNAi) increases the lethality of mat-1/apc-3 embryos**

| Strain               | RNAi  | Average number of embryos/plate | % Embryonic lethality |
|----------------------|-------|---------------------------------|-----------------------|
| N2                   | none  | 26                              | 0                     |
| mat-1(ax161)         | none  | 22                              | 27.8±6.2              |
| N2                   | ubc-2 | 15                              | 8.4±5.4               |
| mat-1(ax161)         | ubc-2 | 17                              | 100±0                 |
| N2                   | ubc-1 | 19                              | 0                     |
| mat-1(ax161)         | ubc-1 | 11                              | 26.6±5.1              |
| N2                   | ubc-20| 28                              | 0                     |
| mat-1(ax161)         | ubc-20| 22                              | 26.7±7.6              |
| N2                   | ubc-18| 23                              | 0                     |
| mat-1(ax161)         | ubc-18| 18                              | 29.0±7.3              |
| N2                   | ubc-7 | 21                              | 0                     |
| mat-1(ax161)         | ubc-7 | 30                              | 23.7±6.4              |
| N2                   | ubc-22| 19                              | 0                     |
| mat-1(ax161)         | ubc-22| 20                              | 29.1±8.5              |
| N2                   | ubc-16| 23                              | 0                     |
| mat-1(ax161)         | ubc-16| 21                              | 30.8±6.9              |
| N2                   | uev-2 | 30                              | 0                     |
| mat-1(ax161)         | uev-2 | 23                              | 26.8±6.8              |
| N2                   | ubc-13| 26                              | 0                     |
| mat-1(ax161)         | ubc-13| 21                              | 27.7±5.7              |

Data are from the first 48 hours of RNAi treatment (16°C). Each condition was tested in at least two trials with five separate plates per trial.

Anti-phosphohistone H3 (pHisH3) antibodies (Upstate Biotechnology) were used to identify cells captured in the process of meiosis or mitosis (Hendzel et al., 1997). The meiotic arrest phenotype seen with **ubc-2 (RNAi)** is strikingly similar to that previously reported for *C. elegans* mutants with defective subunits of the APC/C (Davis et al., 2002; Furuta et al., 2000; Golden et al., 2000). The APC/C was first identified.

**Table 2. ubc-2 (RNAi) increases the sterility of mat-1/apc-3 hermaphrodites**

| Strain               | RNAi | Average number of embryos/plate | % Embryonic lethality |
|----------------------|------|---------------------------------|-----------------------|
| N2                   | none | 22                              | 0                     |
| mat-1(ax161)         | none | 20                              | 23.6±4.3              |
| N2                   | ubc-2| 14                              | 100±0                 |
| mat-1(ax161)         | ubc-2| 0.3                             | 100±0                 |
| N2                   | ubc-1| 18                              | 0                     |
| mat-1(ax161)         | ubc-1| 16                              | 28.5±6.3              |
| N2                   | ubc-20| 27                             | 0                     |
| mat-1(ax161)         | ubc-20| 21                             | 26.0±9.6              |
| N2                   | ubc-18| 24                             | 0                     |
| mat-1(ax161)         | ubc-18| 20                             | 27.3±5.0              |
| N2                   | ubc-7 | 26                              | 0                     |
| mat-1(ax161)         | ubc-7 | 16                              | 23.0±6.3              |
| N2                   | ubc-22| 27                             | 0                     |
| mat-1(ax161)         | ubc-22| 24                             | 30.1±8.6              |
| N2                   | ubc-16| 24                             | 0                     |
| mat-1(ax161)         | ubc-16| 22                             | 29.1±6.7              |
| N2                   | uev-2| 14                             | 6.6±3.3               |
| mat-1(ax161)         | uev-2| 16                             | 60.1±4.8              |
| N2                   | ubc-13| 25                             | 0                     |
| mat-1(ax161)         | ubc-13| 24                             | 27.4±4.3              |

Data are from 64-80 hours after beginning RNAi treatment (16°C). Each condition was tested in at least two trials with five separate plates per trial.

**Fig. 1.** RNAi of **ubc-2** and **apc-11** show indistinguishable embryonic phenotypes in *Caenorhabditis elegans*. Several embryos from each RNAi treatment are shown. The left panels show DAPI staining and the right panels show pHisH3 staining to detect metaphase figures. These embryos have all failed to complete the first cell division and the meiotic arrest phenotype is apparent in both sets of RNAi. Representative oocyte metaphase chromosomes are indicated with arrows and sperm chromatin is indicated by carets. During the meiotic divisions of the oocyte chromosomes, the quiescent, hypercondensed sperm chromatin mass does not stain with pHisH3 antibodies (Golden et al., 2000).
as a regulator of the mitotic cell cycle (King et al., 1995; Sudakin et al., 1995; Zachariaes and Nasmyth, 1996), yet it also functions during the meiotic cell cycle (Cooper et al., 2000; Furuta et al., 2000; Golden et al., 2000; Peter et al., 2000). In *C. elegans*, oocytes depleted for any one of several different APC/C subunits either by mutation or RNAi can be fertilized, but they subsequently arrest at metaphase of meiosis I (Davis et al., 2002; Furuta et al., 2000; Golden et al., 2000). *C. elegans* mutants with this phenotype are called metaphase-to-anaphase transition defective (*mat*). In side-by-side comparisons, embryos from mothers fed double stranded RNA (dsRNA) corresponding to *ubc-2* proved to be indistinguishable from embryos of mothers fed dsRNA corresponding to *apc-11*, the RING finger subunit of the APC/C (Fig. 1). As is typical for RNAi feeding experiments, both the penetrance and the severity of embryonic lethality increased through time as pre-existing stores of *UBC-2* protein were progressively depleted (compare Tables 1 and 2). In fact, the first dead embryos produced by *ubc-2*(*RNAi*) mothers die as multicellular embryos (data not shown) suggesting that *ubc-2* does function in later divisions as has been previously documented for temperature-sensitive APC/C mutants raised under hypomorphic conditions (Shakes et al., 2003). Thus, the meiotic arrest observed in later embryos does not rule out a role for *ubc-2* in either meiosis II or subsequent mitotic divisions.

To test whether *UBC-2* and the APC/C functionally interact, *ubc-2* dsRNA was fed to *mat-1*(*ax161*) worms that contain a temperature-sensitive, hypomorphic allele of the APC/C subunit *apc-3/cdc-27*. Individually, both *ubc-2*(*RNAi*) and *mat-1*(*ax161*) mothers produced largely viable, initial clutches at 16°C (Table 1). Under identical conditions at 16°C, the double hypomorphs produced 100% non-viable progeny. Similar enhancement interactions have also been observed in double hypomorphs of two different APC/C subunits (Shakes et al., 2003). Thus, the meiotic arrest observed in later embryos does not rule out a role for *ubc-2* in either meiosis II or subsequent mitotic divisions.

Together, the indistinguishable RNAi phenotypes of *ubc-2* and APC/C subunits and the observed enhancement in double mutants suggest that UBC-2 is likely to function as the Ub for APC/C during the post-fertilization, meiotic divisions of the embryo.

### ubc-2 RNAi enhances the mitotic germline defects of hypomorphic APC/C alleles

In addition to its role in meiotic cell divisions, the APC/C is required in the mitotic divisions of germ cell nuclei within the distal gonad (Furuta et al., 2000; Golden et al., 2000; Shakes et al., 2003). To investigate whether UBC-2 also plays an essential role in these cell divisions, animals fed *ubc-2* dsRNA were analyzed for mitotic germline defects. Severe loss-of-function alleles of APC/C subunits exhibit excess metaphase figures and elevated numbers of pHisH3 staining nuclei in their distal gonads (Golden et al., 2000). In contrast, *mat-1*(*ax161*) exhibits only slightly elevated levels of pHisH3 staining nuclei and, thus, even at 25°C *ax161* appears to have sufficient levels of functional APC/C activity to support mitotic proliferation in the distal germline (Shakes et al., 2003) (Table 3). Wild-type worms fed *apc-11* or *ubc-2* dsRNA showed little or no increase in pHisH3 staining nuclei (Table 3).

### Table 3. *ube-2 (RNAi)* enhances the mitotic germline defects in *mat-1/apc-3* hermaphrodites

| Strain          | RNAi     | Number of adult gonads | Number of pHisH3 nuclei |
|-----------------|----------|------------------------|-------------------------|
| Wild type       | none     | 13                     | 4.6±2.8                 |
| Wild type       | *ube-2*  | 10                     | 4.4±3.2                 |
| Wild type       | *apc-11* | 16                     | 6.1±2.7                 |
| *mat-1(ax161)*  | none     | 12                     | 7.1±2.1*                |
| *mat-1(ax161)*  | *ube-2*  | 15                     | 22.6±5.9*               |
| *mat-1(ax161)*  | *apc-11* | 17                     | 7.9±4.4*                |

*Statistically different to number in the wild-type control (P<0.005).*

*Statistically different to number in non-RNAi counterpart (P<0.005).*

Animals were examined after RNAi treatment for 40-44 hours at 25°C.

To test for a genetic interaction between *ubc-2* and the APC/C, we fed *ubc-2* dsRNA to *mat-1*(*ax161*) worms. Initial observations showed a decrease in fertility under these circumstances (Table 2). Examination of stained gonads showed that while the mitotic defects of *mat-1*(*ax161*);*apc-11*(*RNAi*) animals were no different from their non-RNAi treated siblings, *mat-1*(*ax161*);*ubc-2*(*RNAi*) worms exhibited significantly elevated numbers of pHisH3 staining nuclei (Table 3, Fig. 2). Similar synergistic interactions were found when mutants of other APC/C subunits (*emb-27/APC6* and *mat-2/APC1*) were fed *ubc-2* dsRNA (Table 4). These results suggest that UBC-2 drives mitotic germ cell proliferation in the distal gonad, probably by serving as a ubiquitin-conjugating enzyme for the APC/C.
Although highly suggestive of a direct interaction between UBC-2 and the APC/C, our genetic studies do not rule out an indirect effect of UBC-2. In fact, the SUMO-conjugating enzyme Ubc9 was found to have this type of indirect interaction with the APC/C in *Saccharomyces cerevisiae* (Dieckhoff et al., 2004). Therefore, we investigated a direct interaction between UBC-2 and the APC/C by testing whether purified UBC-2 and APC-11 proteins could function together in vitro. In these assays, ubiquitination activity is assessed by the formation of high molecular weight ubiquitin conjugates that form because of conjugation of ubiquitin to itself, the RING finger protein APC-11 or the Ubc. Under standard conditions (see Materials and Methods), APC-11 showed robust ubiquitin ligase activity in combination with UBC-2 (Fig. 3A). APC-11 also exhibited ubiquitin ligase activity in combination with UbcH5c, a human ubiquitin-conjugating enzyme that is 92.5% identical to UBC-2 (data not shown). The observed ubiquitination was dependent on the inclusion of both the E1 and Ubc enzymes and was not seen when GST alone was substituted for the ubiquitin ligase. Importantly, a mutant form of APC-11, in which the third cysteine within the critical RING finger region was replaced by a leucine, failed to support ubiquitination in this assay (Fig. 3B).

To assess further the specificity of the Ubc-ligase interaction, we tested whether APC-11 ubiquitination activity could be supported by other ubiquitin conjugating enzymes from *C. elegans*. We chose a group of eight additional Ubcs, including *ubc-13*, the Ubc most closely related to *ubc-2* (Fig. 4C). These proteins were overexpressed in *E. coli* as His-tagged fusions and purified. UBC-2 was the only Ubc from this collection that supported significant APC-11 ubiquitination activity (Fig. 4A). In order to test for the general activity of these Ubcs, in vitro ubiquitination assays were performed using the RING finger protein RBX-1. RBX-1 is another small, RING-H2 ubiquitin ligase that functions with a broad range of Ubcs (Furkawa et al., 2002). In these RBX-1 assays, ubiquitination activity was seen for all Ubcs except UEV-2 (as expected as Uevs are catalytically inactive Ubc variants) and UBC-7 (Fig. 4B). Therefore, the failure of the other worm Ubcs to support APC-11 mediated ubiquitination does not stem from a defect in their enzymatic capabilities.

**Table 4. ubc-2 (RNAi) enhances the mitotic germline defects in multiple APC/C mutants**

| Strain        | RNAi   | Number of adult gonads | Number of pHisH3 nuclei |
|---------------|--------|------------------------|-------------------------|
| Wild type     | none   | 13                     | 5.2±3.4                 |
| Wild type     | *abc-2*| 3                      | 4.3±2.5                 |
| emb-27(p48)   | none   | 9                      | 4.7±1.7                 |
| emb-27(p48)   | *abc-2*| 13                     | 13.9±5.3*               |
| emb-27(ax81)  | none   | 6                      | 5.0±4.6                 |
| emb-27(ax81)  | *abc-2*| 11                     | 12.6±5.6*               |
| *mut-2*(ax102)| none   | 12                     | 7.8±4.5*                |
| *mut-1*(ax161)| none   | 11                     | 6.6±3.4                 |
| *mut-1*(ax161)| *abc-2*| 15                     | 17.6±5.7*               |

Animals were grown on RNAi or control plates at 16°C and then transferred to 25°C for 8-12 hours.

*Statistically different from number in wild-type controls (*P*<0.005).
†Statistically different from number in non-RNAi counterpart (*P*<0.005).

**Fig. 3. In vitro *C. elegans* ubiquitination assays with APC-11 and UBC-2.** Ubiquitination reactions were carried out as described in Materials and Methods. (A) Reactions were performed with GST-APC-11 and UBC-2. Ubiquitination reactions were probed with anti-HA antibody. Activity depends on the presence of both E1 and E2 enzymes, as well as APC-11. In the last lane, *C. elegans* RBX-1 is used as a positive control with the human E2, UbcH5c. (B) APC-11 that contains a mutation in a conserved residue of the RING finger shows no ubiquitination activity. The Coomassie blue-stained gel on the right shows purified wild-type and mutant GST-APC-11 fusion proteins.

The results of the ubiquitination reactions are consistent with the genetic results above indicating that UBC-2 is the only Ubc that showed genetic interactions with *mat-1*(ax161). The results suggest that APC-11 functions specifically with UBC-2 and not other Ubcs in *C. elegans*. Interestingly, the core region encompassing the active site of UBC-2 and UbcH5c, the other Ubc that works with APC-11 in vitro, is identical in UBC-2 and UbcH5c. This result raises the possibility that one or more residues within this active site may play a role in determining the specificity of Ubc-ligase interactions.
ubc-2 RNAi results in germline defects

In *C. elegans*, germ cell development occurs in a temporal-linear progression along the length of a tube-like, largely syncytial gonad (reviewed by Hubbard and Greenstein, 2000; McCarter et al., 1997). A population of mitotically proliferating germ cell nuclei is maintained in the distal gonad in response to signals from the somatic distal tip cell. Nuclei immediately outside this signaling zone transition out of mitosis and into meiosis, passing quickly through the early stages of meiosis and entering an extended pachytene state. In this transition zone, the chromosomes sort into appropriate homologous pairs and undergo extensive chromatin remodeling associated with large-scale changes in gene transcription. In DAPI-stained gonads, nuclei within the transition zone can be recognized by their distinctive ‘half-moon’ morphology.

During the course of the RNAi experiments, we noticed a novel germline phenotype associated with *ubc-2(RNAi)* hermaphrodites grown at 25°C. In the gonads of these hermaphrodites (but not their 20°C siblings), *ubc-2(RNAi)* caused a progressive expansion of the transition zone forming large numbers of half-moon-shaped nuclei (Fig. 5A). The progressive nature of the defect under RNAi feeding conditions suggests that nuclei entering the transition zone arrest in an early meiotic state whereas nuclei that have already progressed beyond this point remain unaffected. The reason for the temperature sensitivity of this RNAi phenotype is unknown but it is likely to reflect a general enhancement of RNAi phenotypes due to increased protein turnover or to reflect the role of ubiquitination in cellular heat shock responses.

Since our standard RNAi feeding protocol begins at the L4 larval stage, we assumed that there might be residual *ubc-2* activity in the germline. In order to achieve complete...
removal of UBC-2 protein, we began feeding experiments in the L1 stage. L1 larvae were transferred to plates and were allowed to feed on \textit{ubc-2} dsRNA bacteria until adulthood. After 3 days at 20°C, worms fed HT115 control bacteria were fertile and embryos were easily seen in all adult hermaphrodite uteri ($n=37$). However, worms subjected to \textit{ubc-2(RNAi)} matured into largely sterile adults (31/37 with no visible embryos) or produced only tiny clutches of dead eggs. Potential causes of this sterility were: a metaphase arrest of mitotically proliferating germ cells, the above described block in early meiosis (transition zone defect) or some other defect. To investigate the nature of this sterility, \textit{ubc-2(RNAi)} adult hermaphrodites from the L1 feeding experiments were cut open, fixed, and stained with DAPI. Examination of metaphase figures in HT115 fed and \textit{ubc-2(RNAi)}-fed worms showed little difference (average of 1 metaphase figure/gonad arm for HT115, $n=8$; average of 0.4 metaphase figure for \textit{ubc-2(RNAi)}, $n=13$). At 20°C, there was no obvious expansion of the transition zone even in L1-fed worms. In fact, the transition zone defect was seen only at 25°C whether feeding commenced at the L4 or the L1 stage. In contrast, analogous transition zone defects were never observed in APC/C RNAi or mutant animals under any conditions. Furthermore, when APC/C mutants were fed \textit{ubc-2} dsRNA at 25°C, the appearance of the transition zone defect was delayed, presumably due to the earlier metaphase block of the mitotically dividing germ cell nuclei (Fig. 5A).

At 20°C, the L1 fed \textit{ubc-2(RNAi)} worms did not exhibit mitotic or meiotic germline defects, but instead exhibited a defect in their maturing oocytes. Half of the adults examined (6/12 gonads) contained oocytes with over-replicated DNA in the proximal arm of their gonads (Fig. 5B). This endomitotic oocytes (Emo) phenotype has been previously reported under conditions of defective ovulation, defective communication between the germline and the overlying somatic sheath cells or cholesterol deprivation (McCarter et al., 1997; Shim et al., 2002). It is possible that \textit{ubc-2} mediated ubiquitination functions in one or more of these pathways. This function for \textit{ubc-2} is presumably independent of APC/C as a similar phenotype was never observed among more than 30 temperature-sensitive APC/C mutants or APC/C RNAi examined to date (Davis et al., 2002; Golden et al., 2000; Shakes et al., 2003). Furthermore, endo-reduplication of unfertilized oocytes in the uteri of wild-type hermaphrodites is suppressed in APC/C mutants (Furuta et al., 2000).

**Discussion**

Our results indicate that UBC-2 is the ubiquitin-conjugating...
enzyme that works with APC/C in *C. elegans*. Three lines of evidence support this conclusion. First, RNAi of *ubc-2* results in meiotic metaphase I-arrested one-cell embryos that are indistinguishable from those generated by the RNAi of individual APC/C subunits (Davis et al., 2002; Furuta et al., 2000; Golden et al., 2000; Moore and Boyd, 2004). Secondly, in double depletion studies *ubc-2* was the only Ubc of the nine tested that exhibited enhancement interactions in combination with APC/C mutants. These studies revealed that UBC-2 works with APC/C to promote the meiotic metaphase to anaphase transition and also to promote mitotic progression in proliferating germ cell nuclei. Lastly, UBC-2 was the only worm Ubc to support in vitro ubiquitination with APC-11, the RING finger of the APC/C.

One interesting implication of our results is that *C. elegans* APC/C specifically functions with UBC-2, but not with other *C. elegans* Ubcs. In contrast, human and fission yeast APC/C can interact with more than one Ubc (Gmachl et al., 2000; Seino et al., 2003; Tang et al., 2001). The human enzymes Ubc4 and UbcH10 have been shown both biochemically and functionally to work with APC/C (Gmachl et al., 2000; Leversen et al., 2000; Stroschein et al., 2001; Tang et al., 2001; Townsley et al., 1997; Yu et al., 1996). In molecular alignments, worm UBC-2 is more closely related to human Ubc4 (93.5% identity) and the highly related UbcH5c (92.5% identity) than to UbcH10 (36.7% identity). UbcH10 is also related to worm UBC-13 (38.4% identity) and UBC-1 (34.6% identity), which both lacked activity in our in vitro assays with APC-11 and our phenotypic enhancement studies. Closely related Ubcs from both clam (E2-C) and *Xenopus* (UbcX) have also been shown to function with APC/C. Interestingly, all three of these (UbcH10, E2-C and UbcX) have unique N-terminal extensions when compared to UBC-2 and other Ubcs. Tang et al. (Tang et al., 2001) have shown that UbcH10 requires both APC11 and the Cullin Homology Domain (CHD)-containing subunit, APC2, for activity in vitro. In contrast, Ubc4 is active in ubiquitination when combined with only APC11. Thus, it may be that the APC2 subunit is required to recruit Ubcs containing this N-terminal extension. *C. elegans* has no Ubc with an N-terminal extension similar to that of UbcH10, Ubc-X or E2-C. Therefore, it is unclear whether *C. elegans* possesses an APC2-dependent Ubc.

In addition to its APC/C mediated functions, *C. elegans ubc-2* was found to drive additional processes within the *C. elegans* germline. Although the expanded transition zone phenotype for *ubc-2(RNAi)* does not automatically implicate its function within a specific cellular pathway, the spectrum of its potential germline functions is intriguing. Within the transition zone, germ cells switch from a mitotic to a meiotic state and experience global changes in chromatin structure and gene transcription. Homologous chromosomes pair and form synaptonemal complexes. Thus, it is notable that in *C. elegans*, expanded transition zones have also been reported for mutant or RNAi-mediated depletions of the post-translational silencing protein EGO-1 (Smardon et al., 2000), the synaptonemal complex protein SYP-2 (Colaiacovo et al., 2003) and DNA topoisomerase IIIα (Kim et al., 2000). Furthermore, expanded transition zones have also been observed upon depletion of two proteins related to the Skp-1 subunit of the SCF ubiquitin-ligase complex (skr-1 and skr-2) (Nayak et al., 2002). Although SCF itself typically uses Cdc34 as its Ubc (Deshaies, 1999), this result may suggest an additional direct or indirect link to *ubc-2*. In other systems, Ubc9, a sumoylating enzyme, associates with the synaptonemal complexes of mouse spermatocytes (Kovalenko et al., 1996) and the ubiquitination and/or sumoylation of histones plays a key role in gene regulation and DNA repair (Citterio et al., 2004; Kagey et al., 2003; Shio and Eisenman, 2003; Sun and Allis, 2002). The germline phenotypes associated with *ubc-2(RNAi)* may involve any of the above-mentioned pathways. One potential way to discern the cellular pathway involved would be to analyze UBC-2 interactions with ubiquitin ligases that exhibit germline phenotypes. A search of the comprehensive *C. elegans* database, WormBase, revealed six potential E3 ligases that exhibit germline phenotypes. A search of the comprehensive *C. elegans* database, WormBase, revealed six potential E3 ligases (four of the RING finger subclass, one of the HECT domain subclass, and one of PHD domain subclass) that are reported to have sterile RNAi phenotypes (WormBase, 2003, http://www.wormbase.org/ WS83). Further studies will reveal whether one or more of these potential E3 ligases function as *ubc-2* partners in either transition zone progression or in preventing endo-reduplication in pre-fertilization oocytes.

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