

Research

Functional and phylogenetic analysis of the ubiquitylation system in *Caenorhabditis elegans*: ubiquitin-conjugating enzymes, ubiquitin-activating enzymes, and ubiquitin-like proteins

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Abstract

Background: The eukaryotic ubiquitin-conjugation system sets the turnover rate of many proteins and includes activating enzymes (E1s), conjugating enzymes (UBCs/E2s), and ubiquitin-protein ligases (E3s), which are responsible for activation, covalent attachment and substrate recognition, respectively. There are also ubiquitin-like proteins with distinct functions, which require their own E1s and E2s for attachment. We describe the results of RNA interference (RNAi) experiments on the E1s, UBC/E2s and ubiquitin-like proteins in *Caenorhabditis elegans*. We also present a phylogenetic analysis of UBCs.

Results: The *C. elegans* genome encodes 20 UBCs and three ubiquitin E2 variant proteins. RNAi shows that only four UBCs are essential for embryogenesis: LET-70 (UBC-2), a functional homolog of yeast Ubc4/5p, UBC-9, an ortholog of yeast Ubc9p, which transfers the ubiquitin-like modifier SUMO, UBC-12, an ortholog of yeast Ubc12p, which transfers the ubiquitin-like modifier Rub1/Nedd8, and UBC-14, an ortholog of *Drosophila* Courtless. RNAi of *ubc-20*, an ortholog of yeast *UBC1*, results in a low frequency of arrested larval development. A phylogenetic analysis of *C. elegans*, *Drosophila* and human UBCs shows that this protein family can be divided into 18 groups, 13 of which include members from all three species. The activating enzymes and the ubiquitin-like proteins NED-8 and SUMO are required for embryogenesis.

Conclusions: The number of UBC genes appears to increase with developmental complexity, and our results suggest functional overlap in many of these enzymes. The ubiquitin-like proteins NED-8 and SUMO and their corresponding activating enzymes are required for embryogenesis.

Background

The ubiquitin-conjugation system is responsible for regulating the rates of turnover of a wide variety of regulatory proteins in eukaryotes, and is also involved in marking damaged proteins for degradation by the 26S proteasome (for reviews, see [1,2]). As well as ubiquitin itself, the central components of this system include ubiquitin-activating enzymes (E1s),

ubiquitin-conjugating enzymes (UBCs or E2s), and ubiquitin-protein ligases (E3s). The E1s activate ubiquitin in an ATP-dependent reaction, resulting in formation of an enzyme-bound ubiquitin thioester; species studied to date have only a few distinct E1 sequences. The E2s or UBCs accept activated ubiquitin from an E1, also forming a thioester, and mediate the covalent attachment of the

activated ubiquityl moiety to an amino group of a lysyl residue on the substrate protein. In contrast to the E1s, the UBCs show considerable sequence and functional divergence: there are 13 different UBC genes, for instance, in the yeast *Saccharomyces cerevisiae* [3]. The E3s, of which there are a wide variety of types, are generally large multisubunit complexes; these complexes provide most of the specificity in the ubiquitylation system, interacting with particular UBCs to recognize and target a wide variety of proteins for ubiquitylation. Although some examples of ubiquitin-dependent protein processing are known [4,5], and in some cases ubiquitylated proteins may be degraded in lysosomes (reviewed in [6]), the fate of most ubiquitylated proteins is degradation by the 26S proteasome.

In addition to ubiquitin, all eukaryotes studied possess a number of ubiquitin-like (UbL) proteins (for recent reviews see [7,8]). The UbL proteins, which, like ubiquitin, are known to be conjugated to other proteins, include SUMO-1 (also known as sentrin, SMT3, PIC1, UBL1 or GMP1) and NEDD8 (also known as RUB-1). SUMO-1 is conjugated to a variety of nuclear proteins, including the Ran GTPase-activating protein (RanGAP1), p53, I κ B, c-Jun and the heat-shock transcription factor HSF2 [9]. SUMO-1 modification does not seem to target proteins for degradation, but mediates protein-protein interactions and subnuclear localization. NEDD8 conjugation is known to occur only on cullins, components of Skip-Cullin-F-Box (SCF) complexes (reviewed in [10]) which degrade cyclins and other regulatory proteins via the ubiquitin system. Here, NEDD8 conjugation may modify the activity of the complex, but its exact function is not yet clear. The activation of SUMO-1 and NEDD8, and their conjugation to targets, are mediated by E1s and E2s that are specific to these UbLs [7,8].

Given the central role of ubiquitylation in the degradation of such key regulators as mitotic and meiotic cyclins, p53, I κ B, many transcription factors, hormone receptors and other proteins, there is widespread interest in the roles of the various component proteins of this system. The availability of the complete sequence of the *C. elegans* genome, together with the powerful genetic, reverse genetic and other analytical tools available in this species, provides an excellent opportunity to examine systematically the roles of all members of any given gene family in this multicellular organism. Here we describe the results of RNA-mediated interference (RNAi) experiments on all identifiable members of the UBC/E2 family, on the E1s and on the ubiquitin-like modifiers NEDD8 and SUMO in *C. elegans*. We also describe the results of phylogenetic analyses comparing all known nematode, *Drosophila* and human UBCs.

Results and discussion

UBC and UEV proteins in *C. elegans*

On the basis of the *C. elegans* genome sequence we have identified 20 ubiquitin-conjugating enzymes. Gene and

protein names, prefaced by *ubc*- or UBC-, respectively, have been assigned to each (Table 1). Two *C. elegans ubc* genes were previously arbitrarily named *ubc-1* and *let-70 (ubc-2)* [11,12]. These gene names do not correspond to the names of the orthologous yeast genes. *C. elegans let-70 (ubc-2)* is the ortholog of *S. cerevisiae UBC4/5* whereas *C. elegans ubc-1* more closely resembles yeast UBC2. To avoid further confusion, we did not use numbers 4 or 5 in the *C. elegans ubc* nomenclature (10 and 11 also were not used as there were no clear *C. elegans* orthologs of the corresponding *S. cerevisiae* genes). Wherever possible, the assigned names do correspond to the numbering system used for yeast orthologs; however, given the disparity in gene numbers between the two species, it proved impracticable to establish a clear correspondence between all members of the family (see below).

The identification of a coding sequence as a member of the UBC family was based on two criteria: the presence of the UBC protein motif (UBCc in SMART, or Uq con in Pfam; see Materials and methods) and, within this motif, the presence of an active-site cysteinyl residue. Fourteen of the 20 *C. elegans* UBCs have corresponding cDNA clones. These clones not only demonstrate that these genes are expressed, but also allow confirmation of the *Caenorhabditis* database (AceDB) gene predictions.

Three predicted genes failed to match their corresponding cDNA sequences: D1022.1 and R01H2.6 had incorrectly predicted amino termini, and Y54G2A.23 proved to be a fusion of two separate genes, of which only the second part corresponded to a *ubc* cDNA. The R09B3.4 gene prediction in AceDB consists of a fusion of a UBC protein with a transthyretin. This prediction agrees with one cDNA, although several other cDNAs encode only the UBC portion. We have therefore used only the UBC portion in this analysis. Given that the algorithms used for gene prediction are not infallible, *ubc* genes that are not confirmed by cDNA sequences should be considered as tentative until backed by experimental evidence.

Sequence alignment of all the putative *C. elegans* UBC proteins (see below) permitted some further refinement of the gene predictions not supported by cDNA sequence. C06E2.3 appeared to have an incorrectly predicted intron, and an alternate splice site nearby eliminated a block of 15 amino acids that did not align with the other sequences. Similar intron boundary changes were made in F49E12.4 and Y94H6A.6, eliminating groups of 6 and 22 unaligned amino acids, respectively. C06E2.3 also appeared to have an incorrectly predicted amino terminus, by comparison with the amino terminus of F40G9.6, to which it is closely related. Accordingly, minor changes were made in these predicted genes before the protein sequences were used in the subsequent analysis. The AceDB gene prediction F52C6.12 is very similar in sequence to *let-70 (ubc-2)* but lacks most of the UBC motif and the active-site cysteinyl residue. This gene was not considered to encode a functional UBC.

Table 1

RNAi with *C. elegans* ubiquitin-conjugating enzyme genes

Cosmid	Gene name	dsRNA source	Fragment size (bp)	Chromosome	Number of RNAi trials	Embryonic lethality %	Secondary phenotype	Embryonic lethality (%) brood A	Embryonic lethality (%) brood B	Secondary phenotype
UBC proteins										
B0403.2	<i>ubc-17</i>	gen PCR	591	X	2	3.4	Normal	-	-	-
C06E2.3	<i>ubc-21</i>	gen PCR	1131	X	2	0	Normal	-	-	-
C06E2.7	<i>ubc-22</i>	gen PCR	1075	X	2	0	Normal	-	-	-
C28G1.1	<i>ubc-23</i>	gen PCR	491	X	2	0	Normal	-	-	-
C35B1.1	<i>ubc-1</i>	pR1.7	593	IV	2	0	Normal	0	0	Normal
D1022.1	<i>ubc-6</i>	yk640 h7	750	II	2	6.9	Normal	-	-	-
F29B9.6	<i>ubc-9</i>	yk312e11	1160	IV	4	60.2	Evl, abnormal morphology	2.7	18.2	Evl, Egl, abnormal morphology
F40G9.3	<i>ubc-20</i>	yk246g3	835	III	4	0	Rare arrest at L3 stage	4.8	0	Some arrest at L3 to L4 stage*
F49E12.4	<i>ubc-24</i>	gen PCR	842	II	2	0	Normal	-	-	-
F58A4.10	<i>ubc-7</i>	yk486g12	500	III	2	0	Normal	-	-	-
M7.1	<i>let-70</i> (<i>ubc-2</i>)	cDNA	444	IV	-	-	-	-	51	L3 stage arrest
R01H2.6	<i>ubc-18</i>	yk461f2	584	III	2	0	Normal	-	-	-
R09B3.4	<i>ubc-12</i>	yk486f10	630	I	6	85.3	Evl, abnormal morphology	5.8	52.3	Evl, abnormal morphology
Y110A2AR.2	<i>ubc-15</i>	yk602e10	644	II	2	0	Normal	-	-	-
Y54E5B.4	<i>ubc-16</i>	yk137b4	679	I	2	0	Normal	1.0	1.4	Normal
Y54G2A.23	<i>ubc-13</i>	yk659d2	499	IV	2	0	Normal	-	-	-
Y69H2.6	<i>ubc-19</i>	yk290g1	635	V	2	0	Normal	0.8	1.1	Normal
Y71G12B.15	<i>ubc-3</i>	yk255b2	1053	I	2	0	Normal	1.6	2.0	Normal
Y87G2A.9	<i>ubc-14</i>	yk573c8	532	I	6	54.1	Abnormal morphology	-	-	-
Y94H6A.6	<i>ubc-8</i>	gen PCR	890	IV	2	0	Normal	-	-	-
UEV proteins										
F39B2.2	<i>uev-1</i>	yk593d12	499	I	2	0	Normal	-	-	-
F56D2.4	<i>uev-2</i>	gen PCR	1074	III	2	0	Normal	-	-	-
F26H9.7	<i>uev-3</i>	RT-PCR	660	I	2	0	Normal	-	-	-

Gene names have been assigned as indicated in the text. DNA fragments of the indicated sizes were obtained from the cDNA sources shown or from fragments obtained by genomic PCR (gen PCR), or by reverse transcription from RNA (RT-PCR). Phenotypes include Evl (everted vulva) and Egl (egg-laying defect). Embryonic lethality for broods A and B are shown separately. A dash indicates experiment not done. *Variable penetrance. Two potential *C. elegans ubc* genes that have recently come to light are not included in this table. F25H2.8 encodes a protein that is 25% identical to UBC-1 over 100 amino acids spanning the active site. This UBC domain is not recognized by Pfam, but is recognized by SMART (UBCc domain). The protein is 56% identical in amino-acid sequence to *Drosophila* BG:25E8.2 and therefore belongs in group I of Figures 1 and 2. RNAi experiments with F25H2.8 produce paralysis in the adult stage and premature death of the hermaphrodites 4 to 5 days after the beginning of oviposition [85]. YAC clone Y110A2AM may contain another *ubc* gene (currently uncurated) encoding a protein 52% identical over 143 amino acids to Y110A2AR.2 (group XVIII).

In addition to the 20 *ubc* genes that meet the criteria described above, the *C. elegans* genome also contains three genes that possess the UBCc motif, but lack the active-site cysteinyl residue (Table 1). These genes have been named ubiquitin E2 variants, abbreviated as *uev* [13]. The UEV-1 sequence was confirmed by existing cDNAs, and cDNA sequences encoding UEV-3 were obtained by reverse

transcription PCR (RT-PCR). We also detected an aberrant splice variant of the latter gene that had a different carboxy-terminal sequence (data not shown). The *uev-2* gene has no corresponding cDNA sequences.

Recent studies using gene microarrays [14-16] enabled us to ask whether *C. elegans* UBC and UEV genes are expressed

under normal growth conditions. The array studies show that some predicted *ubc* and *uev* genes do not produce detectable levels of mRNA during normal development. In general, these genes are the same ones for which there are currently no known cDNA sequences. Interestingly, four *C. elegans ubc* genes (B0403.2, Co6E2.3, Co6E2.7, C28G1.1) that are clustered on the X chromosome have yielded no detectable mRNA, and no corresponding cDNAs have been identified [15]. We carried out extensive RT-PCR using oligonucleotides corresponding to the sequence of two of these genes, Co6E2.3 and Co6E2.7, but no products were obtained. These results do not preclude the possibility that these genes are induced under special conditions, or that their mRNAs are particularly short-lived or rare. The DNA microarray data show, for example, very low message levels for *ubc-1*, even though this gene is expressed throughout development [11].

To date, DNA microarray data are available on *C. elegans* gene expression profiles throughout development [15,16], in males versus hermaphrodites [16], and in germline versus soma [14]. All of the *ubc* genes with measurable transcript levels vary in expression through development, with mRNA levels always highest in the embryonic stages. In most cases, transcript levels drop in the early larval stages, then increase again in the fourth larval stage and in young adults. These results roughly parallel the amount of cell division taking place in the nematode, which is very high in the embryo, then decreases through the larval stages until the maturation of the gonad and commencement of oogenesis in the fourth larval stage. The available DNA microarray data suggest that most of the genes included in the chips do not show raised message levels in the germline or large differences in expression levels in oocytes versus sperm. However, levels of F49E12.4 message are significantly reduced in the male (the ratio in males versus hermaphrodites is 0.18 averaged over four experiments), whereas F29B9.6 and R01H2.6 mRNAs are enriched roughly twofold in oocytes compared to sperm [14,16].

The *C. elegans* UBC and UEV proteins were aligned with the predicted set of human and *Drosophila* UBC proteins using the ClustalW program [17]. The UBC sequences for the latter two species were obtained from FlyBase [18] and from GeneCards [19], with some additional human sequences obtained by a BLAST search of the published human genome using *C. elegans* LET-70 (UBC-2) as the query sequence. These additional human proteins are identified by their GI

(GeneInfo Identifier) numbers and the other human gene names follow the HUGO nomenclature [20]. The NCUBE1 sequence was obtained from Lester *et al.* [21] and is derived solely from cDNA sequence. Twenty-five *Drosophila* proteins and 26 human proteins were included in the analysis, but additional human UBCs will probably be revealed when the genome sequence is fully assembled. Human and *Drosophila* proteins were not included if they lacked the active-site cysteinyl residue. Alignment of the UBC and UEV proteins revealed some interesting differences in the region surrounding the active site. As shown in Figure 1, this region is demarcated by two invariant residues, a proline (P, in green) and a tryptophan (W, in yellow). The active-site cysteinyl residue (C, in red) is present in the UBC but not the UEV proteins. *C. elegans* Co6E2.7 has a cysteinyl residue in the active-site region that does not align well with the other UBCs. Functional studies will be required to determine if this protein is in fact a UBC. The alignment in Figure 1 is separated into groups by horizontal lines which, along with the adjacent Roman numerals, denote branches on the phylogenetic tree described below.

Many UBCs contain the tripeptide motif HPN (single-letter amino-acid nomenclature; Figure 1, in yellow), which is important for proper folding of the active-site region [1]. Variations in the HPN tripeptide occur in several of the *C. elegans ubc* genes. For example, B0403.2 has the sequence NPN, which is shared by two human E2s, BAB14320 and BAB14724 (Figure 1, group II, highlighted in blue). Group V UBCs have the sequence HCN (Figure 1, yellow). The most extreme variation in this region is seen in a group of four proteins that includes *C. elegans* D1022.1 and Y110A2AR.2 as well as *Drosophila* CG5823 and human NCUBE1. These proteins have the sequence T(P/A)NGR (Figure 1, top, group XVIII, blue letters), a variation that also occurs in *S. cerevisiae* Ubc6p. Partly because of this difference, this subgroup has been referred to as non-canonical ubiquitin E2s - NCUBEs [21]. The effect of such a variation on the structure of the active-site region is unknown.

Another striking difference among the predicted UBCs is a ten amino-acid insertion between the active-site cysteine and a highly conserved tryptophan in F58A4.10 (group XIII), Y71G12B.15 (group XIV-UBC3) and Y87G2A.9 (group XV-UBC7). This insertion is common to similar human and *Drosophila* proteins, including human Cdc34 and *Drosophila* Courtless. Other UBCs have smaller sequence insertions (or small deletions) in the same region. The accommodation of

Figure 1 (see figure on the next page)

ClustalW alignment of the region surrounding the active site of the *C. elegans*, *Drosophila* and human UBC proteins. The names of the proteins are color-coded: *C. elegans* (green), *Drosophila* (blue), and human (red). Subgroups of UBCs, separated by horizontal lines, have Roman numerals and UBC designations that correspond to branches shown on the dendrogram in Figure 2. The UBC designations (right) for the subgroups indicate the most similar *S. cerevisiae* UBC. The active-site cysteinyl residue (C) is highlighted in red, and an invariant proline (P, green) and tryptophan (W, yellow) are also indicated. Other features indicated are the UBC tripeptide motif HPN and variants (in yellow and blue) (see text)

		Yeast	
CG5823	KP--FSIYML-----TPNGRFKTNTRLCLSDSDFHP-----DTWNP		
Y110A2AR.2	SP--FAITMI-----TPNGRFQTNTRLCLSDSYHP-----ESWNP	XVIII	UBC6
NCUBE1	KP--FSIILL-----TANGRFVVGKKICLSISGHP-----ETWQP		
D1022.1	KP--FNLILL-----TPNGRFELNKKVCLSIGYHP-----ETWLP		
CG7375	EP--FKVCATQ----VYHPNID-LDGNVCLNLR-----EDWNP	X	UBC12
UBE2M	DP--FKVCETM----VYHPNID-LEGNVCLNLR-----EDWKP		
R09B3.4	VP--VVKCLTK----VWHPNIN-EDGSIKLSILRONS-----LDQYGRP		
UbcD10	KP--FKINFKTR----IYHPNID-EKGQVCLPIIST-----ENWKP		
UBE2L3	KP--FKITFKTK----IYHPNID-EKGQVCLPVIISA-----ENWKP		
Ubc84D	MP--FKILFKTK----IYHPNVD-EKGEVCLPIIST-----DNWKP	VII	
R01H2.6	KP--FKVAFETK----IYHPNVD-EEGKFCPLIVTA-----ENWKP		
UBE2L6	KP--FMKFTTK----IYHPNVD-ENGQICLPIISS-----ENWKP		
CG17030	KP--FRIHINTR----MYHLNVN-ERGQVCPVILEV-----EHWIP		
UbcD4	NP--FKARFITR----IWHPNISSVTGAIKLDILK-----DNWAA	IX	UBC1
HIP2	NP--FKVRFITK----IWHPNISSVTGAIKLDILK-----DQWAA		
F40G9.3	SP--FNKVFSTK----IWHPNVSSQTGVICLDILK-----DQWAA		
C06E2.3	EP--FOAKFVTR----IWHPNISSQTGTICLDILK-----DKWTA		
CG8188	TP--FKAYFLTK----IFHPNVA-ANGEICVNTLKKD-----WKP	VIII	
E2 24kDa	SP--FKGYFLTK----IFHPNVG-ANGEICVNVLKR-----WTA		
CG2257	KS--SIGFVNK----IYHPNIDESSGTVCLDVINQA-----WTA		
UBE2H	KS--SIGFMNK----IFHPNIDEASGTVCLDVINQT-----WTA	XI	UBC8
Y94H6A.6	KS--SIGFLNK----IFHPNIDEASGTVCLDVINQVIGGRSV---WKAWTA		
CG14739	TA--FRVRFVTK----ILHPNIEFITGLVCMNVLQKA-----WSS		
courtless	SP--FKMKFTCD----MFHPNIF-ADGRVCISILHAPGDDPMGYELSAERWSP	XV	UBC7
UBCJ	SP--FKMRFTCE----MFHPNIY-PDGRVCISILHAPGDDPMGYESSAERWSP		
Y87G2A.9	SP--FKMRFTCG----IFHPNIY-ADGRVCISILHAPGDDPTGYELSNERWSP		
CG9602	RP--FKMKFITE----IWHPNID-KAGDVCISILHAPGDDKMGYKAERWLP		
F58A4.10	KP--FKMKFISE----IWHPNID-KEGNVCISILHDPGDDKMGYERPEERWLP	XIII	
UBE2G1	RP--FKMKFITE----IWHPNVD-KNGDVCISILHAPGEDKYGEKPEERWLP		
CDC34	SP--FAFRFLTK----MWHPNYI-ETGDVCISILHPPVDDPQSGELPSEWNP		
FLJ20419	SP--FTFRFLTK----MWHPNYI-ENGDVCISILHPPVDDPQSGELPSEWNP	XIV	UBC3
CG7656	SP--FSIRFLTK----VWHPNVY-ENGDLICISILHPPVDDPQSGELPCERWNP		
Y71G12B.15	SP--SMKFTTK----VWHPNVY-ENGDLICISILHSPIDDPQSGELACERWNP		
UBE2A	KP--FTVRFVSK----MFHPNVY-ADGSIKLDILQN-----RWSP		
UBE2B	KP--FTVRFVSK----MFHPNVY-ADGSIKLDILQN-----RWSP	XVI	UBC2
C35B1.1	KP--FTVRFVSK----MFHPNVY-ADGSIKLDILQN-----RWSP		
UbcD6	KP--FTVRFVSK----VFHPNVY-ADGGIKLDILQN-----RWSP		
lesswright	SP--FKCKFEPP----LFHPNVY-PSGTVCLSLLEEDKD-----WRP		
UBE2I	SP--FKCKFEPP----LFHPNVY-PSGTVCLSLLEEDKD-----WRP	XII	UBC9
F29B9.6	TP--FKCKFEPP----LFHPNVY-PSGTVCLSLLEEDKD-----WKP		
F56D2.4	IP--FICEFKTP----LHHPNVD-LRGSYILKMLEQEH-----WSS		
Dsi/Ubc1	KP--FKVAFTTR----IYHPNIN-SNGSIKLDILR-----SQWSP		
effete	KP--FKVAFTTR----IYHPNIN-SNGSIKLDILR-----SQWSP		
LET-70/M7.1	KP--FKVAFTTR----IYHPNIN-SNGSIKLDILR-----SQWSP	IV	
UBE2D3	KP--FKVAFTTR----IYHPNIN-SNGSIKLDILR-----SQWSP		
UBE2D2	KP--FKVAFTTR----IYHPNIN-SNGSIKLDILR-----SQWSP		
UBE2D1	KP--FKIAFTTK----IYHPNIN-SNGSIKLDILR-----SQWSP		
UBE2E3	KP--FKVTFRTR----IYHCNIN-SQGVICLDILK-----DNWSP		
UBE2E1	KP--FKVTFRTR----IYHCNIN-SQGVICLDILK-----DNWSP	V	
UbcD2	KP--FKVTFRTR----IYHCNIN-SQGVICLDILK-----DNWSP		
CG5440	AP--FVVFRTTP----IYHCNIH-RLGFICLDILK-----EKWSP		
CG2574	RA--FRIRFTTR----IYHCNVD-SRGAICLDVLG-----ERWSP		
CG10862	YP--FYLAFLTK----TYHCNIA-LSGRIKLDILG-----SKWSP		
BG;DS01486	KA--FKVRFVTK----IFHPNID-RVGRICLDILK-----DKWSP		
bendless	SA--FKVRFITK----IYHPNID-RLGRIKLDVLK-----DKWSP	VI	UBC13
UBE2N	AA--FKVRFMTK----IYHPNVD-KLGRIKLDILK-----DKWSP		
Y54G2A.23	AA--FKVRFMTK----IYHPNID-KLGRIKLDILK-----DKWSP		
BAA93711	EP--FOIRFLTP----IYHPNID-SAGRIKLDVLK-----PP-----KGAWRP		
CG10682	AA--FVVKFLTS----CFHPNVD-LQGAICLDILK-----DKWSA	XVII	UBC11
UBE2H10	NA--FTVKFLTP----CYHPNVD-TQGNICLDILK-----EKWSA		
CG7220	DS--FEVTFIG---TNIPVHPHY-SNGHICLSILT-----EDWSP		
BAA91954	DS--FQVMFTG---ENIPVHPHY-SNGHICLSILT-----EDWSP	III	
Y54E5B.4	NS--FEVMFVG---ETIPAPHY-SNGHICLSILS-----DDWTP		
BAB14320	VP--FHFCYLSQCSG--RLNPNLY-DNGKVCVSLGTWIGKG-----TERWTS		
BAB14724	HP--FRVKLMTGNNTVRFNPNFY-RNGKVCVSLGTWIGP-----AWSP	II	
B0403.2	SP--KCAFLTTGSGNVRFNPNLY-NDGKICVSLGTWEGRP-----EKWNP		
F49E12.4	KP--FYLKFCFN----VYHPNVDPTCELCSPMLLQ-----ENWKP		
C28G1.1	KA--FTVKFITK----IWHPSVSPFDGTICLHDVDN-----GVWVPV		
F39B2.2	EP--FTVRFTTK----VHMVGVNQSNGVIDKRNLTTLR-----NWSN	I	
EG;25E8.2	EP--FVVRVHP----ISGGYVLIGGATCMELLTKOG-----WSS		
F26H9.7	LI--FRVCFHTF----IFHPNLG-KYGNWDMRGIQ-----WER		
C06E2.7	AL--FOVCQITN----MYTPFIFIPDQVPNSHLELCGR-----AKHWAS		
Y69H2A.9	ASDLRVIFFEQSN----LFHPLICTKSKEKLNRSFP-----EGWKK		

Figure 1 (see legend on the previous page)

variable numbers of extra amino-acid residues at this position is consistent with the three-dimensional structure of the UBC core domain [22], as this region is expected to lie on the surface of the protein. Several UBCs, including *C. elegans* B0403.2 and human BAB14724, have a smaller insertion on the amino-terminal side of the HPN motif.

A phylogenetic analysis was carried out on the *C. elegans*, human and *Drosophila* UBC proteins using the Phylip package of programs (see Materials and methods), setting *C. elegans* Y69H2A.9 as the outlier of an unrooted tree. Y69H2A.9 is most similar to the mouse *fused-toes* (*Ft1*) gene product [23], being 36% identical in amino-acid sequence over 190 residues. Interestingly, the mouse *Ft1* gene encodes a UEV, whereas Y69H2A.9 has an active-site cysteinyl residue. *C. elegans* UEV-type proteins were included in the tree, but those from the *Drosophila* and human proteomes were not. This analysis (Figure 2) shows that most *C. elegans* UBCs have orthologous *Drosophila* or human proteins, or both. Notably, however, some branches on the tree contain only human and *Drosophila* sequences. For example, human UBE2H10 has an ortholog in *Drosophila* (CG10682) but not in *C. elegans* (Figure 2, group XVII). UBE2H10 is involved in B-type cyclin degradation through its association with the anaphase-promoting complex, and dominant-negative mutants of UBE2H10, in which the active-site cysteine is changed to a seryl residue, arrest cells in M phase [24]. The most closely related yeast protein, Ubc11p, is not a functional ortholog of the mammalian protein [25]. B-type cyclins in *C. elegans* [26] and in yeast must therefore be targeted by a different UBC class.

A second phylogenetic lineage that lacks a *C. elegans* representative consists of the human proteins UBE2E1 and UBE2E3 together with three *Drosophila* proteins (Figure 2, V). These E2s are all structurally related to the yeast Ubc4/5 type sequence (group IV), but differ in having a variant HCN tripeptide in the active-site region (see Figure 1, V) and by the presence of an amino-terminal extension that is rich in seryl residues. For example, nine of the first 20 residues of UBE2E3 are serines. UBE2E1 may interact with the HECT-domain family member E6-AP [27], or with another HECT family protein, RSP5 [28]. E6-AP, as part of a larger complex, mediates ubiquitylation of p53, while yeast Rsp5 and its mammalian counterpart Nedd4 mediate ubiquitylation of a variety of cell-surface proteins that are subsequently degraded in the lysosome. It remains to be determined if the serine-rich regions of group V E2s are involved in phosphorylation-mediated regulation of E2 function as suggested by Matuschewski *et al.* [29]. As in the case of UBE2H10 described above, the function of UBCE2E1 and UBCE2E3 must be carried out by another UBC family member in *C. elegans*.

Most branches on Figure 2 have at least one *C. elegans* representative sequence. An interesting case that includes two

C. elegans UBCs occurs in branch XVIII, corresponding to yeast Ubc6p. Ubc6p has a transmembrane domain in its carboxy-terminal extension that anchors the protein in the membranes of the endoplasmic reticulum (ER). The anchored E2 functions in the ubiquitylation of misfolded proteins that are translocated back out of the ER [30]. The only *C. elegans* UBC containing a transmembrane domain in a carboxy-terminal extension is D1022.1. However, Y110A2AR.2 is closely related to D1022.1 but has only a short carboxy-terminal extension and lacks the membrane anchor. A unifying feature of the proteins in this group is the variant T(P/A)NGRF motif in the active-site region (Figure 1, XVIII). As mentioned above, the same variation is present in yeast Ubc6p. To date, *C. elegans* Y110A2AR is the only member of this group lacking the membrane anchor.

The branch containing human HIP2 and *Drosophila* UbcD4 (Figure 2, IX) also contains three *C. elegans* proteins, two of which (C06E2.3 and C28G1.1) are encoded by genes not yet confirmed by cDNA sequence. Of these three *C. elegans* proteins, only F40G9.3 contains a UBA, or ubiquitin-associated domain [31]. This domain occurs in all the human and *Drosophila* UBCs in the branch, although its significance remains unclear. The UBA domain also occurs in C06E2.7, which is perhaps more closely related to group VII proteins. The 340-residue extension of C28G1.1 is similar in sequence to the carboxy terminus of avian FAS-associated factor 1 (FAF1) which mediates apoptosis in L cells [32].

Two groups in Figure 2 contain type II UBC proteins with acidic carboxy-terminal extensions, implicated in target protein recognition and UBC protein dimerization. In group XI (UBC8-type) proteins, the number of acidic residues is lower than that in group XIV (UBC3-type) proteins. For example, Y94H6A.6 (UBC8-type) has an acidic domain consisting of 17 residues, nine of which are aspartyl or glutamyl residues. Y71G12B.15 (UBC3-type), however, has a domain consisting of 32 residues, 20 of which are acidic. In yeast, Ubc3p/Cdc34p is involved in the ubiquitylation of several cell-cycle-related proteins including cyclin 2 (Cln2) and Cln3 [33]. Other targets of Cdc34-mediated ubiquitylation have recently been discovered, including repressors of cyclin AMP-induced transcription [34] and the oncoprotein B-Myb [35], among others. Much less is known about the UBC8-type proteins, although it was recently shown that yeast Ubc8p regulates the ubiquitylation of the gluconeogenic enzyme fructose-1,6-bisphosphatase [36]. *C. elegans* UBC-1 (group XVI) also has an acidic carboxy-terminal extension [11], but human and *Drosophila* orthologs of this protein lack the acidic domain.

Inhibition of *ubc* and *uev* genes in *C. elegans* by double-stranded RNA

The functions of *C. elegans* UBC and UEV proteins were examined by RNAi. Fire *et al.* [37] have shown that double-stranded (ds) RNA corresponding in sequence to a gene of

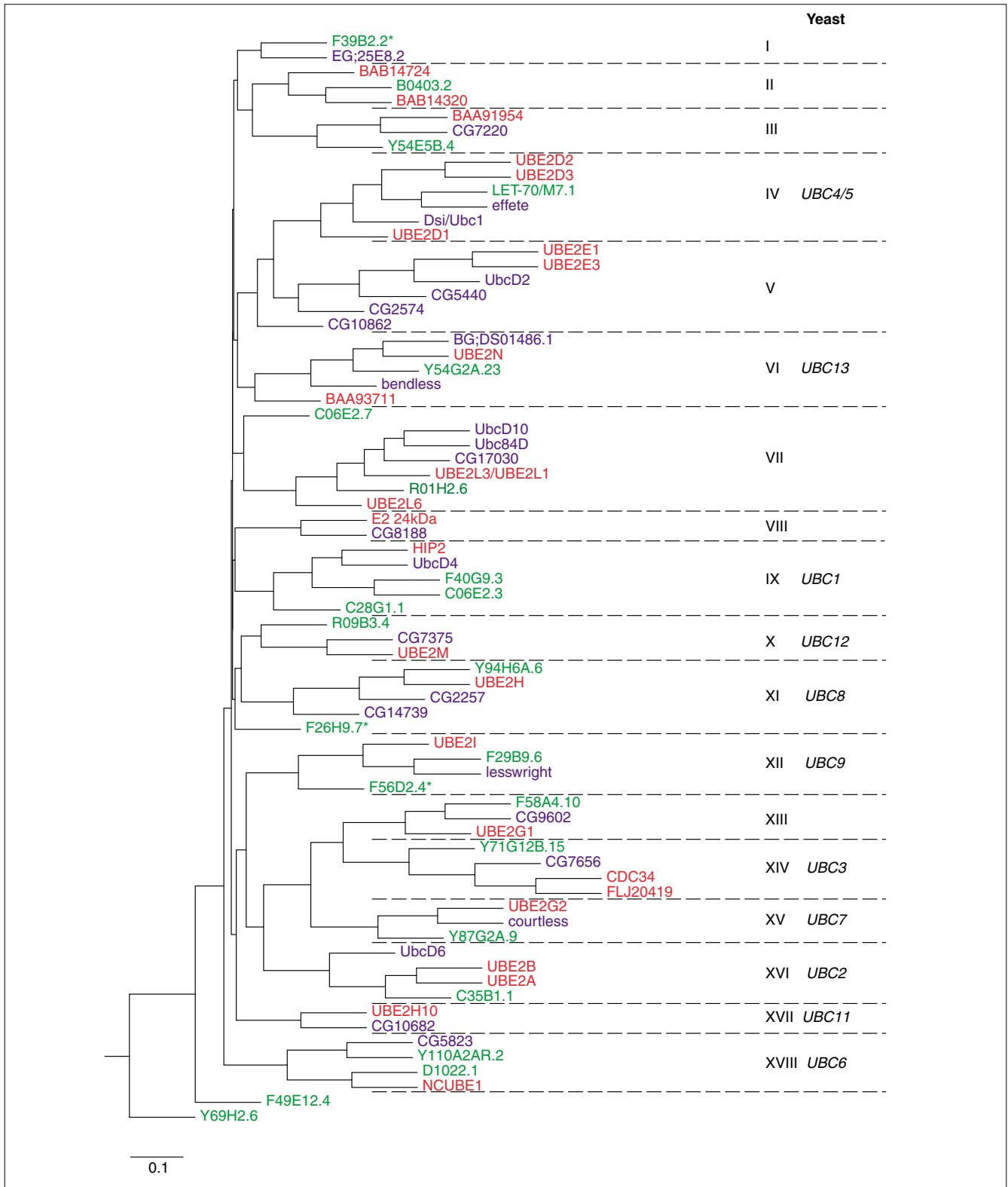


Figure 2
 Phylogenetic relationship of the UBC proteins of *C. elegans* (green), *Drosophila* (blue), and human (red). The dendrogram was prepared using the Phylip package of programs as described in Materials and methods. The major branches are separated by dotted lines and assigned Roman numerals that correspond to the numbering system on Figure 1. Some branches have a UBC label that indicates the most similar *S. cerevisiae* UBC. *C. elegans* UBE proteins are indicated with an asterisk.

interest is effective in producing specific genetic interference in that gene in both the treated animal and its immediate progeny. Variations of this method have been applied to many *C. elegans* genes, and include genome-wide surveys of protein function [38,39]. Here, we use a method in which production of the dsRNA is induced in *Escherichia coli* cells that are then fed to nematodes [40]. In addition, the function of some UBCs was studied by direct injection of dsRNA into young adult hermaphrodites (see Materials and methods for both techniques). The progeny of the treated nematodes were examined for embryonic lethality or any other developmental abnormalities. The average percentage embryonic arrest resulting from interference with expression of each gene is shown in Table 1. Progeny that hatched successfully were allowed to develop to the adult stage and any abnormalities in development noted are summarized in Table 1 as secondary phenotypes. As the primary phenotype for *let-70 (ubc-2)*, *ubc-9*, *ubc-12* and *ubc-14* RNAi was embryonic lethality, the secondary phenotypes probably arise in individuals that escaped the embryonic arrest by maternal rescue. Accordingly, these phenotypes are more commonly seen in embryos that are produced in the first 72 hours of RNAi treatment by the feeding method, when the treated adults may retain some functional UBC protein which they contribute to developing embryos. Secondary phenotypes were also commonly seen in brood A individuals in RNAi injection experiments.

Four of the 20 *C. elegans ubc* genes were found to be essential (Table 1): *let-70 (ubc-2)*, *ubc-9*, *ubc-12* and *ubc-14*. RNAi injection experiments (but not feeding experiments) suggest that *ubc-20* may be essential for larval development. None of the *uev* genes was essential.

LET-70 (UBC-2)

Two recessive lethal alleles are known for *C. elegans ubc-2*: *let-70(s1132)* and *let-70(s689)* [41]. Embryos produced by *let-70 (ubc-2)* RNAi-treated nematodes cease development after gastrulation, at the pre-comma stage. This phenotype is more severe than that seen in either of the lethal alleles, which develop to the second or third larval stage (L2 or L3). These *let-70 (ubc-2)* larvae have defects in intestinal maturation, sarcomere assembly, somatic gonad and vulval development and germ-cell maturation. It is possible that development of *let-70 (ubc-2)* animals to the larval stages is due to maternal rescue of the developmental block in embryogenesis. Alternatively, as both alleles carry point mutations, they may not show the complete null phenotype. A complete description of the *let-70 (ubc-2)* phenotype will be presented elsewhere (T.A.S. and E.P.M.C., unpublished results).

C. elegans LET-70 (UBC-2) is a functional homolog of yeast Ubc4p and Ubc5p [12]. These yeast proteins are essential under stress conditions [42]. The human Ubc4p ortholog UBE2D2 is implicated in the ubiquitylation of I κ B α [43]

and, probably, many other short-lived proteins. The role of Ubc4p in degradation of such short-lived proteins is likely to be due to its association with the SCF complex [44].

UBC-9

The predicted UBC-9 protein is a fusion of a UBC and a transthyretin, and is based on a single cDNA (yk312e11) which encodes both protein domains. However, several other cDNAs appear to encode only the UBC-9 domain. We therefore designed two RNAi constructs to analyze the function of UBC-9: one containing only the coding sequence of the UBC domain, and one containing both the UBC and the transthyretin parts of the predicted protein. The RNAi results were very similar with both constructs. It remains possible that there are two alternate splice variants of this protein.

RNAi with *ubc-9* resulted in embryonic arrest after gastrulation but before any muscle movements. The frequency of embryonic arrest was much higher when the RNAi was applied by the feeding method (Table 1). Lack of UBC-9 also resulted in pleiotropic defects in larval development in animals that completed embryogenesis (probably due to maternal rescue). The most common abnormality was vulval eversion in the fourth larval stage (L4). These eversions were uniformly shaped and resulted either in an egg-laying deficient (Egl) phenotype or in rupture at the vulva during the L4-to-adult molt. A small percentage of *ubc-9* RNAi animals were sterile. Abnormal tails, with a hooked or bent tailspike, were common on animals treated with *ubc-9* RNAi (Figure 3a). In addition, some adults showed small gaps in the alae (raised ridges in the cuticle extending the length of the nematode above the lateral seam cells; see Figure 3f).

S. cerevisiae Ubc9 is essential for growth and binds and transfers the ubiquitin-like modifier SUMO to a number of substrates including I κ B α [45], Ran-GAP1 [46], and p53 [47]. The function of SUMO modification is not clear, but mounting evidence suggests that it is primarily a mechanism for regulating the activity of certain nuclear proteins [48]. *Drosophila ubc9/lesswright* mutants are recessive lethal, the larvae having reduced numbers of thoracic and abdominal segments [49]. The functions of *ubc-9* in *C. elegans* are as yet unexplored.

UBC-12

Embryos from *ubc-12* RNAi-treated nematodes arrested at the comma to tadpole stage, with some muscle-cell movement evident [50]. The frequency of arrest was 85% by feeding and 52% by injection of *ubc-12* dsRNA (Table 1). Pleiotropic defects induced by *ubc-12* RNAi have been previously noted [50]. Lack of UBC-12 resulted in formation of an everted vulva in the L4 stage with the subsequent rupture of the animal during the L4-to-adult molt. UBC-12-deficient adults have very abnormal alae that diverge around a central space or have irregular, disorganized regions appearing as

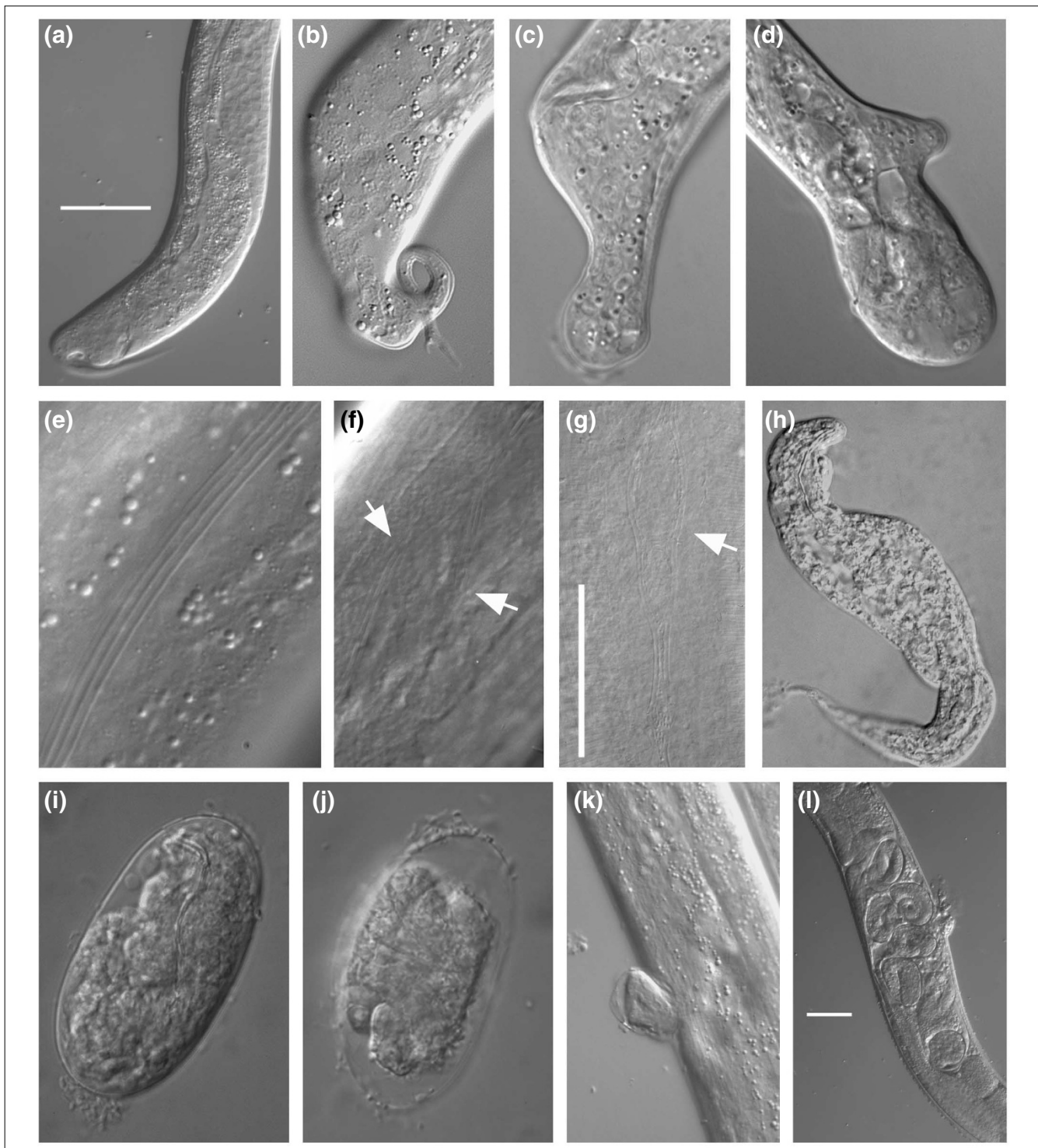


Figure 3

RNAi phenotypes of *C. elegans* *ubc* and related genes. Nematodes were treated with RNAi by the feeding method. **(a)** Reduced and bent tail spike produced by *ubc-9* RNAi. **(b)** Curled and branched tail spike produced by *ubc-12* RNAi. **(c,d)** *ubc-14* RNAi produces a blunt and rounded tail region with a protrusion near or surrounding the anus. Both specimens are young larvae. **(e)** Normal alae on an untreated adult. **(f)** Branched alae (arrows) produced by *ubc-9* RNAi on an adult specimen. **(g)** Irregular alae that diverge around a granular deposit induced by *ubc-12* RNAi. **(h)** Typical morphologically abnormal L1 seen with *ubc-9* RNAi. **(i)** Arrested embryogenesis produced by *ubc-14* RNAi with advanced development of the pharynx. Most specimens treated with *ubc-14* RNAi arrest at an earlier stage, without pharyngeal development. **(j)** Arrested embryogenesis produced by *sumo* RNAi. **(k)** Vulval eversion on *sumo* RNAi-treated young adult. **(l)** Everted vulva and egg-laying defect produced by *uba-2* RNAi. The scale bars indicate 25 μ m. Magnification: **(a,b,k)** $\times 400$; **(c-j)** $\times 1,000$; **(l)** $\times 200$.

granular deposits. Hooked or bent tails were also characteristic of *ubc-12* RNAi animals (Figure 3b).

S. cerevisiae Ubc12p binds and transfers the ubiquitin-like modifier Rub1p, known as Nedd8 in mammals and NED-8 in *C. elegans*. *Caenorhabditis* UBC-12 is highly specific for NED-8 and will not accept activated ubiquitin in the active site [50]. The only known substrates of Rub1/Nedd8 modification (neddylation) are the cullins, at least two of which are scaffolding proteins in E3 ubiquitin-ligase protein complexes [51]. Knockout *ubc12* *S. cerevisiae* are normal, although deletion of the homologous gene in the fission yeast *Schizosaccharomyces pombe* is lethal [52]. Recent studies have suggested that neddylation of human Cullin1 is required for degradation of p27^{Kip1} [53] and for activation of I κ B α ubiquitylation by the SCF ^{β TRCP} (β TRCP, β -transducin repeat-containing protein) [54].

UBC-14

Embryos from nematodes treated with *ubc-14* RNAi arrest post-gastrulation but before any muscle movements. Surprisingly, some *ubc-14* RNAi-treated embryos developed a well-organized pharynx despite the fact that they did not otherwise develop past the comma stage (Figure 3i). The only other defect induced by *ubc-14* RNAi treatment was a blunt abnormal tail with a swelling at the tip that was usually accompanied by an abnormal protrusion of tissue around the anal opening (Figure 3c,d).

The *Drosophila* ortholog of *Caenorhabditis* UBC-14 is Courtless, mutation of which results in abnormal male courtship behavior and sterility [55]. The *S. cerevisiae* ortholog, Ubc7p, is recruited to the surface of the ER by the membrane-bound protein Cue1p, where it functions in the degradation of abnormal ER proteins [56]. *S. cerevisiae* Ubc6p, which is a membrane-bound protein, is also implicated in the degradation of abnormal ER proteins [30]. However, the *C. elegans* ortholog, D1022.1, was nonessential in our RNAi experiments. The closely related protein Y110A2AR.2 (see branch XVIII of Figure 2), which lacks the transmembrane domain, was also nonessential. To check for functional redundancy of the latter two *C. elegans* proteins, we carried out an injection experiment using a mixture of dsRNAs representing the two sequences. This also showed no phenotype, suggesting that *C. elegans* UBC6 is not required under normal growth conditions.

UBC-20

With respect to nematode *ubc* genes, the results obtained by the RNAi feeding method and by dsRNA injection were concordant in all cases but one. Injection of *ubc-20* (F40G9.3, Figure 2, group IX) dsRNA resulted in a variable and, on average, weakly penetrant developmental arrest at the L3-to-L4 stage (Table 1). No embryonic arrest was seen. RNAi treatment by the feeding method, on the other hand, initially showed no phenotype. The feeding experiments with *ubc-20*

were therefore repeated to see if a weak phenotype had been overlooked, and a very low frequency of developmental arrest at the L3 stage was seen (fewer than 1% of the progeny were affected).

Deletion of *S. cerevisiae* *UBC1*, an ortholog of *Caenorhabditis* *ubc-20* (Figure 2, group IX), results in slow mitotic growth and in severely impaired growth following ascospore germination [57]. Overexpression of yeast *UBC1* can partially complement a *ubc4/ubc5* knockout, suggesting that these three UBCs have overlapping functions [57]. *HIP2*, the human ortholog of *Caenorhabditis* *ubc-20*, was isolated in a yeast two-hybrid screen using huntingtin as bait and could possibly be involved in the selective degradation of huntingtin [58]. *Caenorhabditis* *ubc-20* is closely related to Co6E2.3 (Figure 1, group IX) and an attempt was therefore made to knock out both of the corresponding genes simultaneously. Accordingly, dsRNAs representing both genes were mixed and applied by microinjection. This treatment did not result in any increase in the frequency of larval arrest. The marginal phenotype produced by RNAi with *ubc-20* may depend on the concentration of the applied RNA, and any dilution of the RNA (by mixing with a second RNA) may abolish its effectiveness. In addition, previous studies have suggested that nematode larval stages may have some resistance to dsRNA inhibition. Timmons *et al.* [59] noted that when *C. elegans* were cultured continuously on a lawn of bacteria expressing *unc-54* (body-wall myosin) dsRNA, some L1 and L2 stage larvae showed near-normal movement, whereas later larval and adult stages displayed typical paralysis. The lack of effectiveness of *ubc-20* RNAi by the feeding method might therefore be explained by such a larval resistance to RNAi.

Ubiquitin-activating enzymes of *C. elegans*

The *C. elegans* genome includes five genes encoding ubiquitin-activating enzymes - UBAs or E1s (Table 2). These genes have been named according to their counterparts in *S. cerevisiae*, on the basis of BLAST search scores. One of these genes, *Caenorhabditis* *uba-1*, encodes a holoenzyme whose ortholog in yeast activates ubiquitin [60]. The other four genes encode two heterodimeric E1s; in yeast, Uba2p and Aos1p together activate the ubiquitin-like protein SUMO, while Uba3p and Ula1p together activate Rub1 (see [61] for a review). In these heterodimeric enzymes, the ATPase and active-site domains are located in the UBA moieties.

dsRNAi with E1 genes

All of the E1 components tested by RNAi in *C. elegans* were essential (Table 2). RNAi with *uba1* resulted in a very severe phenotype, with essentially no embryos being produced by the treated adults. In fact, the treated adults died after 4 days exposure to the bacterial lawn expressing *uba-1* RNAi. RNAi knockout of *uba-2* or *uba-3* caused arrested embryogenesis, with secondary phenotypes similar to those seen with RNAi of *ned-8* or *SUMO*, namely, vulval eversion (Evl) and Egl

Table 2

RNAi with *C. elegans* ubiquitin-activating enzyme genes and ubiquitin-like genes

Cosmid	Gene name	dsRNA source	Chromosome	Number of RNAi trials	Embryonic lethality (%)	Secondary phenotype
Ubiquitin-activating enzymes						
F11H8.1	<i>uba-3</i>	gen PCR	III	2	41	Evl
C26E6.8	<i>ula-1</i>	yk433e12	III	2	6	Evl
W02A11.4	<i>uba-2</i>	yk632h1	V	2	40	Evl
C47E12.4	<i>uba-1</i>	yk9g12	IV	2	100	Low brood size, death
C08B6.9	<i>aos-1</i>	-	V	Not done	-	-
Ubiquitin-like fusion proteins						
B0303.4		yk490f10	III	2	0	None
C16C8.4		yk262b4	II	2	0	None
F49C12.9		yk162f5	IV	2	0	None
ZK688.5		yk16d10	III	2	2.5	None
Small ubiquitin-like proteins						
F45H11.2	<i>ned-8</i>	T01966	I	1	2	Evl, abnormal morphology
K12C11.2	<i>sumo</i>	yk222g1	I	2	100	Evl, abnormal morphology
F46F11.4		gen PCR	I	2	0	None
F32H5.3		yk534f3	V	2	0	None

DNA fragments used to generate the dsRNA were obtained from the indicated sources, some fragments were generated by genomic PCR (gen PCR). Phenotypes include Evl (everted vulva). A dash indicates experiment not done.

phenotypes (Figure 3l). RNAi with *ula-1* did not cause embryonic arrest, but the treated animals displayed similar secondary phenotypes to those seen with *ubc-12* RNAi.

Ubiquitin-like proteins in *C. elegans*

Two genes in *C. elegans* encode ubiquitin: *ubq-1* encodes polyubiquitin [62], and *ubq-2* encodes a fusion of ubiquitin and a ribosomal protein [63]. Two other *C. elegans* genes encode a fusion of a ubiquitin-like and a ribosomal protein: *ubl-1* (H016I04.6, [64]) and *rps-30* (C26F1.4). Previous RNAi experiments have shown that *ubq-1* and *ubq-2* are essential genes [39]. However, as *ubl-1* and *rps-30* encode fusion proteins that undergo post-translational processing into separate ubiquitin-like and ribosomal protein portions, RNAi does not permit examination of the separate function(s) of the ubiquitin-like portion. Thus, no attempt was made to examine these two genes further by RNAi. However, the *C. elegans* genome encodes at least 17 other proteins containing a ubiquitin-like domain that is either not processed from, or is not fused to, other protein sequences. We have examined eight of these sequences and found that only the ubiquitin-like modifiers NED-8 and SUMO are essential for embryogenesis (Table 2), with secondary phenotypes that are very similar to those produced by eliminating their corresponding conjugating or activating enzymes. For example, although *sumo* RNAi causes embryonic arrest in 100% of progeny (Figure 3j), survivors in the earlier brood show everted vulvae (Figure 3k) and abnormalities in hermaphrodite tail morphology. Similar results with *sumo* RNAi have been previously observed [38]. RNAi with *ned-8*

was ineffective by feeding, perhaps because of the small size of the fragment used (150 bp), although the same fragment size was effective when used in the injection method [50].

Conclusions

Common *ubc* knockout phenotypes in *C. elegans*

A remarkable result in our study is the overt similarity in phenotype produced by RNAi with several different *ubc* genes. Abnormalities in development of the hermaphrodite tail produced by *ubc-9*, *ubc-12* and *ubc-14* RNAi overtly resemble the phenotype of alleles of the *C. elegans* posterior-group HOX gene *nob-1* (no back end [65]). In *nob-1* alleles such as *ct223*, some individuals arrest in the L1 stage with a severely disorganized posterior end. Similar disorganization of the posterior end of the worm is also produced by mutant alleles of the HOX gene *egl-5* [66] and the homeodomain protein-encoding genes *vab-7* [67] and *pal-1* [68]. A complex signaling pathway involving both positive and negative regulatory proteins appears to regulate the formation of the posterior region of *C. elegans*. The current study suggests that three of the *ubc* genes are essential in this process, perhaps being required in the selective degradation of negative regulators.

Alae are present in the L1 stage, in the dauer larva and in the adult, but are absent from the other larval stages [69]. Mutations in other *C. elegans* genes, such as *clh-1*, a calcium-channel gene, also result in small gaps in the alae [70]. Formation of the alae in the adult depends upon the fusion of the underlying seam cells, which occurs after the L4 molt

[69], and laser ablation of seam cells inhibits the formation of the alae overlying the ablated cell [71]. This suggests that the alae abnormalities seen with *ubc-9* and *ubc-12* RNAi may be caused either by a defect in the fusion of seam cells in the L4 or by the absence or displacement of individual cells. Recent studies have indicated that the ubiquitin-conjugation system is essential for myoblast cell fusion [72] and studies are underway to determine if seam-cell fusion events are abnormal in the absence of either *ubc-9* or *ubc-12*.

Comparison of UBC function in *C. elegans* and other organisms

There are surprisingly few correlates between the phenotypes produced by knockouts of the UBC or related genes in *S. cerevisiae* and the phenotypes seen when orthologous genes are knocked out by RNAi in *C. elegans*. Notable differences occur for the yeast genes *CDC34/UBC3*, *UBC12*, and the ubiquitin-like protein-encoding gene *RUB1*. The *C. elegans* ortholog of *S. cerevisiae CDC34/UBC3*, *ubc-3* (Y71G12B.15), appears to be nonessential even though *CDC34* is essential in yeast [73]. Many targets of ubiquitylation by Cdc34p are known, including the G1 cyclins Cln2p [33] and Cln3p [74], and the S-phase cyclin-CDK inhibitor Sic1p [75]. The action of Cdc34p in the degradation of these and other substrates is mediated by the involvement of Cdc34p in the SCF. Furthermore, at least part of the acidic carboxy-terminal extension of Cdc34p is required for its activity [76] and other E2s can substitute for Cdc34p if they are modified to include the Cdc34p carboxyl extension [77]. In *C. elegans*, perhaps the three UBCs that have acidic carboxy extensions, UBC-1, UBC-3 and UBC-8, are functionally redundant.

Components of the Rub1/Nedd8 conjugation pathway are nonessential in *S. cerevisiae* [78], although *rub1*, *ubc12*, *ula1* and *uba3* null mutants show a synthetic lethality when combined with temperature-sensitive mutant *ubc3/cdc34* [79]. In *C. elegans*, RNAi of the corresponding NED-8 conjugation-pathway components results in embryonic lethality or severe developmental abnormalities. This difference probably reflects an enhanced role for NED-8 modification in more complex organisms, although NED-8 is also required for cell viability in the fission yeast *S. pombe* [52].

The number of UBC-coding genes in eukaryotes appears to increase with increasing developmental complexity: 13 UBCs in *S. cerevisiae*, 20 in *C. elegans*, and 25 in *Drosophila*. There are probably in excess of 30 UBCs in the human proteome, although only 26 were fully annotated at the time of this study. Much of the increase in diversity of UBCs has occurred in branches of the UBC family that have no identifiable orthologs in yeast, an example being the UBCs with serine-rich amino-terminal extensions (group V of Figure 1). The increasing divergence in ubiquitin-conjugating enzymes over time may explain the discrepancies in requirements for individual UBC and related proteins in yeast versus nematode. Perhaps the increased number of genes has resulted in

functional redundancy, or in the diversification or even exchange of roles of individual UBCs since the divergence of yeast and animal cells from a common ancestor. In this interpretation, the detection of phenotypes for only five of the UBC genes in *C. elegans* would be due not to redundancy within a CLUSTALW grouping, but to functional 'cross-talk' between members of different groupings.

Materials and methods

Nematode culture

C. elegans Bristol (N2) strain nematodes were cultured by standard techniques [80]. NGM agar plates used for RNAi experiments (see below) contained 1 mM isopropyl β -D-thiogalactopyranoside and 25 μ g/ml carbenicillin.

Polymerase chain reaction

Polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) were carried out as previously described [64].

RNAi assays

Feeding method

The dsRNA was applied to nematodes by the feeding method of Kamath *et al.* [40], which is a modification of the procedure developed by Timmons *et al.* [59]. Either a cDNA copy of each gene (where available), or a DNA fragment generated by PCR (Table 1) were subcloned into pPD129.36 and used as templates for dsRNA production. Each template was tested in at least two separate experiments. Three fourth larval (L4) stage nematodes were introduced to plates spread with dsRNA-containing bacteria and allowed to develop into adults. After 72 h at 15°C, individual treated adults were moved to fresh plates spread with dsRNA-containing bacteria and allowed to lay eggs overnight at 15°C. The following day, the adults were removed and the eggs counted. The percentage of embryonic arrest was determined by counting the number of unhatched eggs remaining 24 h after the removal of the treated adults from the test plates.

Injection method

The dsRNA was prepared by the method of Fire *et al.* [37] with modifications [50]. Injected individuals were allowed to lay eggs for 5 h in order to purge untreated embryos. The adults were then moved to fresh plates and were allowed to lay eggs for approximately 17 h. This brood of eggs (brood A) may be expected to show maternal-rescue phenotypes [50]. The adults were then transferred to another set of fresh plates and allowed to lay eggs for an additional eight hours. This brood of eggs (brood B) is generally free of maternal rescue effects.

Microscopy

Individual nematodes or eggs were picked from plates onto 2% agarose pads containing 10 mM sodium azide as anesthetic and photographed using a Zeiss Axioplan 2 microscope with differential interference contrast optics.

Bioinformatics methods

Some protein sequences were obtained by BLAST searches of the public databases [81]. Protein structural motifs were analyzed using web-based versions of the programs Pfam [82] and SMART [31]. Protein sequence alignments were generated with a web-based version of the CLUSTALW program located at the European Bioinformatics Institute site [83], using the default settings. For phylogenetic analysis, the output for the alignment was set to Phylip, and analyzed with the Phylip package of programs [84] using the neighbor-joining method to construct trees from 1,000 bootstrap replicates of the dataset. The program CONSENSE was used to generate the consensus tree. The output of the Phylip package was converted to its final form using the Phylodendron program developed by D.G. Gilbert, Indiana University, Bloomington, USA.

Accession numbers

UBCs

B0403.2, Q11076; C06E2.3, T15432; C06E2.7, T15431; C28G1.1, T15691; C35B1.1, T32959; D1022.1, T34195; F29B9.6, T29929; F40G9.3, T33629; F49E12.4, T22449; F58A4.10, S40982; M7.1, T23820; R01H2.6, T16646; R09B3.4, T24069; Y110A2AR.2, AAF60411; Y54E5B.4, T27167; Y54G2A.23, AAK93864; Y69H2.6, CAB63403; Y71G12B.15, T21439; Y87G2A.9, CAB60431; Y94H6A.6, AAF60891

UEV proteins

F39B2.2, T21984; F56D2.4, T16479; F26H9.7, AAF60891

EIs

F11H8.1, T16037; C26E6.8, AAA21162; W02A11.4, CAB04891; C47E12.4, T20014; C08B6.9, T19082

Ubiquitin-like fusion proteins

B0303.4, P34256; C16C8.4, T29404; F49C12.9, T22421; ZK688.5, S44920

Small ubiquitin-like proteins

F45H11.2, T22249; K12C11.2, AAK18969; F46F11.4, T25763; F32H5.3, T21684

Additional data files

These include the oligonucleotide primer sequences used in PCR, where relevant, and genomic coordinates for fragments used in the RNAi experiments. Also shown are the complete alignments of the sequences analyzed, which formed the basis for the partial alignment presented in Figure 1.

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