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A HECT domain ubiquitin ligase closely related to the mammalian protein WWP1 is essential for *Caenorhabditis elegans* embryogenesis

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Abstract

The highly conserved ubiquitin/proteasome pathway controls the degradation of many critical regulatory proteins. Proteins are posttranslationally conjugated to ubiquitin through a concerted set of reactions involving activating (E1), conjugating (E2), and ligase (E3) enzymes. Ubiquitination targets proteins for proteolysis via the proteasome and may regulate protein function independent of proteolysis. We describe the cloning and functional analysis of new members of the HECT domain family of E3 ubiquitin ligases. Murine *Wwp1* encoded a broadly expressed protein containing a C2 domain, four WW domains, and a catalytic HECT domain. A *Caenorhabditis elegans* gene was cloned encoding a HECT domain protein (CeWWP1), which was highly homologous to murine and human WWP1. Disruption of *CeWwp1* via RNA interference yielded an embryonic lethal phenotype, despite the presence of at least six additional *C. elegans* genes encoding HECT domain proteins. The embryonic lethality was characterized by grossly abnormal morphogenesis during late embryogenesis, despite normal proliferation early in embryogenesis. CeWWP1 must therefore have unique and nonredundant functions critical for embryogenesis.

Keywords: Gene family; Morphogenesis; Ubiquitination; WW domain

1. Introduction

The conjugation of the 76-amino-acid polypeptide ubiquitin to proteins is a common posttranslational modification that targets protein degradation by the proteasome (Varshavsky, 1997; Hershko and Ciechanover, 1998). Ubiquitination can also regulate protein function by a mechanism independent of proteolysis (Finley et al., 1989; Shih et al., 2000). Three enzymes function in a coordinated manner to mediate ubiquitination — an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase (Scheffner et al., 1995). E3 enzymes physically interact with substrates and are therefore critical determinants of the specificity of ubiquitination. Multiple families of E3 ubiquitin ligases exist, including SCF (Skp1, CDC53/Cullin, F box receptor) ligases (Deshaies, 1999), Ring finger ligases (Kwon et al., 1998), the anaphase promoting complex (Morgan, 1999), and HECT (homologous to E6AP carboxy terminus) domain ligases (Huibregtse et al., 1995). The HECT domain is a novel protein module containing a cysteine residue that forms a thioester bond with ubiquitin and is critical for catalytic activity (Scheffner et al., 1995).

The family of human HECT domain ubiquitin ligases contains at least 20 members, some of which have unique expression patterns in adult tissues (Schwartz

Abbreviations: HECT, homologous to E6 carboxy terminus; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RNAi, RNA interference; RT, reverse transcriptase.

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et al., 1998). A subfamily of HECT domain proteins contains modules known as WW domains that bind tightly to polypeptides containing the amino acid motif, PPXY (PY motif), and certain other proline-rich motifs (Kay et al., 2000). Mammalian HECT/WW proteins include WWP1, WWP2, Itch, AIP4, NEDD4, and RPF1 (Huibregtse et al., 1993; Imhof and McDonnell, 1996; Kumar et al., 1997; Pirozzi et al., 1997; Perry et al., 1998; Wood et al., 1998). Based on the multiple members of the HECT protein family, individual members may have unique and dedicated roles in specific biological pathways. Examples of HECT domain ubiquitin ligases with diverse functions include NEDD4, E6–AP, Itch, and RPF1. NEDD4 mediates the ubiquitination and downregulation of an epithelial cell sodium channel that maintains electrolyte homeostasis (Staub et al., 1996). The physiological importance of NEDD4 is apparent from the human disease Liddle's syndrome, a hypertensive condition resulting from a mutation of the sodium channel, rendering it incompetent to associate with NEDD4. E6–AP mediates papillomavirus-induced ubiquitination and degradation of the tumor suppressor protein p53 (Scheffner et al., 1993). Itch appears to be important for regulating immune and inflammatory responses, as a paracentric inversion of murine *Itch* is associated with hyperplasia of certain hematopoietic cell types and severe inflammation (Perry et al., 1998). Lastly, RPF1 potentiates transactivation mediated by progesterone and glucocorticoid receptors in mammalian cells (Imhof and McDonnell, 1996), and its *Saccharomyces cerevisiae* ortholog RSP5 mediates DNA damage-inducible degradation of the large subunit of RNA polymerase II (Huibregtse et al., 1997). Thus, HECT domain ubiquitin ligases appear to have diverse functions.

Additional members of the HECT/WW domain family of ubiquitin ligases have been cloned, but have not been assigned specific functions. Pirozzi et al. (1997) cloned a cDNA encoding a HECT domain ubiquitin ligase, WWP1, from a human bone marrow library. Although the clone was incomplete, four WW domains and a HECT domain were evident. The broad expression of human *Wwp1* (*hWwp1*) in adult and fetal tissues suggests that it has a fundamentally important role in diverse cell types (Mosser et al., 1998). The peptide binding specificity of WW domains from WWP1 has been characterized (Pirozzi et al., 1997), and one of the WW domains binds with high affinity in vitro to PY motifs within the activation domain of the hematopoietic cell-specific transcription factor NF-E2 (Mosser et al., 1998). However, the endogenous proteins interacting with WWP1 and its physiological role are unknown.

To investigate the function of WWP1, we cloned murine (*mWwp1*) and a *C. elegans* (*CeWwp1*) ortholog of *hWwp1*. Disruption of *CeWwp1* by RNA interference revealed it to be essential for morphogenesis during late embryogenesis, but not for general cell proliferation or metabolic processes necessary for survival.

2. Materials and methods

2.1. cDNA synthesis, 5′ rapid amplification of cDNA ends (RACE) and cloning

mWwp1 was cloned using 5′- and 3′-RACE with a murine heart Marathon-Ready cDNA library (Clontech) according to the manufacturer's instructions. After digestion with *Not*I and *Pst*I, the RACE products were subcloned into pBluescript SK+ (Stratagene). Fragments containing *mWwp1* were identified by Southern blotting with an *hWwp1* partial cDNA probe, and the positive clones were subjected to double-stranded sequencing.

2.2. Interspecific mouse backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *mWwp1* locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blotting and hybridization were performed as described (Jenkins et al., 1982). The probe, a ~450 bp fragment of mouse intronic genomic DNA, was labeled with [α^{32}P]-dCTP by random priming; washing was done to a final stringency of 0.5 × SSCP, 0.1% SDS, 65°C. A fragment of 5.8 kb was detected in *Pst*I-digested C57BL/6J DNA and an 8.5 kb fragment was detected in *Pst*I-digested *M. spretus* DNA. The presence or absence of the 8.5 kb *Pst*I *M. spretus*-specific fragment was followed in backcross mice. A description of the probes and RFLPs for the loci linked to *mWwp1* including *Gem*, *Pou3f2*, and *Cga* was reported previously (Avraham et al., 1993; Santoro et al., 1995). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

2.3. Northern blotting analysis

A blot containing mRNA (2 μg) from adult mouse tissues was obtained from Clontech (murine multiple tissue northern blot). A 0.55 kb *mWwp1* restriction fragment including WW domains 1.3 and 1.4 (nucleotides 1368–1923 of Fig. 1**A**) was random primed with [α^{32}P]-dCTP, and the blots were hybridized under stringent conditions with 2×10^7 cpm of probe in 10 ml of ExpressHyb (Clontech). After stringent washing for 10 min with 2 × SSC–1% SDS, followed by 2 h at 68°C

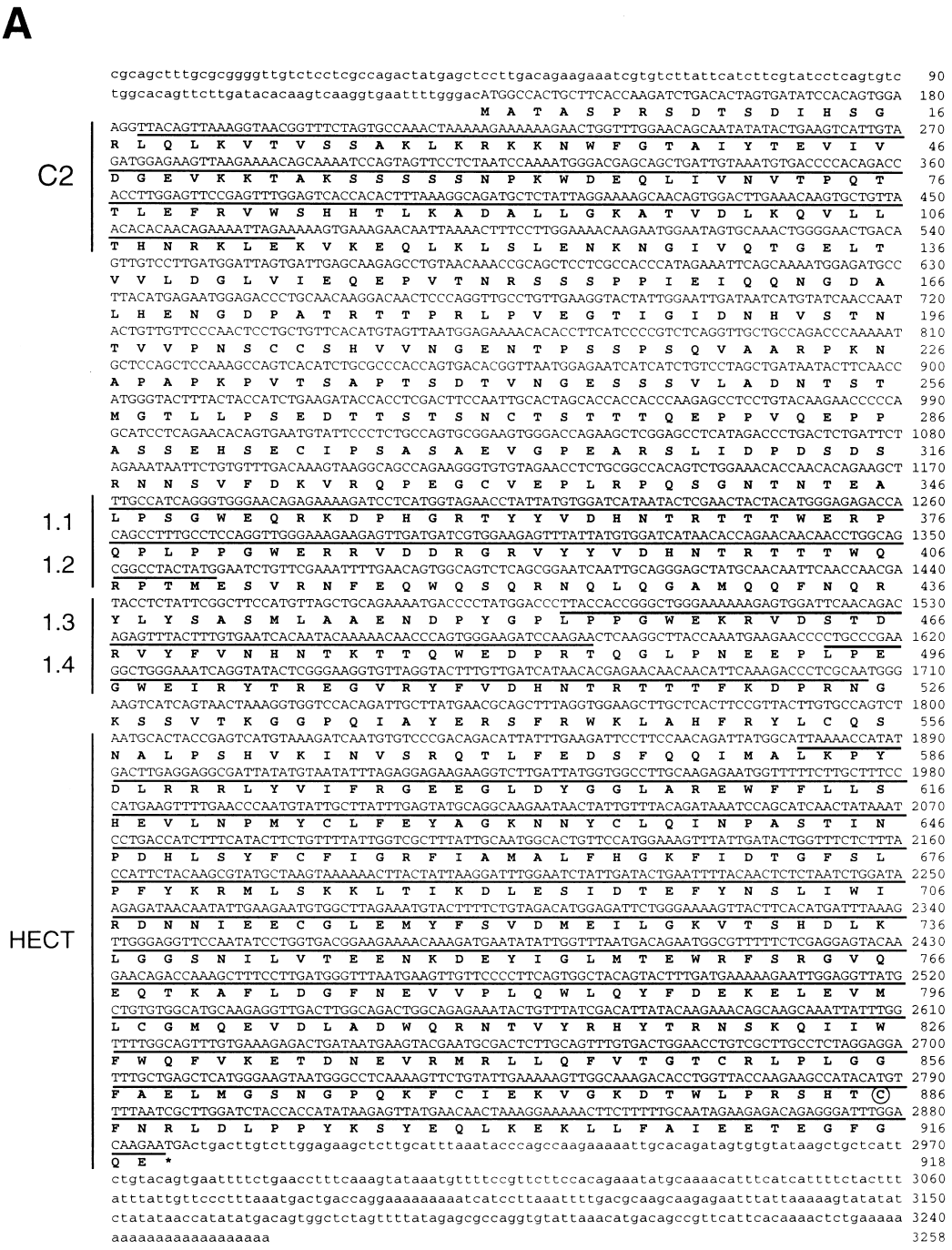

Fig. 1. cDNA structure of mouse *Wwp1*. (**A**) The *mWwp1* cDNA nucleotide sequence including partial 5′ and 3′ untranslated sequences. The deduced amino acid sequence of WWP1 is also shown with a C2 domain, WW domains (1.1–1.4) and HECT domain overlined. The cysteine residue required for ubiquitin binding is circled and the stop codon is indicated by an asterisk. (**B**) The diagram depicts the domain structure of WWP1, including a C2 domain, four WW domains and a HECT domain.

with 0.2 × SSC–0.1% SDS, bands were visualized by exposing the blot to a PhosphorImager for 24 h. The blot was stripped and reprobed with a mouse β-actin probe.

2.4. Phylogenetic analysis

Amino acid sequences of the mouse WWP1 protein and 15 WWP and Smurf proteins obtained through BLAST searching were aligned pair-wise using the 'BLAST 2 Sequences' search tool (Tatusova and Madden, 1999). The sequence alignments were refined by eye using the conserved core WW domains and conserved amino acid motifs within the C-terminal and N-terminal domains as guides. The aligned sequences were analyzed for relationships using both the neighbor-joining (NJ) methodology and the maximum parsimony methodology. Analyses were performed using components of the Phylogenetic Analysis Using Parsimony (PAUP) program, version 4.0 beta (Swofford, 1998).

NJ trees were computed from mean character distances between sequences, with gaps treated as missing data. A consensus NJ tree was computed from the results of 500 bootstrap replicates from the original sequences. Parsimony trees were computed using a heuristic search strategy that employed initial trees generated by random, step-wise addition (100 trees held at each step) of sequences with branch swapping using the tree-bisection-reconnection (TBR) algorithm. For these analyses, gaps were treated as a new character state (= 21st amino acid). Equal weights were given to each character and character state changes were unordered and unweighted. Trees were computed unrooted and then rooted to the Smurf sequences for presentation. A consensus parsimony tree was computed from 500 bootstrap replicates of the original sequences.

The Smurf proteins (two sequences) do not share homology in the N-terminal domain with the WWP proteins (14 sequences). Thus, the NJ and parsimony analyses were performed in two ways. One set of analyses used all aligned characters (1258 characters) with the N-terminus of the Smurf proteins given missing values, whereas the second set of analyses used only the aligned WW domain and C-terminal segments (825 characters); both analyses yielded identical results.

2.5. RNA interference

The *CeWwp1* clone Y65B4BR was identified from a BLAST search (Altschul et al., 1997) of the Sanger Center *C. elegans* genome database for sequences related to *mWwp1*. The SMART program (http://smart.embl-heidelberg.de) (Schultz et al., 1998) was used to evaluate the domain structure of the predicted CeWWP1 protein. Additional *C. elegans* HECT domain proteins were identified by a search of the Wormpep database of predicted proteins. Alignment of HECT domains was done using the 'BLAST 2 Sequences' program (Tatusova and Madden, 1999) to determine percent identity.

Disruption of *CeWwp1* was accomplished by RNA

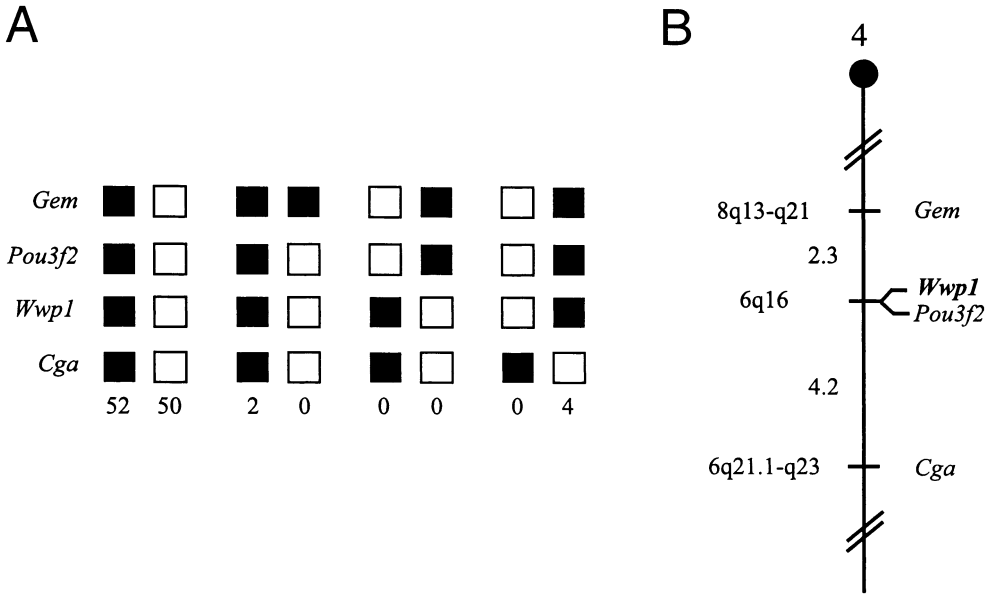

Fig. 2. Chromosomal localization of *mWwp1*. *mWwp1* was placed on mouse chromosome 4 by interspecific backcross analysis. (**A**) The segregation patterns of *mWwp1* and flanking genes in 108 backcross animals that were typed for all loci. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F_1 parent. Alleles of C57BL/6J and *M. spretus* are represented by shaded and white boxes respectively. (**B**) A partial chromosome 4 linkage map showing the location of *mWwp1* in relation to linked genes. The chromosome is drawn to scale with recombination distances given in centimorgans. Loci have been positioned on the interspecific backcross map with respect to the centromere.

interference (RNAi) (Bosher and Labouesse, 2000). To prepare double-stranded RNA for RNAi, an 869 bp region of *CeWwp1* encompassing exons for WW domains 2–4 and 79 amino acids of the HECT domain was amplified by PCR from genomic DNA. In this region, no nucleotide sequence similarity was evident between *CeWwp1* and clones of other *C. elegans* HECT domain proteins. Thus, it is highly unlikely that the activities of other E3 ubiquitin ligase genes would be influenced by the RNAi. PCR primers were 5′-AAAACTCGAGCTTGAAGCACACGAACAATGG and 5′-AAAAGAATTCGGCAATGAATCGTCCAATATACTC. The PCR product was cloned into pBluescript SK+ as a *Xho*I–*Eco*RI fragment. *CeWwp1* double-stranded RNA (dsRNA) was transcribed from the pBluescript clone using a Megascript kit (Ambion). *CeWwp1* dsRNA was introduced either by microinjection or by soaking. Similar phenotypes were observed by both methods. For microinjections, young N2 adults were injected with 2 μg/μl dsRNA. For soaking, L4 animals were soaked in M9 buffer (22 mM KH_2PO_4, 42 mM NaH_2PO_4, 85 mM NaCl, 1 mM $MgSO_4$) containing 2 μg/μl dsRNA overnight, then transferred to plates to lay eggs, a protocol similar to that described by Subramanian and Seydoux (1999).

3. Results and discussion

3.1. Cloning, chromosomal localization, and expression of mWwp1

A 3.2 kb *mWwp1* cDNA was assembled from 5′- and 3′-RACE products obtained from a heart cDNA library (Fig. 1**A**). The cDNA sequence encodes a protein of 918 amino acids. A partial *hWwp1* clone was described previously lacking complete 5′ and 3′ ends (Pirozzi et al., 1997). Like hWWP1, mWWP1 contains four WW domains and a HECT domain (Fig. 1**B**). In addition, mWWP1 contains a C2 domain (Perin et al., 1990) at the amino terminus, which was not evident from the partial human cDNA. C2 domains bind Ca^{2+} and phospholipids and are present in a subset of the HECT domain ubiquitin ligases.

The mouse chromosomal location of *Wwp1* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2900 loci, which are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse genomic DNA probe. The 8.5 kb *Pst*I *M. spretus* RFLP was used to follow the segregation of the *Wwp1* locus in backcross

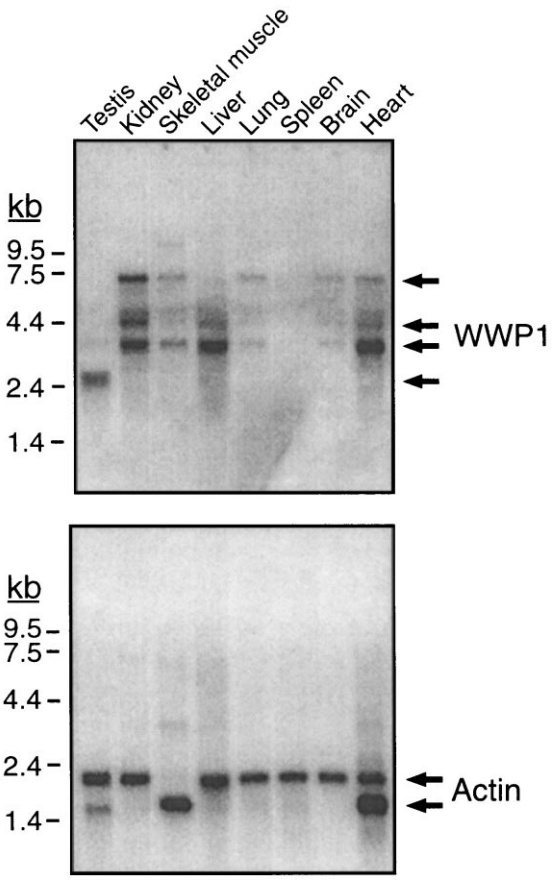

Fig. 3. *mWwp1* expression in adult mouse tissues. *mWwp1* expression was measured by northern blotting using a multiple tissue northern blot. Multiple *mWwp1* transcripts were detected under high-stringency conditions (arrows). The blot was stripped and hybridized with a β-actin probe as a control. The 2.0 kb β-actin transcript was detected in all tissues, whereas the 1.8 kb muscle-specific α-actin transcript was detected in skeletal muscle and heart. The size of the transcripts was determined based on the mobility of RNA size standards (as indicated to the left of the blot).

mice. The mapping results indicated that *Wwp1* is located in the proximal region of mouse chromosome 4 linked to *Gem*, *Pou3f2* and *Cga*. Although 108 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 2), up to 172 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere — *Gem* — 4/172 — *Pou3f2* — 0/167 — *Wwp1* — 5/118 — *Cga*. The recombination frequencies [expressed as genetic distances in centimorgans (cM) ± the standard error] are: *Gem*, 2.3 ± 1.2; [*Pou3f2*, *Wwp1*, 4.2 ± 1.9 — *Cga*]. No recombinants were detected between *Pou3f2* and *Wwp1* in 167 animals typed in common, suggesting that the two loci are within 1.8 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosome 4 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (Mouse Genome Database, Jackson Laboratory). *Wwp1* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown).

Like *hWwp1*, which is broadly expressed in adult and fetal tissues (Mosser et al., 1998), northern blotting showed that *mWwp1* is expressed in multiple adult tissues, with the weakest expression in spleen (Fig. 3). A predominant transcript of 3.7 kb was detected in most tissues, and variable levels of 4.6 and 7.1 kb transcripts were detected. The major transcript in testis was 2.6 kb, smaller than the 3.2 kb cDNA shown in Fig. 1. In this regard, Kwon et al. (1998) described a testis-specific transcript for the murine Ubr1 ubiquitin ligase, which was smaller than the corresponding transcripts in other tissues.

3.2. Evolutionary relationships between HECT domain proteins

A BLAST search of the *C. elegans* genome database for genes encoding homologs of mWWP1 identified an uncharacterized gene present on clone Y65B4BR from chromosome I. The deduced 792 amino acid product of the gene contains a C2 domain, four WW domains and a HECT domain. Alignment of the WW/HECT region of the predicted *C. elegans* protein with the corresponding regions of mWWP1, hWWP1 and hWWP2 revealed an average of 66% identity (79% similarity) to each of the proteins (Fig. 4). Thus, we designated this clone *CeWwp1*.

Phylogenetic analyses were conducted to assess the relatedness of mWWP1 and CeWWP1 to other HECT domain proteins from several organisms. All parsimony and NJ phylogenetic analyses produced trees with similar topology and groupings bearing only minor branch length and branch support differences. A consensus tree generated from 500 NJ bootstrap analyses indicates several important relationships (Fig. 5). The mWWP1 protein is most closely related to the hWWP1 and is also highly related to hWWP2 and mItch. Analyses absolutely support the clade consisting of the hWWP1, mWWP1, hWWP2, mItch and hAIP4 proteins, indicating that these share a single common ancestor.

Although the NEDD4 proteins are homologous to the WWP protein group, they constitute a distinct clade sharing a unique common ancestor separate from the WWP proteins. The homologous fungal proteins also cluster together and are supported as a unique clade more closely related to the WWP group in the NJ tree. However, the parsimony bootstrap analyses do not support this topology or these relationships and place all three clades (WWP, NEDD4 and Fungal proteins) at an unresolved polytomy. These results indicate that the WWP/NEDD4-type gene was established before the split of the fungi and animals with the duplication event giving rise to the WWP and NEDD4 groups occurring near the time of the divergence of these groups.

The Smurf proteins (Zhu et al., 1999) appear to be related to the WWP/NEDD4 proteins. However, they have low homology within the N-terminal region to the

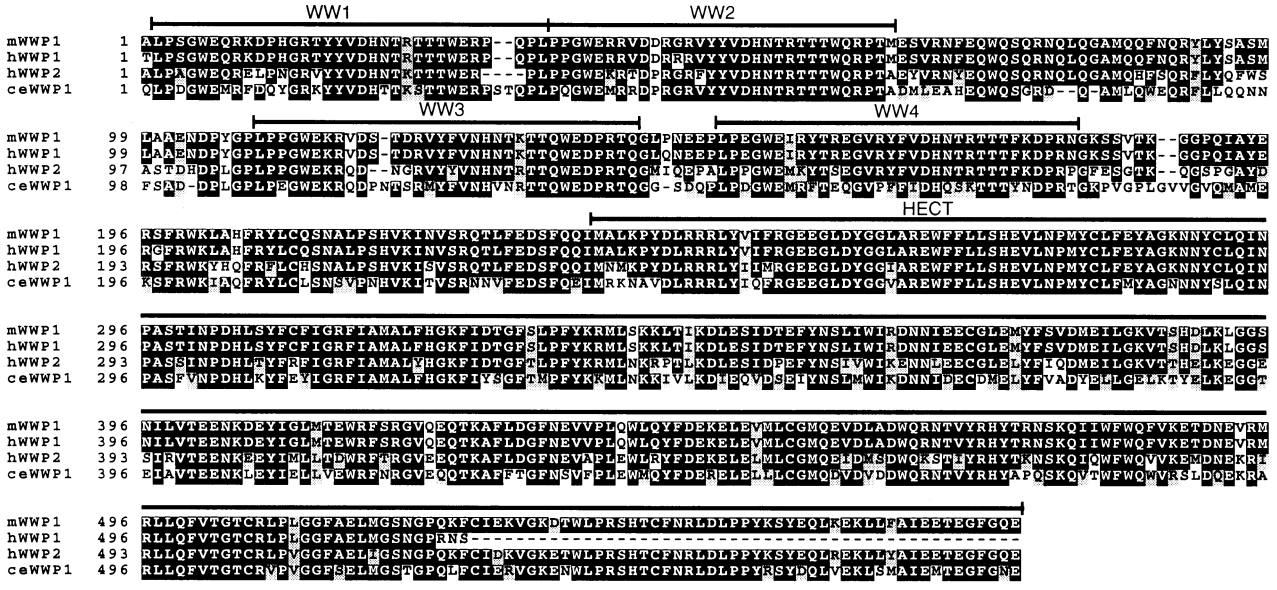

Fig. 4. Strong conservation of HECT domain proteins from nematodes to humans. Alignment of WW and HECT domains of WWP1 and WWP2 proteins from human, mouse and *C. elegans*. Domains were predicted using the SMART program and aligned using the Clustal W program. Identities and similarities are shaded black and gray respectively. Position 1 corresponds to the amino terminus of WW1 and not to the first amino acid of the proteins.

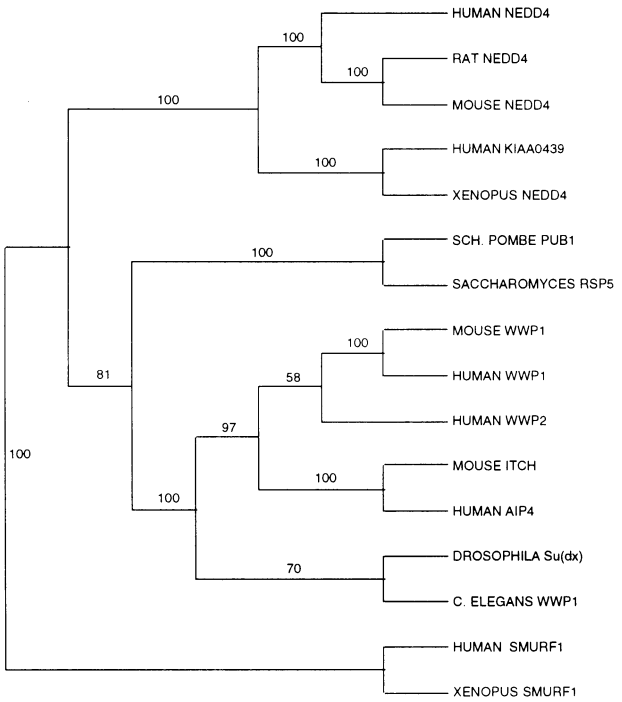

Fig. 5. NJ analysis of WWP and related proteins. The NJ tree was determined using the PAUP program from mean character differences among sequences calculated using only the conserved WW domains and HECT domain. The NJ tree was contained within the 50% majority-rule consensus tree from 500 bootstrap replicates. The percent branch support from the bootstrap analyses is indicated above each branch.

WWP/NEDD4 proteins and have a reduced number of WW domains. These differences may have resulted from independent losses or independent gains of these domains onto an ancestor bearing a HECT domain. The HECT domain of E6–AP proteins cluster as the nearest neighbors to the HECT domain of the Smurf proteins in independent analyses (data not shown), suggesting a possible swapping of WW domains into a gene expressing the common HECT domain with independent acquisition or retention of unique N-termini. Current sequence information is insufficient to resolve these possibilities.

3.3. A family of C. elegans HECT domain proteins

In addition to CeWWP1, sequences were obtained for six uncharacterized HECT domain proteins from the Wormpep database of *C. elegans* predicted proteins. The predicted proteins contain a HECT domain and diverse protein–protein interaction domains (Table 1). Unlike human and mouse WWP1s, which are highly conserved throughout the HECT domains with CeWWP1 (71% and 72% identity respectively), the HECT domains of these *C. elegans* proteins share no more than 37% identity with CeWWP1; thus, these sequences were not included in the phylogenetic analysis of Fig. 5. The poor sequence conservation and diversity of protein–protein interaction domains suggest that these proteins have unique and nonredundant functions.

3.4. CeWWP1 is essential for morphogenesis but not for general cell proliferation and metabolism

To define the physiological role of CeWWP1, we disrupted *CeWwp1* by RNAi (Fig. 6). Regardless of whether the *CeWwp1* dsRNA was introduced by injection or soaking, the predominant phenotype was embryonic lethality (84%). However, some animals escaped embryonic lethality and either arrested at an early larval stage (L1–L2, 7%) or matured into fertile adults (9%). Lethality occurred late in embryogenesis, as *CeWwp1* RNAi embryos developed to the comma stage without gross abnormalities (Fig. 6**B**, compare panels a and b). In wild-type embryos, most cell divisions are completed by the comma stage of development. As *CeWwp1* RNAi embryos develop normally to this stage, WWP1 is not essential for general cell proliferation or metabolic processes required for survival. Differentiated tissues were detected (intestine, pharyngeal muscles, body wall

Table 1
C. elegans HECT domain proteins

Protein[a]	Size[b] (amino acids)	Chromosome	% identity[c] (HECT domains)	Domain[d]	Domain function
CeWWP1	792	I	100	WW(4)	Interaction with specific proline-rich sequences
F45H7.6	889	III	34	WW(2)	
D2085.4	1001	II	26	IQ(1)	Calmodulin binding
C34D4.14	2761	IV	32	ANK(2)	Protein–protein interactions
F36A2.13	2899	I	24	ZnF UBR1 (1)	N-end rule recognition
Y39A1C.2	1066	III	27		
Y54G2A.A	875	IV	37		

[a] CDS identifier for Wormpep database.
[b] Predicted size.
[c] Percent identities of HECT domains were determined by 'Blast 2 Sequences' and are relative to CeWWP1.
[d] The number of domains is indicated in parentheses.

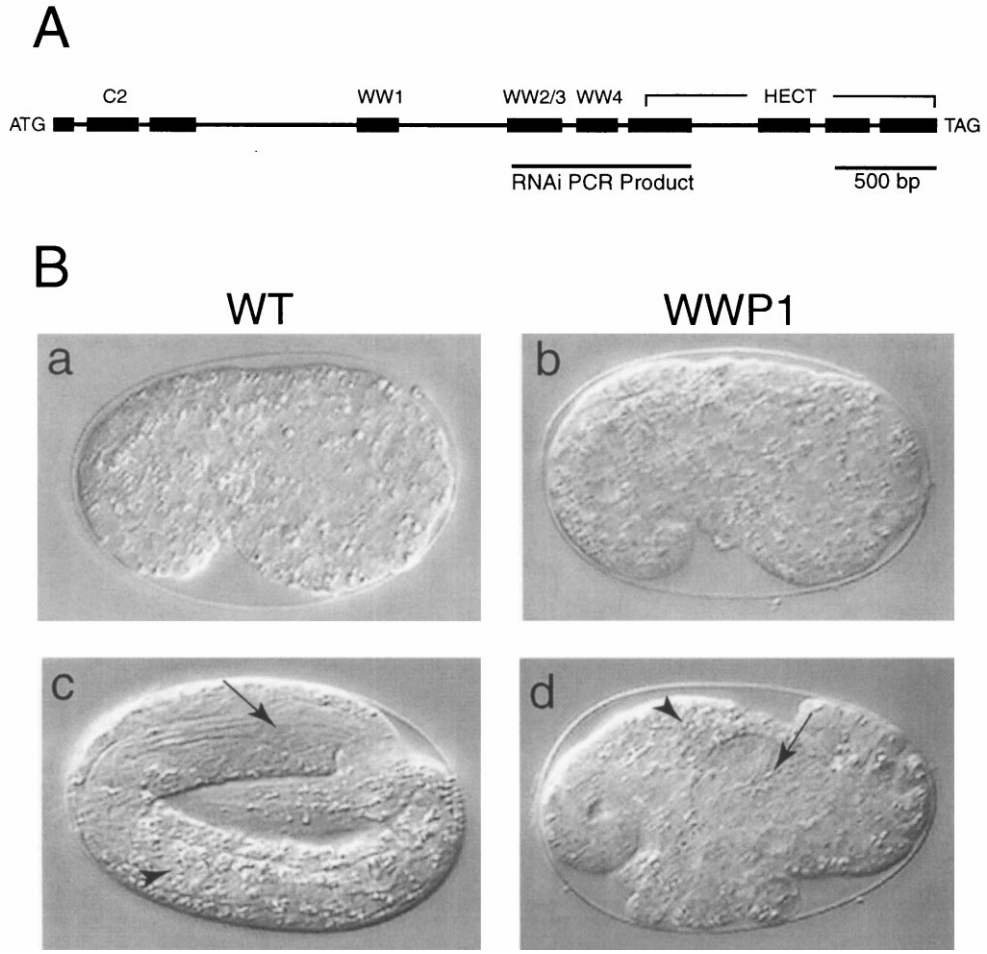

Fig. 6. Disruption of *CeWwp1* expression by RNAi results in severe morphological defects and late embryonic lethality. (**A**) Predicted genomic structure of *CeWwp1*. Genomic sequence was obtained from a BLAST search of the *C. elegans* genome database for matches to *mWWP1*. The region corresponding to the interfering dsRNA is underlined. Boxes and lines denote exons and introns respectively. (**B**) Wild-type embryos and *CeWwp1* RNAi embryos at the comma stage (panels a and b) and just prior to hatching (panels c and d). Arrows indicate pharyngeal tissue, and arrowheads point to intestinal granules.

muscles) in the arrested *CeWwp1* RNAi embryos, although massive tissue disorganization was evident. For example, even though pharyngeal tissue was present, it did not organize into a morphologically wild-type pharynx (Fig. 6**B**, compare panels c and d). Thus, CeWWP1 is essential for morphogenesis. Although one can rule out a general role for CeWWP1 in cell proliferation, CeWWP1 may be required for a specialized subset of cell divisions.

Functional analyses of mammalian HECT domain ubiquitin ligases have revealed diverse physiological roles. Consistent with this, the requirement for CeWWP1 in morphogenesis is clearly unique and nonredundant. Interestingly, the only HECT domain protein of *S. cerevisiae* that contains WW domains, RSP5, is also the only one shown to be essential (Huibregtse et al., 1995). The results described herein represent the only report of a HECT domain ubiquitin ligase essential for morphogenesis in any organism. It is unclear whether this developmental function is unique or will also be seen with other HECT domain ubiquitin ligases of unknown function. Given the strong conservation of CeWWP1 with mouse and human WWP1, an attractive hypothesis is that the mammalian ortholog of CeWWP1 may also be critical for morphogenesis. The developmental requirement for CeWWP1 may reflect the control of a morphogenetic regulator(s) via CeWWP1-mediated ubiquitination. The power of *C. elegans* genetics should allow one to define the physiological roles of CeWWP1 and other members of this small HECT domain family. Further analyses with this system may reveal whether individual ubiquitin ligases have unique substrates and are committed to specific biological pathways.

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