

# Distinct and Redundant Functions of $\mu$ 1 Medium Chains of the AP-1 Clathrin-Associated Protein Complex in the Nematode *Caenorhabditis elegans*

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In the nematode *Caenorhabditis elegans*, there exist two  $\mu$ 1 medium chains of the AP-1 clathrin-associated protein complex. Mutations of *unc-101*, the gene that encodes one of the  $\mu$ 1 chains, cause pleiotropic effects (Lee *et al.*, 1994). In this report, we identified and analyzed the second  $\mu$ 1 chain gene, *apm-1*. Unlike the mammalian homologs, the two medium chains are expressed ubiquitously throughout development. RNA interference (RNAi) experiments with *apm-1* showed that *apm-1* and *unc-101* were redundant in embryogenesis and in vulval development. Consistent with this, a hybrid protein containing APM-1, when overexpressed, rescued the phenotype of an *unc-101* mutant. However, single disruptions of *apm-1* or *unc-101* have distinct phenotypes, indicating that the two medium chains may have distinct functions. RNAi of any one of the small or large chains of AP-1 complex ( $\sigma$ 1,  $\beta$ 1, or  $\gamma$ ) showed a phenotype identical to that caused by the simultaneous disruption of *unc-101* and *apm-1*, but not that by single disruption of either gene. This suggests that the two medium chains may share large and small chains in the AP-1 complexes. Thus, *apm-1* and *unc-101* encode two highly related  $\mu$ 1 chains that share redundant and distinct functions within AP-1 clathrin-associated protein complexes of the same tissue.

## INTRODUCTION

Clathrin-coated pits and vesicles are ubiquitous organelles found in all the eukaryotic cells that mediate intracellular protein trafficking (Keen, 1990; Robinson, 1994; Hirst and Robinson, 1998). Clathrin-coated vesicles are composed of membrane fraction, selected membrane proteins, clathrin, and clathrin-associated proteins (APs). While clathrin is a structural unit common to all the clathrin-coated vesicles, APs can vary depending on the localization of the vesicles at the cellular and subcellular level (for example, Ahle *et al.*, 1988; Dell'Angelica *et al.*, 1997), and are thought to be important in selecting cargoes in the vesicles. There are four AP complexes identified so far in various species. All four complexes are similar in their composition and structure in that they are hetero-tetramers of two large chains, one small chain, and one medium chain. The medium chains of the clathrin AP complexes are known to interact with the tyrosine or dileucine residues of their cargo proteins (Ohno *et al.*, 1995; Rodionov and Bakke, 1998; Hofmann *et al.*, 1999). AP-1 complex contains  $\beta$ 1 and  $\gamma$  adaptin as large chains,  $\mu$ 1A or  $\mu$ 1B as a medium chain, and  $\sigma$ 1 (AP19) as a small chain. AP-2 has  $\alpha$ - and  $\beta$ 2 adaptin as large chains,  $\mu$ 2 (AP50) as a medium chain, and  $\sigma$ 2 (AP17) as a small chain. AP-3

complex consists of  $\beta$ 3A or  $\beta$ 3B and  $\delta$  adaptins as large chains,  $\mu$ 3A or  $\mu$ 3B as medium chains, and  $\sigma$ 3 as a small chain (Dell'Angelica *et al.*, 1997; Simpson *et al.*, 1997). AP-4 complex is a recently identified complex that consists of  $\beta$ 4 and  $\epsilon$  adaptins as large chains,  $\mu$ 4 as a medium chain, and  $\sigma$ 4 as a small chain (Dell'Angelica *et al.*, 1999a; Hirst *et al.*, 1999). Some of the large chains share some similarity in their amino acid sequence (for example, Kirchhausen *et al.*, 1989; Robinson, 1989), as do those of medium chains and small chains (Kirchhausen *et al.*, 1991; Nakai *et al.*, 1993; Nakayama *et al.*, 1991; Phan *et al.*, 1994; Thuriereau *et al.*, 1988). The localization of the AP-1 and AP-2 complexes is well known. AP-1 is at the *trans*-Golgi, AP-2 on the plasma membrane. The medium chains of the mammalian AP-1 complexes show tissue-specific expression (Ohno *et al.*, 1999). AP-3 complexes were reported to be present in most cells, but some components of the AP-3 complexes were tissue-specific (Dell'Angelica *et al.*, 1997; Pevsner *et al.*, 1994). AP-4 was associated with the *trans*-Golgi network or with an adjacent structure in all cell types (Dell'Angelica *et al.*, 1999a; Hirst *et al.*, 1999).

Genetic analysis of medium chains has been reported in many systems. In yeast, mutations in  $\mu$ 1 is known to enhance the temperature-sensitive growth phenotype and the  $\alpha$ -factor processing defect caused by a temperature-sensitive allele of the clathrin heavy chain gene (Stepp *et al.*, 1995). AP-3 is necessary for proper sorting of vacuolar alkaline

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**A**

APM-1	1	<b>MSISGLFILD</b> LKGNVVISRNYRGD <b>VD</b> MSCIEK <b>F</b> MP <b>LL</b> VE <b>KE</b> DEGSASPVLVHQGISYTYI	60
UNC-101	1	<b>MATSAMFILD</b> LKGTIISRNYRGD <b>ID</b> MTAIDKF <b>I</b> HLL <b>ME</b> KEEGSAAPVLT <b>Y</b> QDTNFVFI	60
AP47	1	<b>MSASAVYVLD</b> LKGVLCRNYRGD <b>VD</b> MSEVEHF <b>MP</b> IL <b>ME</b> KEEGMLSPILAHGGVRFMWI	60
APM-2	1	MTGGFLVYVNHKGEVLSRIYRDDVTRNAVDAFRVNV <b>I</b> HARQQ-VRSPVTNMARTSFFHV	58
		* * * * *	
APM-1	61	<b>KYMN</b> VYLVTISKKNTN <b>V</b> LVLSALY <b>K</b> I <b>VE</b> VFCE <b>Y</b> FK <b>T</b> LE <b>EE</b> AVRDN <b>F</b> VI <b>I</b> YEL <b>F</b> DE <b>ML</b> DF	120
UNC-101	61	<b>KHTN</b> IYLV <b>S</b> ACRSNN <b>V</b> VT <b>M</b> IL <b>S</b> FLY <b>K</b> CV <b>E</b> VF <b>S</b> E <b>Y</b> FK <b>D</b> VE <b>EE</b> SVRDN <b>F</b> VI <b>I</b> YEL <b>L</b> DE <b>MM</b> DF	120
AP47	61	<b>KHNN</b> LYLV <b>A</b> TSKKNAC <b>V</b> SLV <b>S</b> FLY <b>K</b> V <b>V</b> Q <b>V</b> F <b>S</b> E <b>Y</b> FK <b>E</b> LE <b>EE</b> SI <b>R</b> DN <b>F</b> VI <b>I</b> YEL <b>L</b> DE <b>L</b> MD <b>F</b>	120
APM-2	59	KRGNV <b>W</b> ICX <b>V</b> TRQNV <b>N</b> AAM <b>V</b> FA <b>L</b> K <b>R</b> FAD <b>T</b> M <b>Q</b> SY <b>F</b> G <b>K</b> L <b>NE</b> EN <b>V</b> K <b>N</b> N <b>F</b> VL <b>I</b> YEL <b>L</b> DE <b>I</b> L <b>D</b> F	118
		* * * * *	
APM-1	121	<b>GYPQT</b> TESK <b>IL</b> Q <b>E</b> FI <b>T</b> Q <b>Q</b> GN <b>L</b> -----ETVRPPMAV <b>T</b> NAV <b>S</b> WR <b>S</b> EG <b>I</b> K <b>Y</b> R <b>K</b> NE <b>V</b> F	170
UNC-101	121	<b>GFPQT</b> TESR <b>IL</b> Q <b>E</b> Y <b>I</b> T <b>Q</b> E <b>G</b> Q <b>K</b> L-----ISAPRXPMAV <b>T</b> NAV <b>S</b> WR <b>S</b> EG <b>I</b> K <b>Y</b> R <b>K</b> NE <b>V</b> F	171
AP47	121	<b>GYPQT</b> TD <b>S</b> K <b>IL</b> Q <b>E</b> Y <b>I</b> T <b>Q</b> E <b>G</b> H <b>K</b> L-----TGAPRPPAT <b>V</b> NAV <b>S</b> WR <b>S</b> EG <b>I</b> K <b>Y</b> R <b>K</b> NE <b>V</b> F	172
APM-2	119	GYPQ <b>NT</b> DP <b>G</b> VL <b>K</b> TF <b>I</b> T <b>Q</b> Q <b>V</b> RTADAP <b>V</b> VP <b>V</b> T <b>K</b> EE <b>S</b> Q <b>I</b> T <b>S</b> Q <b>V</b> T <b>G</b> Q <b>I</b> G <b>W</b> RR <b>E</b> G <b>I</b> K <b>Y</b> RR <b>N</b> E <b>L</b> F	178
		* * * * *	
APM-1	171	<b>LDV</b> IESVN <b>L</b> ANA <b>Q</b> GT <b>V</b> L <b>R</b> SE <b>I</b> VG <b>S</b> I <b>R</b> FR <b>V</b> VL <b>S</b> GM <b>P</b> EL <b>R</b> L <b>GL</b> ND <b>K</b> V <b>F</b> F <b>Q</b> Q <b>S</b> G <b>A</b> SS <b>R</b> ---R	227
UNC-101	172	<b>LDV</b> IESVN <b>L</b> AS <b>A</b> NG <b>T</b> V <b>L</b> Q <b>S</b> E <b>I</b> VG <b>S</b> V <b>K</b> MR <b>V</b> YL <b>T</b> GM <b>P</b> EL <b>R</b> L <b>GL</b> ND <b>K</b> V <b>L</b> F <b>E</b> G <b>S</b> GR <b>G</b> K-----	226
AP47	173	<b>LDV</b> IEAV <b>N</b> LL <b>V</b> S <b>A</b> NG <b>N</b> V <b>L</b> R <b>S</b> E <b>I</b> VG <b>S</b> I <b>K</b> MR <b>V</b> FL <b>S</b> GM <b>P</b> EL <b>R</b> L <b>GL</b> ND <b>K</b> V <b>L</b> FD <b>N</b> T <b>G</b> R <b>G</b> K-----	227
APM-2	179	LDVIEY <b>V</b> N <b>L</b> LM <b>N</b> Q <b>Q</b> Q <b>V</b> L <b>S</b> A <b>H</b> V <b>G</b> R <b>K</b> V <b>A</b> M <b>K</b> S <b>Y</b> L <b>S</b> GM <b>P</b> E <b>C</b> K <b>F</b> G <b>I</b> ND <b>K</b> I <b>T</b> IE <b>G</b> K <b>S</b> K <b>P</b> G <b>S</b> DD <b>P</b> N	238
		* * * * *	
APM-1	228	<b>GNSG</b> K <b>G</b> VE <b>L</b> E <b>D</b> I <b>K</b> F <b>H</b> Q <b>C</b> V <b>R</b> L <b>S</b> R <b>F</b> D <b>S</b> E <b>R</b> T <b>I</b> S <b>F</b> I <b>P</b> P <b>D</b> G <b>E</b> F <b>E</b> L <b>M</b> S <b>Y</b> R <b>L</b> T <b>T</b> Q <b>V</b> K <b>P</b> L <b>I</b> W <b>E</b> A <b>A</b> V <b>E</b>	287
UNC-101	227	--- <b>SK</b> S <b>V</b> E <b>L</b> E <b>D</b> V <b>K</b> F <b>H</b> Q <b>C</b> V <b>R</b> L <b>S</b> R <b>F</b> D <b>T</b> D <b>R</b> T <b>I</b> S <b>F</b> I <b>P</b> P <b>D</b> G <b>A</b> F <b>E</b> L <b>M</b> S <b>Y</b> R <b>L</b> T <b>T</b> V <b>V</b> K <b>P</b> L <b>I</b> W <b>I</b> E <b>T</b> S <b>I</b> E	283
AP47	228	--- <b>SK</b> S <b>V</b> E <b>L</b> E <b>D</b> V <b>K</b> F <b>H</b> Q <b>C</b> V <b>R</b> L <b>S</b> R <b>F</b> E <b>N</b> D <b>R</b> T <b>I</b> S <b>F</b> I <b>P</b> P <b>D</b> G <b>E</b> F <b>E</b> L <b>M</b> S <b>Y</b> R <b>L</b> N <b>T</b> H <b>V</b> K <b>P</b> L <b>I</b> W <b>I</b> E <b>S</b> V <b>I</b> E	284
APM-2	239	KASRA <b>A</b> V <b>A</b> I <b>D</b> D <b>C</b> Q <b>F</b> H <b>Q</b> C <b>V</b> L <b>K</b> F <b>E</b> T <b>E</b> H <b>A</b> I <b>S</b> F <b>I</b> P <b>P</b> D <b>G</b> E <b>Y</b> E <b>L</b> M <b>R</b> Y <b>R</b> T <b>T</b> K <b>D</b> I <b>Q</b> L <b>P</b> F <b>R</b> V <b>I</b> P <b>L</b> V <b>R</b>	298
		* * * * *	
APM-1	288	<b>RHA</b> HSR <b>V</b> E <b>M</b> V <b>K</b> A <b>K</b> S <b>Q</b> F <b>K</b> R <b>S</b> V <b>A</b> N <b>H</b> V <b>E</b> V <b>I</b> I <b>P</b> V <b>P</b> S <b>D</b> V <b>S</b> A <b>P</b> K <b>F</b> K <b>T</b> G <b>A</b> G <b>T</b> A <b>K</b> Y <b>V</b> P <b>E</b> L <b>N</b> A <b>I</b> V <b>W</b> S	347
UNC-101	284	<b>RHS</b> SR <b>V</b> S <b>F</b> I <b>I</b> <b>K</b> A <b>K</b> S <b>Q</b> F <b>K</b> R <b>R</b> S <b>T</b> A <b>N</b> N <b>V</b> E <b>I</b> I <b>I</b> P <b>V</b> P <b>S</b> D <b>A</b> D <b>S</b> P <b>K</b> F <b>K</b> T <b>S</b> I <b>G</b> S <b>V</b> K <b>Y</b> T <b>P</b> E <b>Q</b> S <b>A</b> F <b>V</b> W <b>T</b>	343
AP47	285	<b>KHS</b> SR <b>I</b> E <b>Y</b> M <b>V</b> <b>K</b> A <b>K</b> S <b>Q</b> F <b>K</b> R <b>R</b> S <b>T</b> A <b>N</b> N <b>V</b> E <b>I</b> H <b>I</b> P <b>V</b> P <b>N</b> D <b>A</b> D <b>S</b> P <b>K</b> F <b>K</b> T <b>T</b> V <b>G</b> S <b>V</b> K <b>W</b> V <b>P</b> E <b>N</b> S <b>E</b> I <b>V</b> W <b>S</b>	344
APM-2	299	E <b>V</b> S <b>R</b> N <b>K</b> M <b>E</b> V <b>K</b> V <b>V</b> V <b>K</b> S <b>N</b> F <b>K</b> P <b>S</b> L <b>L</b> A <b>Q</b> K <b>L</b> E <b>V</b> R <b>I</b> P <b>T</b> P <b>P</b> N <b>T</b> S <b>G</b> V <b>L</b> I <b>C</b> M <b>K</b> G <b>K</b> A <b>K</b> Y <b>K</b> A <b>G</b> E <b>N</b> A <b>I</b> V <b>W</b> K	358
		* * * * *	
APM-1	348	<b>IRS</b> F <b>P</b> G <b>G</b> R <b>E</b> Y <b>I</b> M <b>R</b> S <b>S</b> F <b>M</b> L <b>P</b> S <b>I</b> G <b>S</b> E <b>E</b> L <b>E</b> --GR <b>P</b> P <b>I</b> N <b>V</b> K <b>F</b> E <b>I</b> P <b>Y</b> T <b>T</b> S <b>G</b> L <b>Q</b> V <b>R</b> Y <b>L</b> K <b>I</b> I <b>E</b> K--	403
UNC-101	344	<b>IKN</b> F <b>P</b> G <b>G</b> K <b>E</b> Y <b>L</b> L <b>T</b> A <b>H</b> L <b>S</b> L <b>P</b> S <b>V</b> M <b>S</b> E <b>E</b> S <b>E</b> --GR <b>P</b> P <b>I</b> K <b>V</b> K <b>F</b> E <b>I</b> P <b>Y</b> F <b>T</b> T <b>S</b> G <b>I</b> Q <b>V</b> R <b>Y</b> L <b>K</b> I <b>I</b> E <b>K--</b>	399
AP47	345	<b>VKS</b> F <b>P</b> G <b>G</b> K <b>E</b> Y <b>L</b> M <b>R</b> A <b>H</b> F <b>L</b> P <b>S</b> V <b>E</b> A <b>E</b> D <b>K</b> E--G <b>K</b> P <b>P</b> I <b>S</b> V <b>K</b> F <b>E</b> I <b>P</b> Y <b>F</b> T <b>T</b> S <b>G</b> I <b>Q</b> V <b>R</b> Y <b>L</b> K <b>I</b> I <b>E</b> K--	400
APM-2	359	<b>IKR</b> M <b>A</b> G <b>M</b> K <b>E</b> S <b>Q</b> I <b>S</b> A <b>E</b> I <b>D</b> L <b>L</b> S <b>T</b> G <b>N</b> V <b>E</b> K <b>K</b> W <b>N</b> R <b>P</b> P <b>V</b> S <b>M</b> N <b>F</b> E <b>V</b> P--F <b>A</b> P <b>S</b> G <b>L</b> K <b>V</b> R <b>Y</b> L <b>K</b> V <b>F</b> E <b>P</b> K <b>L</b>	417
		* * * * *	
APM-1	404	-- <b>SG</b> Y <b>Q</b> A <b>L</b> P <b>W</b> V <b>R</b> Y <b>V</b> T <b>Q</b> N <b>G</b> D <b>Y</b> Q <b>M</b> R <b>M</b> T--	426
UNC-101	400	-- <b>RG</b> Y <b>Q</b> A <b>L</b> P <b>W</b> V <b>R</b> Y <b>I</b> T <b>Q</b> N <b>G</b> E <b>Y</b> E <b>M</b> R <b>M</b> K--	422
AP47	401	-- <b>SG</b> Y <b>Q</b> A <b>L</b> P <b>W</b> V <b>R</b> Y <b>I</b> T <b>Q</b> N <b>G</b> D <b>Y</b> Q <b>L</b> R <b>T</b> Q--	423
APM-2	418	<b>NYS</b> D <b>H</b> D <b>V</b> I <b>K</b> W <b>V</b> RY <b>I</b> G <b>R</b> S <b>G</b> L <b>Y</b> --	437
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**Figure 1.** (A) Comparison of amino acid sequences of APM-1, UNC-101, APM-2, and mouse AP47. The amino acids that are conserved in the three APM-1 homologs are in bold and shaded letters, and the asterisks underneath the sequences represent the amino acids conserved in all four proteins. Some of the amino acids are conserved in all four proteins, but others are conserved only in the AP-1 medium chains. (B) Diagram showing the evolutionary relationship among medium chains of clathrin AP complexes. Medium chains of all four types of complexes are compared. The circle on the center indicates a hypothetical ancestral medium chain gene. The prefix C indicates the sequence is from *C. elegans*, H from humans, R from rat, M from mouse, D from *Drosophila*, Y from yeast. For example, CAPM-1 is *C. elegans* APM-1 and DAPM-1 is the *Drosophila* APM-1 homolog. This dendrogram clearly shows that APM-1 is a member of the AP-1 medium chain family, together with UNC-101. (C) Genomic structures of the *apm-1* and *unc-101* genes. Exons are drawn as thick lines, and introns are drawn as thin lines outside the exon structures. Introns are not drawn in scale. The numbers indicate the numbers of nucleotides in each exon or intron.

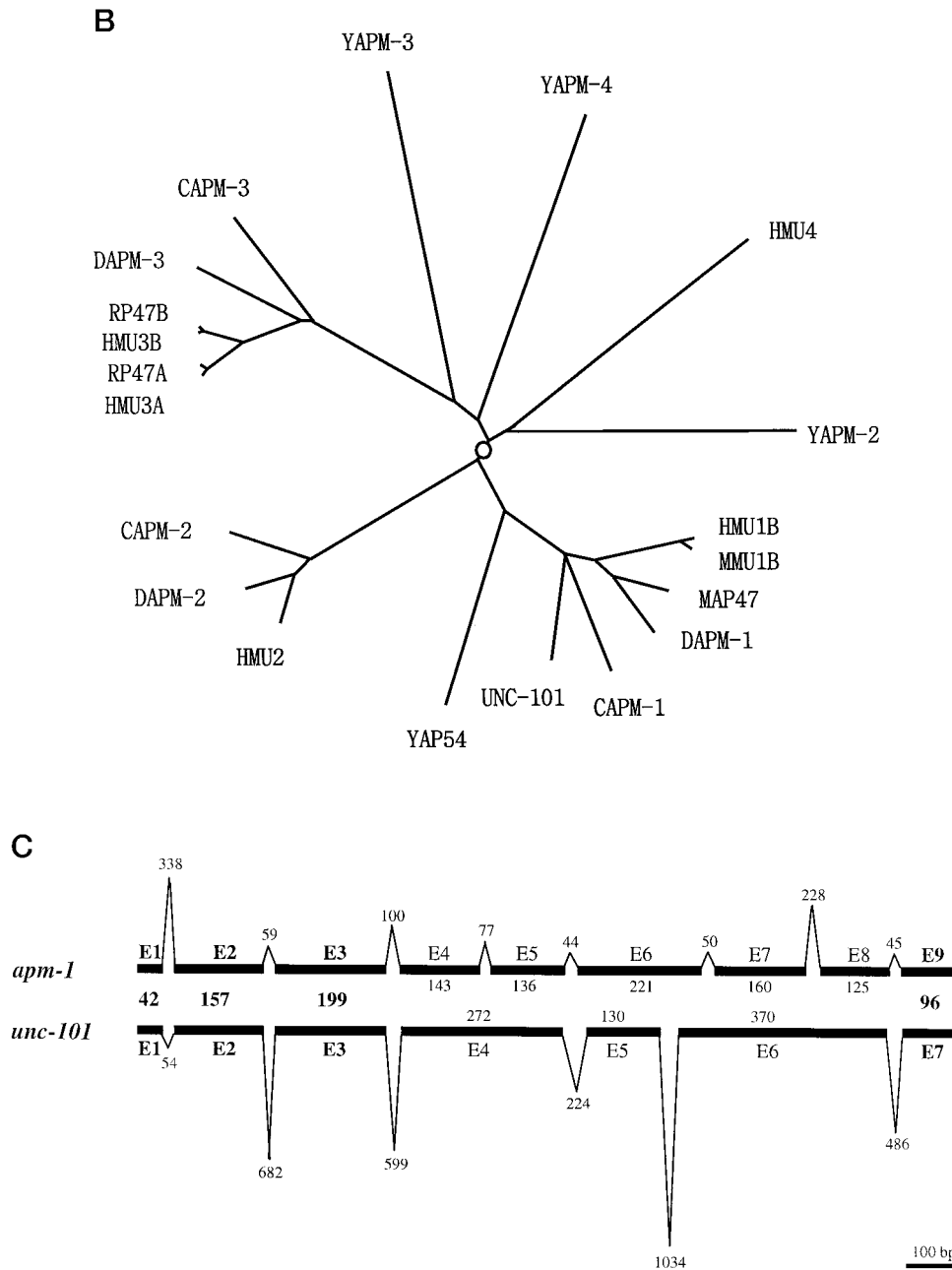


Figure 1 (cont).

phosphatase (Stepp *et al.*, 1997). These phenotypes are mild in terms of viability of the organism. In the mammalian system, two  $\mu 1$  chains have been characterized; one of them,  $\mu 1B$ , is expressed in epithelial cells and is required for basolateral targeting in these cells (Folsch *et al.*, 1999, Ohno *et al.*, 1999).  $\gamma$ -Adaptin was essential for embryonic development in mice by analyzing knockout mice (Zizioli *et al.*, 1999).  $\beta 3A$  was mutated in patients with human Hermansky-Pudlak syndrome (HPS; Dell'Angelica *et al.*, 1999b) and in the mouse hypopigmentation mutant pearl (Feng *et al.*,

1999), indicating that AP-3 functions in protein sorting to lysosomes. In the nematode *C. elegans*, one medium chain of AP-1, encoded by *unc-101*, was identified (Lee *et al.*, 1994). Genetic analysis of *unc-101* showed that mutations in this gene caused pleiotropic effects, including subviability, uncoordinated movement, a defect in neuronal dye uptake, male spicule defect, defecation defect, and suppression of a reduction-of-function mutation in the epidermal growth factor receptor (*let-23EGFR*) gene (Lee *et al.*, 1994). Interestingly, putative null mutations of *unc-101* do not cause 100%

lethality, but only 50% lethality—50% of progeny from a homozygous mother of *unc-101/unc-101* genotype will survive (Lee *et al.*, 1994). This suggests that another gene, encoding the medium chain of AP-1, can replace the essential function of *unc-101* in individuals with defects in *unc-101*. Consistent with this, *unc-101* mutations do not cause vulval defect, while *unc-101* mutations have been isolated as extragenic suppressors of the *let-23(sy1)* vulvaless phenotype. In vulval tissues, there must therefore exist other genes that act redundantly with *unc-101* as negative regulators. One such gene is *sli-1*. An *unc-101; sli-1* double mutant displays greater-than-wild-type vulval induction (Sternberg *et al.*, 1994). It is conceivable that there may be more redundant negative regulators of vulval development, because not all *unc-101; sli-1* double mutants show multivulval phenotypes (Sternberg *et al.*, 1994).

Here we report identification of a homolog of *unc-101*, which we named *apm-1* (associated protein complex medium chain-1). We show that *unc-101* and *apm-1* are both ubiquitously expressed throughout development and that *apm-1* plays redundant roles with *unc-101* in embryogenesis and vulval development. We show that *apm-1* also has distinct functions from those of *unc-101*. Finally, we report characterization of the functions of the two medium chains and compare them to those of the other AP-1 complex subunits,  $\alpha 1$ ,  $\beta 1$ , and  $\gamma$ .

## MATERIALS AND METHODS

### Strains and Culture

The Bristol strain N2 was used as the standard wild-type strain. The mutations used for *apm-1* and *unc-101* functional analysis are *unc-101(sy108)* (Lee *et al.*, 1994) and *let-23(sy1)* (Aroian *et al.*, 1990). *rol-6(su1006)* DNA and *dpy-20(e1282)* was used as selection markers for DNA microinjection as described below. The culture of *C. elegans* was previously described (Brenner, 1974).

### cDNA Screening

We used the cDNA clone CEED20 (accession number T00259) from the GenBank database as a start point for cloning a full-length cDNA clones. We used the CEED20 DNA as probe in a cDNA screening for full-length clones. We used a standard hybridization procedure (Sambrook *et al.*, 1989). We isolated three cDNA clones from a *C. elegans* cDNA library (Barstead and Waterston, 1989), all of which contained inserts of the same length. We determined the sequence of one of the clones, CEED20-3. Sequencing reactions were performed using Sequenase 2.0 and reagents from United States Biochemical (Cleveland, OH).

### Sequence Analysis

Compiling of DNA and amino acid sequences were carried out using the Macvector program (IBI, Oxford Molecular Group, Hunt Valley, MD) and the GCG package v7.0, a software of the Genetics Computer Group (Madison, WI; Devereux *et al.*, 1984). The BLAST program of the GCG software was used to search and compare homologies of the sequences. The Pileup and Gap programs were used to generate the comparisons of the amino acid sequences. The clustal w program was used to analyze and calculate genetic distances among the medium chain homologs. Tree view 1.5 (Win 32) version 1.5.2. (Page, 1998) was used to construct the dendrogram of the medium chain homologs. The sequences that were used in the sequence comparison and dendrogram were from *C. elegans* (CAPM-1, in this study; UNC-101, CAPM-2, Lee *et al.*, 1994,

CAPM-3, Cosmid F53H8.1, *C. elegans* genome project), humans ( $\mu 1B$ , NP 005489.1;  $\mu 2$ , sp P20172;  $\mu 3A$ , gb AAD43328.1;  $\mu 3B$ , sp P53677;  $\mu 4$ , gb AAD43328.1), mouse ( $\mu 1B$ , gb AAD28085.1; AP47, sp P35585), rat (p47A, sp P53676; p47B, sp P53678), fly (APM-1, emb CAA06918.1; APM-2, emb CAA06785.1; APM-3, emb CAA08768.1), and from yeast (YAP54, sp Q00776; APM-2, sp P38700; APM-3, emb CAA97989.1; APM-4, sp Q99186).

### Expression Studies

To construct an *unc-101* GFP reporter gene, we used the pJL1 plasmid and the vector pPD95.77 from Andy Fire (Carnegie Institute of Washington, Baltimore, MD). We amplified *unc-101* genomic DNA from the K11D10 cosmid, using two PCR primers, and produced the pJL271 plasmid by replacing the *unc-101* genomic region of pJL1 with PCR product. The 5' and 3' subcloning sites were *HindIII* and *BamHI*. The two PCR primers were K11-1, 5' CTCGTC-GACCTGAT CCGTGTC 3' and 101-C, 5' GGGATCCGATTCTC-CATTTTGTAG 3'. Next, the 1.8 kb *gfp* fragment from pPD95.77 was subcloned into pJL271. The 5' and 3' subcloning sites were *BamHI* and *SpeI*. To construct the *apm-1* GFP reporter, we subcloned amplified 6.0 kb genomic DNA using two PCR primers into the Fire vector pPD95.79. The subcloning sites were *SallI* and *BamHI*. The two PCR primers were F55-1, 5' GTGAAACTGCTGAAGGAAGC 3' and CE19, 5' GGGATCCTCATTGATAATCTCCG 3'.

### Construction of Hybrid Genes

Construction of the *unc-101* hybrid genes was described (Lee *et al.*, 1994). To construct an APM-1 hybrid gene, we amplified the APM-1 cDNA from nucleotide #325 through #1229 using two PCR primers. Both the 5' and 3' subcloning sites, *NruI* and *EcoRV*, are conserved in APM-1. The two PCR primers are: CE-6, 5'CGATAATTCGT-TATTATTIA TG3', and CE-7, 5'ATCCAGATTTCCTATGAT TTT3'. The amplified DNA was ligated to the 7.2kb *NruI/EcoRV* fragment of pJL2. The resulting plasmid is the APM-1 hybrid gene. This construct contains the 5' promoter region of *unc-101*, the 5' coding region of *unc-101* up to the *unc-101* cDNA nucleotide #388, the *apm-1* cDNA from *NruI* site to *EcoRV* site (corresponding nucleotides in *unc-101* are #389 to #1281, the *unc-101* 3' region from #1282 to the end of cDNA, and the untranscribed 3' region of *unc-101*). The predicted protein from this construct contains 301 amino acid residues from APM-1, and 123 amino acid residues from UNC-101. A positive control construct, the *unc-101* hybrid gene, contained all amino acids for UNC-101. A negative control construct contains only 123 amino acids from UNC-101, and the remaining amino acids from APM-2.

### Microinjection Experiments and Double Strand RNA Interference

Microinjection of DNA into the gonad of *C. elegans* hermaphrodite adults was previously described (Mello *et al.*, 1991). For expression studies, the GFP reporter constructs were coinjected into N2 wild-type animals with the pRF4 plasmid containing a dominant mutant gene for *rol-6*. The total concentration of injected DNA was 140  $\mu\text{g}/\text{ml}$  (reporter 100  $\mu\text{g}/\text{ml}$  and pRF4 40  $\mu\text{g}/\text{ml}$ ). The transgenic animals were selected by their rolling behaviors, and the animals were observed for GFP expression. For hybrid constructs, we used *unc-101(sy108); let-23(sy1); dpy-20(e1282)* animals as the host for microinjection. We coinjected the hybrid genes with a *dpy-20(+)* clone as a selection marker. The host animals have a dumphy body shape (*Dpy* phenotype), and the selection marker DNA can rescue the *Dpy* phenotype to wild-type body shape, enabling the selection of transgenic animals containing the microinjected genes. Following microinjection, we selected non*Dpy* transgenic animals, established stable lines that inherited the transgenes, and examined the phenotype of vulval differentiation. For RNAi, templates for RNA synthesis were produced by PCR amplification of full-length cDNA using



T3 and T7 primers. RNAs were synthesized using a commercially available *in vitro* transcription kit (Promega, cat.#P2075, P2083) with T3 and T7 RNA polymerases. Unmodified RNA was resuspended for injection at 10  $\mu$ g/ml to 500  $\mu$ g/ml concentration in DEPC-treated water. Following microinjection of double strand RNA, the injected animals (P0) were transferred to new plates every 12 h and F1 progeny were counted and analyzed.

### Microscopy

Differential interference contrast (DIC) microscopy (Nomarski optics) and fluorescence microscopy were used to observe the phenotypes and expression patterns. For DIC microscopy, we treated the worms with sodium azide at 1 mM concentration; for fluorescence microscopy, we treated the worms with levamisole at 100 ng/ml.

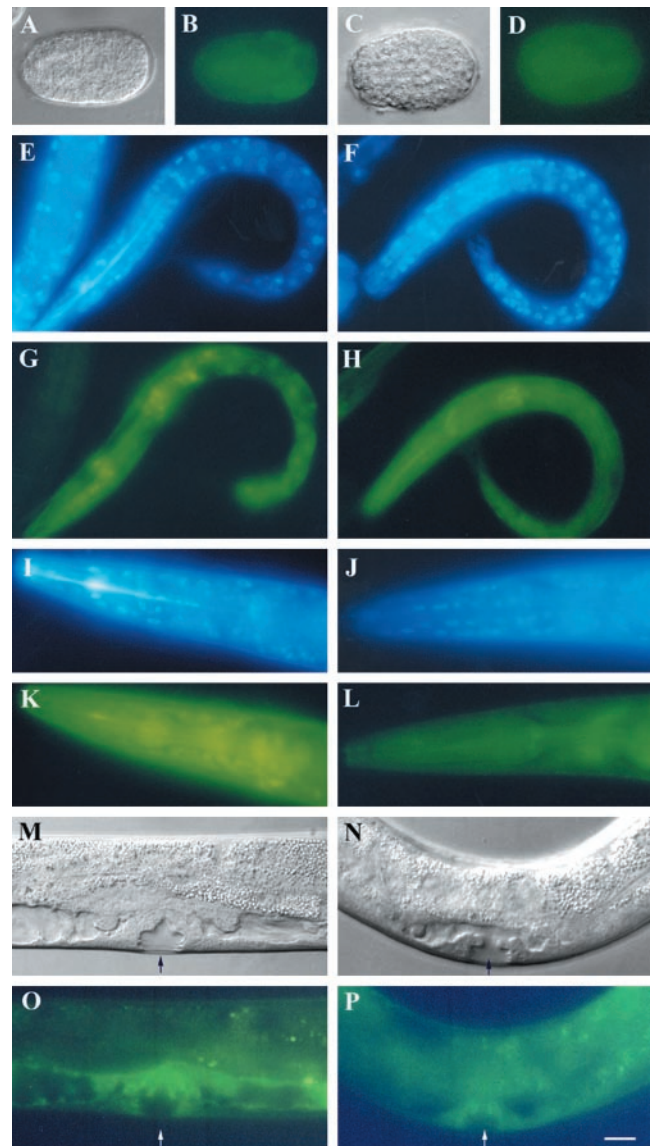
## RESULTS

### Molecular cloning of *apm-1*, a homolog of the *unc-101* gene, encoding a medium chain of trans-Golgi clathrin-associated protein complex AP-1

A *C. elegans* cDNA sequencing project identified a cDNA clone (CEED20) containing a partial sequence similar to *unc-101* (accession number T00259). We screened a cDNA library with probes made from the cDNA of CEED20. We isolated three full-length cDNA clones and determined the sequence of one of them. The sequence would encode a putative protein of 426 amino acids. We named this gene *apm-1* (associated protein complex medium chain-1). Comparison of the sequence of APM-1 with other medium chain homologs indicated that this protein is more related to AP47 than to AP50 (Figure 1A). APM-1 has the same degree of similarity to mammalian AP47 as it has to UNC-101 in *C. elegans* (72% identity in both cases). Comparison of the amino acid sequences among the medium chain homologs from yeast to humans showed that APM-1 and UNC-101 are clearly grouped within the same subfamily of AP-1 medium chains (Figure 1B). DNA sequence comparison between *apm-1* and *unc-101* showed that the discrepancies are biased toward the third bases of codons (our unpublished results). There was minimal sequence identity in the 5' nontranslated region or in the 3' nontranslated region (our unpublished results), suggesting that these two genes might be subject to different types of regulation. The genome project later revealed that the genomic clone K11D2 (accession number Z83115) contained the full length *apm-1* gene. A comparison of the genomic structures of the two genes showed that the boundaries of the first three exons and the last exon are conserved between the two genes, but the boundaries of the central exons are divergent (Figure 1C). The number of exons is also different: *apm-1* has nine exons while *unc-101* has only seven exons.

### *apm-1* and *unc-101* Are Expressed Ubiquitously throughout Development

To determine the expression patterns of the medium chains, we constructed GFP reporter constructs (see MATERIALS AND METHODS) and examined the GFP expression patterns in transgenic animals containing these reporter constructs. We found that *unc-101* was expressed in most cells, if not all, at most embryonic and postembryonic stages. The highest level of expression was observed in muscles and



**Figure 2.** Expression patterns of *unc-101* and *apm-1*. The two genes are expressed ubiquitously throughout development. The left panels show expression patterns of *unc-101* and the right panels those of *apm-1*. (A-D) show embryos consisting of ~ 100 cells, (E-H), animals at their L1 stage, (I-L), the head region of animals at their L4 stage, and (M-P) show the vulval regions of L4 stage animals. A, C, M, and N are Nomarski images of the embryos; E, F, I, and J are DAPI staining images showing locations of the nuclei. All others show GFP expression. Both genes are strongly expressed during embryogenesis and at the L1 stage. At the L4 stage, *unc-101* is strongly expressed in the pharynx and in the vulval cells, and *apm-1* is strongly expressed in the nerve ring, as well as in the vulval cells. The arrows in (M-P) are the invaginations of the vulvae. The scale bar is 10  $\mu$ m.

pharyngeal regions (Figure 2B, G, K). While *apm-1* was also expressed ubiquitously throughout development, its expression was stronger in neurons, as demonstrated by the bright fluorescence in the nerve ring (Figure 2, D, H, L). When

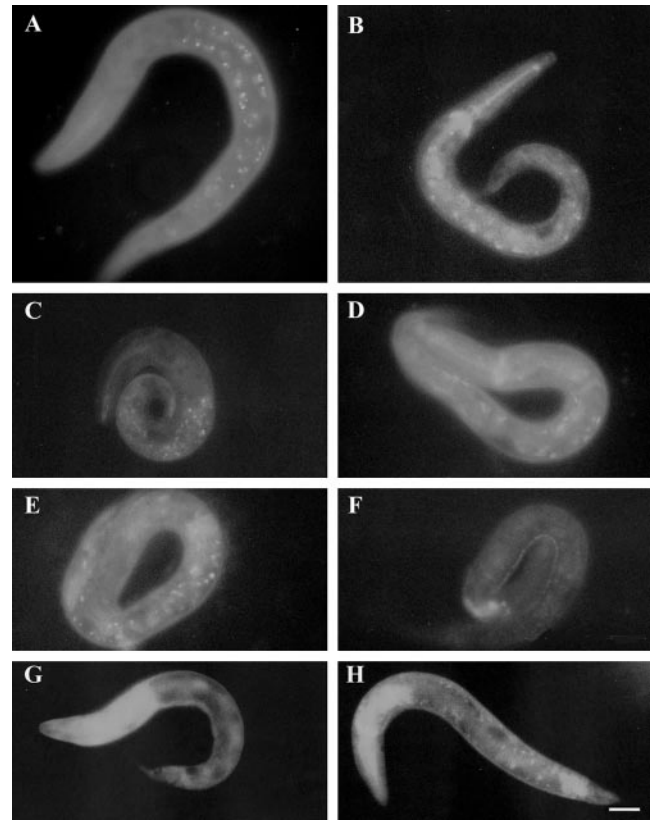
examining the *apm-1* expression by lacZ reporter assays, we also observed high expression of *apm-1* in the intestine (our unpublished results). The expression of both genes was strong in the vulval cells when vulval tissues were undergoing morphogenesis (Figure 2, M-P). The overlapping expression pattern of *apm-1* and *unc-101* implied that these two medium chains were present in the same cells, possibly interacting with both shared and distinct cargo proteins during transport.

### RNAi of the Medium Chain Genes Specifically Phenocopies Reduction-of-Function Mutations of Corresponding Genes

To dissect the functions of *apm-1* compared with those of *unc-101*, as well as other components of the AP-1 complex, we wanted to examine the phenotypes associated with disruption of *apm-1* function. As there is no available mutation in the *apm-1* gene, we used double-strand RNAi to phenocopy *apm-1* reduction-of-function mutations. As APM-1 and UNC-101 share approximately 72% identity at the amino acid level, the possibility existed that the RNAi of one of these genes might interfere with the function of the other gene. We examined whether double-strand RNAs of *apm-1* and *unc-101* specifically and exclusively interfered with their respective targets. As shown in Figure 3, *unc-101* RNAi caused reduction of GFP expression driven by the *unc-101* gene while it did not cause any reduction in GFP expression driven by the *apm-1* gene (Figure 3, C and D). Likewise, *apm-1* RNAi interfered specifically with *apm-1*, not with *unc-101* (Figure 3, E and F). In a control experiment, RNAi of *apm-2*, the medium chain of AP-2 complexes in the nematode, did not cause any reduction of either *apm-1* or *unc-101* expression (Figure 3, G and H). We therefore concluded that we could specifically phenocopy reduction-of-function mutations of either of these two medium chain genes by RNAi using the complementary RNAi or phenocopy double mutations of *apm-1* and *unc-101* by using double-strand RNAs of both genes simultaneously.

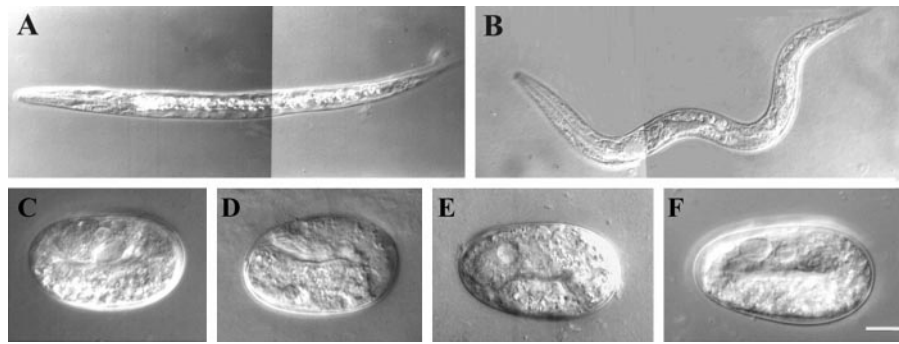
### Disruption of *apm-1* Alone Causes Larval Lethality, and Simultaneous Disruption of *apm-1* and *unc-101* Causes Embryonic Lethality

We first examined the function of *apm-1* during early development by RNAi. In a control experiment, we injected *unc-101* double-strand RNA into N2 wild-type animals. At a high concentration (500  $\mu\text{g/ml}$ ), double-strand RNA caused, at most, 50% larval lethality compared with the putative null mutation of *unc-101*(*sy108*) animals, confirming that the null phenotype of *unc-101* would not lead to 100% lethality. The animals showed arrested development at various stages of development. Most of the surviving animals showed the uncoordinated (Unc) phenotype (as is the case in *unc-101* mutant animals) and did not show any embryonic lethality (Figure 4A). We then injected a high concentration of *apm-1* double-strand RNA (200  $\mu\text{g/ml}$ ) into N2 animals and examined the phenotypes of the F1 progeny. We found that 100% of the F1 animals showed arrested development as L1 larvae (Table 1, Figure 4B); also, they did not show any embryonic lethality. Most animals displayed the typical phenotype consisting of movement at first after hatching, displacement over a short distance, then sudden arrest of movement in



**Figure 3.** Specific RNAi effect of *unc-101* and *apm-1*. The left panels show animals expressing the UNC-101::GFP reporter protein, and the right panels show animals expressing the APM-1::GFP reporter protein. (A and B) wild-type expression of *unc-101* and *apm-1*, respectively. (C and D) GFP reporter expression after RNAi of *unc-101*. Only UNC-101::GFP expression is reduced, while APM-1::GFP is not affected. (E and F) GFP reporter expression after RNAi of *apm-1*. Only APM-1::GFP expression is reduced while UNC-101::GFP is not affected. (G and H) GFP reporter expression after RNAi of *apm-2*. APM-1::GFP and UNC-101::GFP expressions are not affected by *apm-2* RNAi. The Dpy phenotype reportedly caused by disruption of *apm-2* gene function is obvious as the animals are shorter and fatter (Shim and Lee, 2000).

any direction. In addition, they had bloated anterior intestines, as if pumping was normal but that the ingested bacterial stream had stopped at the anterior intestine. The arrested L1 animals had very slow pumping motions with head and tail moving very little. The shape of intestine was abnormal in the animals with arrested development, with exaggerated curvature and uneven thickness of the intestine tubes. In addition, animals with arrested development had thinner posterior bodies than wild-type animals. Next, we injected the same concentration of *apm-1* double-strand RNA into *unc-101*(*sy108*) animals to phenocopy double mutations of *apm-1* and *unc-101* and found that the F1 animals showed up to 100% embryonic lethality due to arrested development at the twofold stage within the eggshells (Table 2, Figure 4C). Thus, removal of both of the AP-1 medium chains leads to a synthetic embryonic lethal phenotype that cannot be achieved by the removal of either one of the two genes. This



**Figure 4.** Redundancy of *unc-101* and *apm-1* during embryogenesis. Lethal phenotypes caused by RNAi of *unc-101*, *apm-1*,  $\sigma 1$ ,  $\beta 1$ , or  $\gamma$ . (A) F1 progeny from a wild-type animal injected with *unc-101* double-strand RNA. About half of the animals arrested at various stages of development, and this specific figure shows an animal arrested at the L1 stage. (B) F1 progeny from a wild-type animal injected with *apm-1* double-strand RNA. All the animals arrested as L1 larvae, and the anterior intestine was bloated, suggesting that, although pharyngeal pumping was not affected, the bacterial stream was blocked due to some malfunction or structural defect in the intestine. L1 animals arrested by *apm-1* RNAi have a slightly different posture when they arrest. This is because they can move and feed on bacteria for a short period of time before suddenly stopping. On the other hand, arrested L1 animals of *unc-101* mutants hardly move, if at all, and arrest in a straight posture. (C) F1 progeny from an *unc-101(sy108)* animal injected with *apm-1* double-strand RNA. All the animals arrested as embryos, as shown in this figure. (D) F1 progeny from an N2 animal injected with  $\sigma 1$  double-strand RNA. (E) F1 animal from a wild-type animal injected with  $\beta 1$  double-strand RNA. (F) F1 animal from a wild-type animal injected with  $\gamma$  double-strand RNA. As can be easily noticed in (C-F), the phenotypes caused by double RNAi of both *apm-1* and *unc-101* are identical to those caused by single RNAi of  $\sigma 1$ ,  $\beta 1$ , or  $\gamma$  chain gene. The scale bar is 10  $\mu$ m.

indicates that one of the two genes must be present for embryonic development, but that either one of the two genes is redundant in the presence of the other. In contrast to the redundant functions of *apm-1* and *unc-101* in embryogenesis,

each medium chain may play a distinct role after hatching, because most animals in which only one of the two genes was disrupted could hatch but could not survive after the early larval stages.

**Table 1.** Larval lethality caused by RNAi of *apm-1* in N2 background

High concentration			
Time after injection (h)	Eggs collected (n)	Arrested larvae (n)	Larval lethality (%)
0-12	60	54	90
12-24	363	363	100
24-36	340	340	100
36-48	117	117	100
48-60	76	76	100
Low concentration			
Time after injection (h)	Eggs collected (n)	Arrested larvae (n)	Larval lethality (%)
0-12	86	43	50
12-24	254	196	77
24-36	408	92	23
36-48	268	37	14
48-60	170	12	8

*apm-1* double-strand RNA was injected into the gonads of N2 animals at either high (200  $\mu$ g/ml) or low (20  $\mu$ g/ml) concentration, and the arrested larvae were counted. Animals that did not arrest developed into adults without any significant defects. Note that there was no embryonic lethality.

**Table 2.** Embryonic lethality caused by RNAi of *apm-1* in the *unc-101(sy108)* background

High concentration			
Time after injection (h)	Eggs collected (n)	Arrested embryos (n)	Embryonic/total lethality <sup>a</sup> (%)
0-12	51	17	34/76.5
12-24	238	225	94.5/98.7
24-36	240	237	100/100
36-48	213	213	100/100
48-60	76	76	100/100
Low concentration			
Time after injection (h)	Eggs collected (n)	Arrested embryos (n)	Embryonic/total lethality <sup>a</sup> (%)
0-12	98	23	23.5/71.2
12-24	207	99	43.0/100
24-36	261	55	21.8/87.8
36-48	212	10	4.8/67.5
48-60	116	1	0.9/39.7

The same concentrations of *apm-1* double strand RNA were injected into *unc-101(sy108)* animals.

<sup>a</sup> Because *unc-101(sy108)* animals alone show about 45% larval lethality, total lethality is higher than embryonic lethality in some cases.



### ***apm-1* and *unc-101* Are Redundant Negative Regulators of an EGF-Mediated Signaling Pathway**

*Unc-101* mutations suppress the vulvaless phenotype of *let-23* EGFR reduction-of-function mutations, indicating that the normal function of *unc-101* might be negative regulation of the EGF signaling pathway in vulval development (Lee *et al.*, 1994). Because *unc-101* single mutant animals do not show any abnormal vulval induction (Lee *et al.*, 1994), the existence of redundant negative regulators of vulval development, acting in parallel with *unc-101*, has been suggested. One such gene is *sli-1* (Yoon *et al.*, 1995). Single mutants of either *sli-1* or *unc-101* do not exhibit any vulval phenotype, but double mutants for *unc-101* and *sli-1* have greater-than-wild-type vulval induction (Sternberg *et al.*, 1994). We wished to examine if *apm-1* was another redundant negative regulator of the signaling mediated by LET-23 EGFR.

We have previously shown that a hybrid construct, which contained the *unc-101* promoter region and most of the mammalian AP47, could rescue phenotypes associated with an *unc-101* mutation when introduced into the mutant animals by microinjection (Lee *et al.*, 1994). To test whether APM-1 protein could complement functions of UNC-101 protein when overexpressed, we constructed and examined a hybrid gene (see MATERIALS AND METHODS). In the hybrid gene, we substituted two thirds of the UNC-101 protein from the C terminal end with APM-1. The rationale for this substitution was that the N-terminal region of the medium chains was required for interaction with the  $\beta$  chain, while the C-terminal region was important for interaction with target proteins (Aguilar *et al.*, 1997). We microinjected this hybrid gene into the gonads of *unc-101(sy108); let-23(sy1); dpy-20(e1284)* animals with cloned DNA of *dpy-20(+)* as a selection marker. The resulting nonDpy transgenic animals were examined for their ability to complement the *unc-101* mutations that result in suppression of the vulvaless phenotype of the *let-23(sy1)* mutation. The suppression of the vulvaless phenotype of the *let-23(sy1)* mutation by *unc-101* mutations was rescued in the transgenic animals containing extra copies of the hybrid gene as 4 out of 8 transgenic animals at the L3 molt stage, observed under Nomarski optics, restored the vulvaless phenotype (Figure 5E). Our results indicate that overexpressed APM-1 may complement the functions of UNC-101 in the absence of functional, endogenous UNC-101 in the vulval cells.

We then asked whether *apm-1* would act redundantly in the vulval induction pathway by examining the vulval phenotype of *unc-101(sy108)* animals injected with low concentrations of *apm-1* double strand RNA. If the two genes are indeed redundant negative regulators of the pathway *in vivo*, we would expect to see the greater-than-wild-type vulval induction observed in *unc-101; sli-1* double mutants. We used low concentrations of *apm-1* double strand RNA for this experiment since a high concentration leads to 100% embryonic lethality. Out of 67 F1 postRNAi survivors, 15 animals showed greater-than-wild-type induction as observed using Nomarski optics (for example, Figure 6B), whereas *unc-101(sy108)* animals without the RNAi of *apm-1* did not show increased induction compared with the wild-type (Figure 6A), indicating that *apm-1* indeed acts redundantly with *unc-101* in negatively regulating the vulval induction pathway. Next, we wished to determine whether the reduced function of *apm-1* can directly suppress the

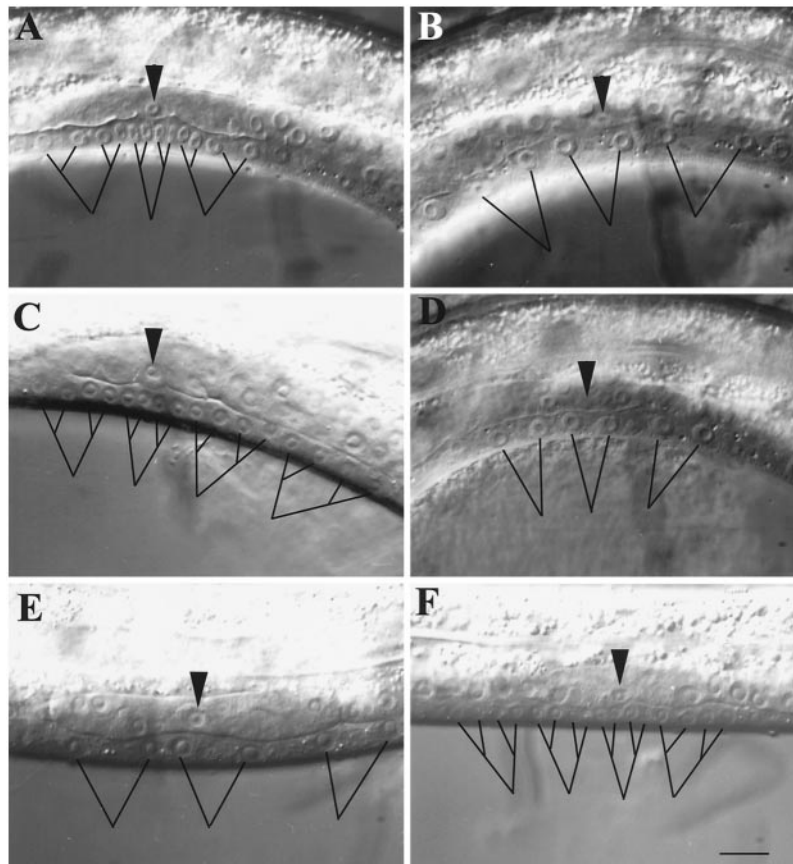
vulvaless phenotype caused by the *let-23(sy1)* mutation even in the presence of the wild-type gene activity of *unc-101*. We injected double strand RNA of *apm-1* into *let-23(sy1)* animals at low concentration. We found that at 20  $\mu\text{g}/\text{ml}$  microinjected double strand RNA, 73% of F1 postRNAi survivors ( $n = 19$ ) were suppressed for the vulvaless phenotype, thus exhibiting morphologically wild-type or greater-than-wild-type vulvae (for example, Figure 6C). 67% of F1 postRNAi survivors ( $n = 12$ ) had functional vulvae, with which they could lay eggs. In contrast, in the *let-23(sy1)* animals without RNAi, only 10% showed morphologically wild-type vulvae, and 9% had functional vulvae ( $n = 231$  and 222, respectively). These data indicated that the *apm-1* gene acts as another negative regulator of the vulval induction pathway. Surprisingly, we also observed greater-than-wild-type induction of vulval precursor cells (VPCs) in transgenic N2 animals containing an *apm-1::GFP* reporter construct (Figure 6D), indicating that this reporter construct may have acted as a dominant negative mutation and may have interfered with the wild-type function of *apm-1*. This would have caused the VPCs to be induced excessively compared with wild-type. Thus, we infer that *apm-1* and *unc-101* are redundant negative regulators of the vulval induction pathway in the nematode.

### **The Two Medium Chains Are Shared by Other Components of AP-1 Complex in the Nematode**

Thorough database searches failed to identify more than one homolog each for the large and small chains of AP-1 complexes in the nematode. We identified only single homologs of  $\sigma 1$ ,  $\beta 1$ , and  $\gamma$  chain genes. To determine if the two medium chains, APM-1 and UNC-101, share other components when constituting the AP-1 complexes, we examined the phenotypes caused by RNAi of other components of the AP-1 complex. The F1 progeny from wild-type animals injected with double-strand RNA of the small chain  $\sigma 1$  gene showed 100% embryonic lethality at the concentration of 100  $\mu\text{g}/\text{ml}$  (table 3, Figure 4D). At lower concentration, development of most F1 progeny was arrested at the embryonic stage or at the larval stages (our unpublished results). RNAi of the  $\beta 1$  or  $\gamma$  chain displayed identical phenotypes (our unpublished results and Figure 4, E and F). These results clearly imply that the removal of the single small chain of the AP-1 complex caused phenotypes as severe as those caused by the removal of both of the medium chains.

To further confirm the result, we examined the effect of RNAi of  $\sigma 1$ , the small chain of the AP-1 complex, on the expression patterns of *apm-1* and *unc-101*. It has been reported that loss of one component of the clathrin-associated protein complex led to destabilization of the whole complex (Dell'Angelica *et al.*, 1999b, Zizioli *et al.*, 1999). We reasoned that the stability of both APM-1 and UNC-101 proteins would be decreased by loss of one of the components of the AP-1 complex if they indeed shared this component. We microinjected low concentration of  $\sigma 1$  double-strand RNA into transgenic animals containing either an APM-1::GFP or an UNC-101::GFP reporter gene and obtained RNAi-affected embryos and animals at different larval stages. RNAi of  $\sigma 1$  caused reduction in expression of both *apm-1* and *unc-101* genes in embryos and larvae (Figure 7, F, H, K, L), while  $\sigma 2$  RNAi did not result in any difference of expression (Figure 7, M and N). This result indicates that the disruption of  $\sigma 1$





**Figure 5.** An APM-1 hybrid protein can complement UNC-101 proteins in vulval development: The *apm-1* hybrid gene can rescue the suppression of vulvaless phenotype of *unc-101(sy108)*. The animals in the photographs are at the L3 molt stage, which corresponds to the period of vulval induction. The arrowheads indicate the locations of the anchor cell that induces the vulval precursor cells (VPCs). The lines indicate the lineage of each VPC. The genotypes are: (A) wild-type; (B) *let-23(sy1)*; (C) *unc-101(sy108); let-23(sy1)*; (D) *unc-101(sy108); let-23(sy1)::unc-101 minigene*; (E) *unc-101(sy108); let-23(sy1)::apm-1 hybrid gene*; and (F) *unc-101(sy108); let-23(sy1)::apm-2 hybrid gene*. (A), wild-type vulval induction in which three vulval precursor cells (VPCs) have divided twice to make four progeny each. (B) There is no vulval induction because of the reduction-of-function mutation in the *let-23EGFR* gene. All VPCs have divided only once. (C) The *unc-101* mutation suppresses the *let-23(sy1)* vulvaless mutant phenotype to greater-than-wild-type vulval induction. Four VPCs, instead of three VPCs as in normal induction, have divided twice. (D) The transgenic *unc-101* minigene rescued the endogenous *unc-101 (sy108)* mutation, and thus no vulval induction occurred, as in the single mutant of *let-23(sy1)*. (E) The *apm-1* hybrid gene complemented the endogenous *unc-101 (sy108)* mutation, thus no vulval induction occurred, as in the single mutant of *let-23(sy1)*. (F) Four VPCs were induced because the *apm-2* hybrid gene could not complement the endogenous *unc-101 (sy108)* mutation. This phenotype is identical to that of *unc-101(sy108); let-23(sy1)* mutant animals. The scale bar is 10  $\mu$ m.

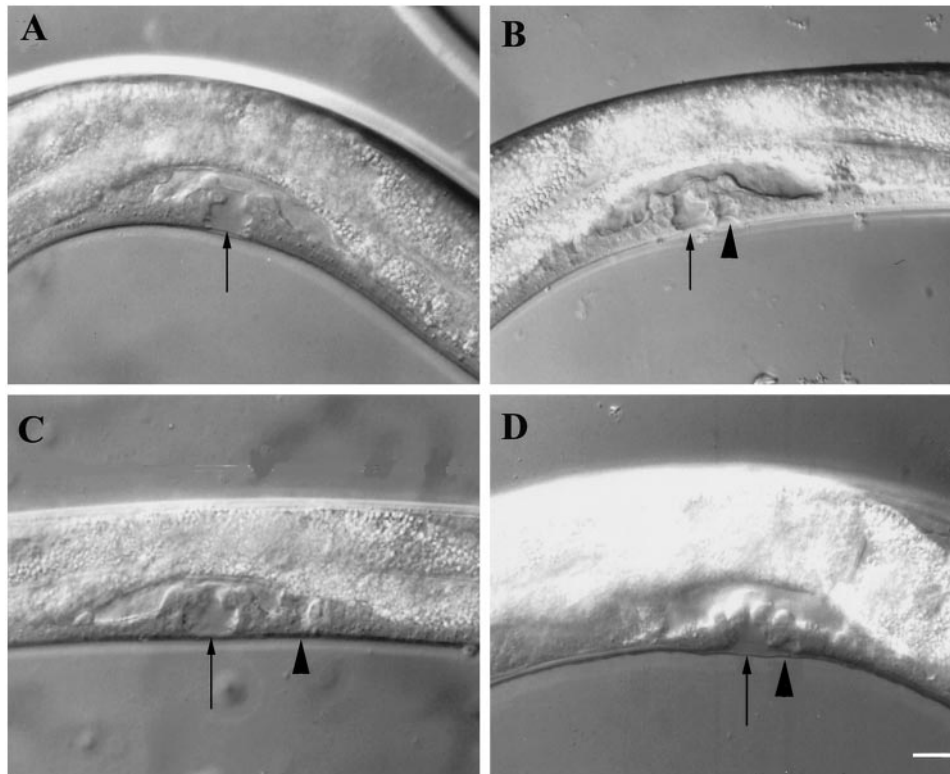
destabilized both AP-1 complexes containing APM-1 or UNC-101 as their medium chain. It is therefore conceivable that a  $\sigma$ 1-containing AP-1 complex can have either APM-1 or UNC-101 as its medium chain.

## DISCUSSION

In this article, we have identified a second homolog of AP47, the medium chain of the *trans*-Golgi clathrin-associated protein complex AP-1. Our functional analysis suggested that, on their own, *apm-1* and *unc-101* are dispensable for embryonic development as single disruption of either gene did not cause any embryonic lethality. However, when both of these genes are disrupted, embryogenesis is affected, indicating

that the combined functions of APM-1 and UNC-101 proteins are essential for embryogenesis. Our results also indicated that the nematode AP-1 complexes can contain either UNC-101 or APM-1 as their medium chains along with other components, probably serving both shared and distinct functions. We propose that the AP-1 complex in the nematode can employ either one of the two medium chains as its medium chain for cargo transport.

Comparison of the genomic DNA sequences of the *apm-1* and *unc-101* genes revealed interesting structural features. The boundaries of the first three exons and the last one exon are conserved in both genes, whereas exon/intron boundaries as well as the number of central introns vary. It is possible that the genes were initially duplicated during evo-



**Figure 6.** Redundancy of *unc-101* and *apm-1* in vulval development. The arrows indicate normal invagination of vulval tissues at the L4 stage in all figures. (A) An *unc-101(sy108)* animal showing wild-type vulval induction as indicated by a single invagination of the vulval tissue composed of the progeny of three VPCs. (B) An *unc-101(sy108)* animal injected with *apm-1* double-strand RNA. An additional invagination by vulval cells, due to greater-than-wild-type induction, is indicated by an arrowhead. (C) *Alet-23(sy1)* animal injected with *apm-1* double strand RNA. As in (B) an extra invagination is shown, as indicated by an arrowhead. (D) A transgenic animal of wild-type genetic background containing the *apm-1::GFP* reporter construct. An extra invagination is shown next to the normal vulval invagination. The scale bar is 10  $\mu\text{m}$ .

lution and that the introns in the central part of the genes were introduced later, at different loci, contributing to novel, distinct functions for each gene.

Mutations in the *unc-101* locus cause pleiotropic effects, suggesting that *unc-101* is not equivalent to *apm-1* and that *unc-101* has distinct functions from those of *apm-1*. However, several previous observations suggested that *unc-101* may not be fully distinct from *apm-1*. For example, the phenotypes of *unc-101* putative null mutations are not identical in all animals although they bear the same mutations in *unc-101*. The lethality associated with the putative null mutations is not complete as only 50% of the progeny of homozygous hermaphrodites actually die. The defecation defect shows more variety even in a single animal model (Thomas, 1990). Each defecation cycle in *C. elegans* is composed of an anterior body muscle contraction (aBoc), a posterior body muscle contraction (pBoc), and an expulsion (Exp) step. In an *unc-101* null mutant animal, the aBoc step is missing in half of the defecation cycles, while in the other half of the defecation cycles, the aBoc is normal. Therefore, it is conceivable that a gene may exist that shares partial redundancy with *unc-101*. We infer that *apm-1* may be one such gene. In this report, we have shown that *apm-1* and *unc-101*

on their own are redundant for embryonic development and for regulating an EGF-mediated signaling pathway.

Concerning the extent of redundancy between *apm-1* and *unc-101*, one possibility is that *unc-101* and *apm-1* have identical functions, and that full expression of both the genes is required for production of a sufficient amount of proteins. This is unlikely for the following reasons. Mutations in the *unc-101* locus were not dosage-dependent but were fully recessive, indicating that the loss of one copy of *unc-101* does not cause any defect. However, one still cannot exclude the possibility that there exists a threshold level of expression of these genes required for their proper functioning. Another prediction of the above hypothesis is that the *apm-1* mutant animals will have the same phenotype as *unc-101* mutant animals, but this is not the case either. Animals affected by RNAi of *apm-1* displayed different phenotypes from those with *unc-101* mutations. A second possibility is that APM-1 and UNC-101 are expressed in different types of cells, although they have the same functions. This possibility is unlikely either, since our expression studies did not reveal any difference in the pattern of *unc-101* and *apm-1* expression throughout development (Figure 2). A third hypothesis,

**Table 3.** Embryonic lethality caused by RNAi of  $\sigma 1$  in the wild type background

High concentration (100 $\mu\text{g}/\text{ml}$ )			
Time after injection (h)	Eggs collected (n)	Arrested embryos (n)	Embryonic/total lethality <sup>a</sup> (%)
0–12	107	10	9.3/86.9
12–24	284	225	79.3/100
24–36	390	390	100/100
36–48	337	337	100/100
48–60	345	345	100/100
Low concentration (10 $\mu\text{g}/\text{ml}$ )			
Time after injection (h)	Eggs collected (n)	Arrested embryos (n)	Embryonic/total lethality <sup>a</sup> (%)
0–12	113	1	0.88/83.2
12–24	250	150	60.0/93.2
24–36	416	182	43.8/74.0
36–48	396	50	12.6/38.4
48–60	351	22	6.27/23.3

<sup>a</sup> Total lethality includes both the arrested embryos and the larvae arrested at various stages of development.

which we prefer, is that APM-1 acts as a counterpart of UNC-101 (or vice versa) within the context of the AP-1 trans-Golgi clathrin-associated protein complex, and it may interact with distinct sets of target proteins from those of UNC-101 depending on the tissue type and the stage of development. It is possible that *apm-1* can somehow compensate for the UNC-101 function when *unc-101* is mutated. If this were the case, single mutants of *apm-1* would probably have different phenotypes from those of *unc-101* and double mutants for *unc-101* and *apm-1* would be 100% lethal. As a matter of fact, RNAi of *apm-1* in the *unc-101* background caused 100% embryonic lethality, while RNAi of *apm-1* in the wild-type background resulted in different phenotypes from those of *unc-101* mutants. As our results clearly showed, RNAi of the small or large chain of the AP-1 complex ( $\sigma 1$ ,  $\beta 1$ , and  $\gamma$ ) caused 100% embryonic lethality, indicating that UNC-101 and APM-1 function as two alternative forms of the medium chain in the AP-1 complex.

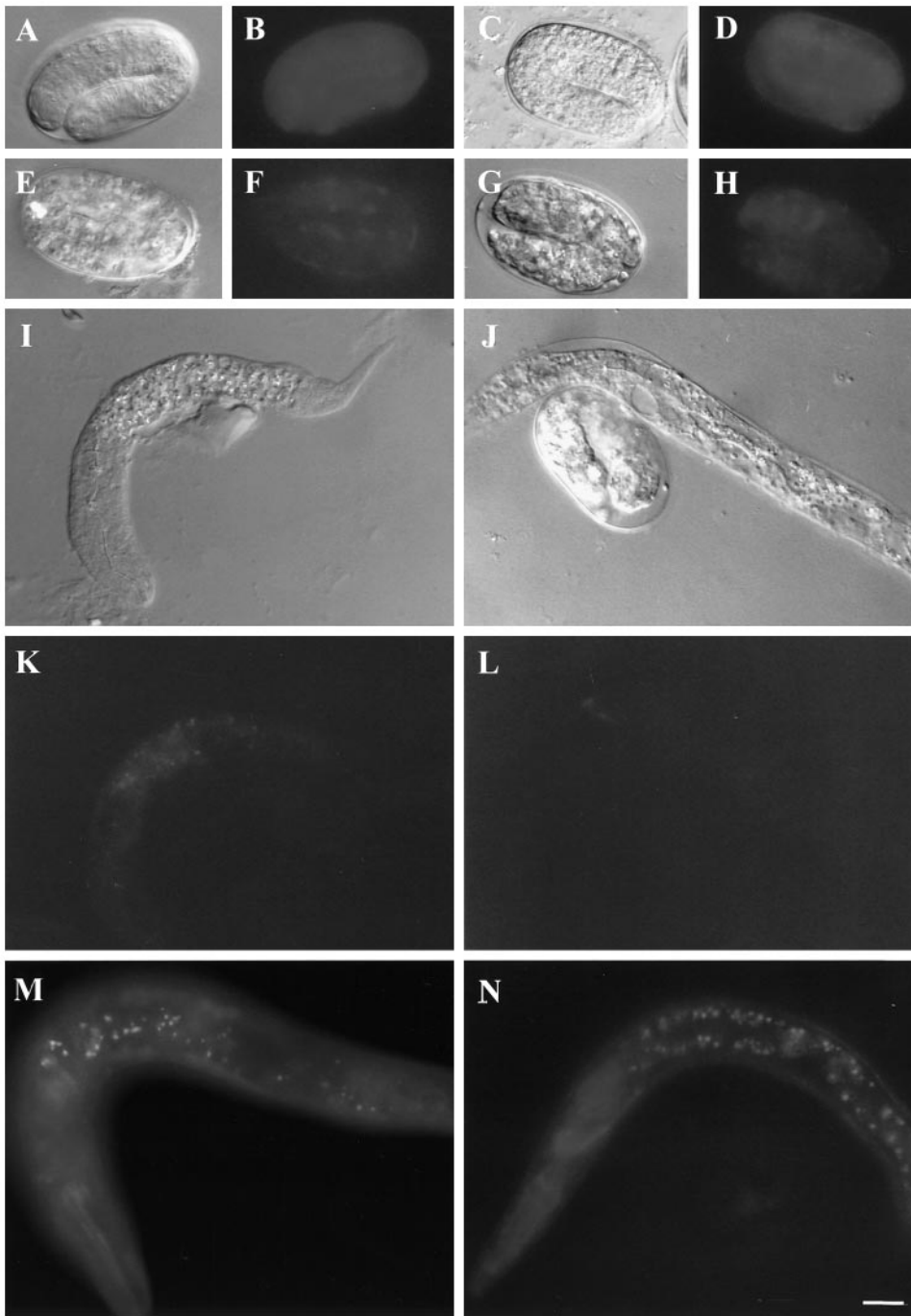
One interesting feature of *apm-1* function is its role in vulval development, which is mediated by the LIN-3 (EGF)-LET-23 (EGFR) signaling pathway. We propose that *apm-1* is another negative regulator of vulval development on the bases of the following observations. First, a hybrid protein, composed of two thirds of the *apm-1* gene and one third of the *unc-101* gene, can complement a defective UNC-101 protein function in vulval development, when expressed under the control of the *unc-101* promoter. Second, reduction in *apm-1* function can suppress the vulvaless phenotype of *let-23* (*sy1*) even in the *unc-101* (+) background. This is also observed when *unc-101* mutations are introduced into the *apm-1*(+) background. Third, disruption of both *apm-1*

and *unc-101* caused greater-than-wild-type vulval induction. The phenotypes observed after the disruption of different sets of genes, *let-23*, *unc-101*, and/or *apm-1* are summarized in table 4. These results suggest that *apm-1* shares redundant functions with *unc-101* for normal vulval development in wild-type animals.

Why were *apm-1* mutations not identified in the genetic screen for suppressors of the vulvaless phenotype of the *let-23*(*sy1*) mutation? A possible answer is that the screen was not applied extensively enough to identify a reduction-of-function allele of a gene whose null phenotype is complete lethality. Nevertheless, a putative *unc-101* null mutation was isolated in a screen (Lee *et al.*, 1994). The fact that the *apm-1*:GFP reporter construct, which presumably over-expressed APM-1::GFP in the wild-type background, caused greater-than-wild-type induction of VPCs indicates that *apm-1* might play a more important role in the vulval induction pathway than *unc-101*, although *apm-1* was not isolated by a conventional genetic screen.

It is now clear that *apm-1* and *unc-101* play redundant roles in embryogenesis and in vulval development. However, as a single disruption of *apm-1* or *unc-101* clearly showed, a decreased function in either gene led to distinct phenotypes and therefore, the *apm-1* and *unc-101* genes have distinct functions. As described in the RESULTS section, *apm-1* RNAi animals were arrested at the L1 larval stage with abnormal anterior intestine bloating although the larvae seemed normal after hatching. This indicates that *apm-1* gene activity is required even in the presence of wild-type *unc-101* activity during larval development. The intestinal phenotype that appears only in *apm-1* RNAi animals may imply that APM-1 function is important in intestine cells. A new  $\mu 1$  gene ( $\mu 1\text{B}$ ) has been identified in mouse and humans and recent studies showed that the  $\mu 1\text{B}$  medium chain is epithelial cell-specific and important for polarized transport of proteins (Ohno *et al.*, 1999, Folsch *et al.*, 1999). It is possible that APM-1 may likewise have important roles in polarized cells, such as in the intestinal cells of the nematode. Another distinct function of *apm-1* and *unc-101* was drawn from the behavioral phenotype associated with a single disruption of each gene. While *apm-1* RNAi animals do not seem to be Unc (uncoordinated movement), as they move for a short time after hatching, *unc-101* mutant animals are severe Unc, indicating that UNC-101 protein may have major functions in the nervous system or muscles. Our RNAi results indicated that the Unc phenotype of *unc-101* mutants may be due to muscle defects, since RNAi often does not interfere with neuronal gene activity (Travnerakis *et al.*, 2000). Supporting this, GFP fluorescence in the neurons persisted after *apm-1* RNAi in our RNAi experiment. Although *apm-1* and *unc-101* seem to be expressed in overlapping cells at all developmental stages, they exhibited different phenotypes, suggesting that both APM-1 and UNC-101 proteins interact with distinct target proteins.

In summary, we showed in this report that there exist two medium chains of the AP-1 complex in the nematode. Both are expressed ubiquitously throughout development and play redundant roles in embryogenesis and vulval development, but they appear to have distinct functions during early larval stages after hatching. The two medium chains are shared by other components of AP-1 complexes



**Figure 7.** RNAi of  $\sigma 1$  destabilizes both the UNC-101 and APM-1 proteins. The left panels show UNC-101::GFP expression and the right panels show APM-1::GFP expression. (A-H) show animals at their 2–3 fold embryonic stages, and (I–N) show animals at their L1 stage. A, C, E, G, I, and J are Nomarski images and all the others are GFP fluorescence images. (A–D) show the wild-type expression patterns of *unc-101* and *apm-1*, and (E–L) show the results of RNAi of the  $\sigma 1$  gene. Both UNC-101::GFP and APM-1::GFP expression are reduced at the embryonic stage and the L1 stage by the RNAi as shown in F, H, K, and L. On the contrary, RNAi of  $\sigma 2$ , the AP-2 small chain gene, neither reduced UNC-101::GFP nor APM-1::GFP expression as shown in M and N. Note that the nerve ring still contains APM-1::GFP expression even after RNAi of  $\sigma 1$  in L.

as in mammals. An important future direction of research should be to characterize specific functions for these two AP-1 complexes in more detail. The medium chains of AP complexes are known to recognize tyrosine signals and dileucine signals in specific target proteins. In order to elucidate the distinct functions of AP-1 complexes, it will be crucial to identify the cargo proteins that interact with UNC-101 and/or APM-1 protein.

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**Table 4.** Summary of the phenotypes of the animals with various *apm-1* and *unc-101* genetic backgrounds

Genotype <sup>a</sup>	Vulval phenotype
Wild-type	Wild type (WT)
<i>let-23</i>	Vulvaless
<i>unc-101</i>	WT
<i>unc-101; let-23</i>	WT or Greater than WT <sup>b</sup>
<i>unc-101; let-23; [Ex unc-101]</i>	Vulvaless
<i>unc-101; let-23; [Ex apm-1 hybrid]</i>	Vulvaless
<i>unc-101; apm-1 RNAi</i>	WT or Greater than WT <sup>b</sup>
<i>let-23; apm-1 RNAi</i>	WT or Greater than WT <sup>b</sup>
<i>wild type; [Ex apm-1::GFP]</i>	WT or Greater than WT <sup>b</sup>

<sup>a</sup> The exact genotypes of *unc-101* and *let-23* are *unc-101(sy108)* and *let-23(sy1)*. *Unc-101(sy108)* is a putative null allele of *unc-101*, and *let-23(sy1)* is a reduction-of-function allele of *let-23*. [Ex gene] indicates that this gene was introduced as a transgene.

<sup>b</sup> *Unc-101* mutations can suppress the vulvaless phenotype of *let-23(sy1)* to yield wild-type vulval induction in some cases and greater-than-wild-type vulval induction in other cases (Lee *et al.*, 1994). We found that *apm-1* RNAi can also cause greater-than-wild-type vulval induction in various experimental schemes.

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## REFERENCES

Aguilar, R.C., Ohno, H., Roche, K.W., and Bonifacino, J.S. (1997). Functional domain mapping of the clathrin-associated adaptor medium chains  $\mu 1$  and  $\mu 2$ . *J. Biol. Chem.* 272, 27160–27166.

Ahle, S., Mann, A., Eichelsbacher, U., and Ungewickell, E. (1988). Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *Embo. J.* 7, 919–929.

Aroian, R.V., Koga, M., Mendel, J.E., Ohshima, Y., and Sternberg, P.W. (1990). The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* 348, 693–699.

Barstead, R.J., and Waterston, R.H. (1989). The basal component of the nematode dense-body is vinculin. *J. Biol. Chem.* 264, 10177–85.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71–94.

Dell'Angelica, E.C., Mullins, C., and Bonifacino, J.S. (1999a). AP-4, a novel protein complex related to clathrin adaptors. *J. Biol. Chem.* 274, 7278–7285.

Dell'Angelica, E.C., Ohno, H., Ooi, C.E., Rabinovich, E., Roche, K.W., and Bonifacino, J.S. (1997). AP-3: an adaptor-like protein complex with ubiquitous expression. *Embo. J.* 16, 917–998.

Dell'Angelica, E.C., Shotelersuk, V., Aguilar, R.C., Gahl, W.A., and Bonifacino, J.S. (1999b). Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. *Mol. Cell.* 3, 11–21.

Devereux, J., Haeberli, P., Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12, 387–395.

Feng, L., Seymour, A.B., Jiang, S., To, A., Peden, A.A., Novakm, E.K., Zhen, L., Rusiniak, M.K., Eicher, E.M., Robinson, M.S., Gorin,

M.B., and Swank, R.T. (1999). The beta3A subunit gene (*Ap3b1*) of the AP-3 adaptor complex is altered in the mouse hypopigmentation mutant pearl, a model for Hermansky-Pudlak syndrome and night blindness. *Hum. Mol. Genet.* 8, 323–330.

Folsch, H., Ohno, H., Bonifacino, J.S., and Mellman, I. (1999). A novel clathrin adaptor complex mediates basolateral targeting in polarized epithelial cell. *Cell* 99, 189–198.

Hirst, J., Bright, N.A., Rous, B., and Robinson, M.S. (1999). Characterization of a fourth adaptor protein complex. *Mol. Biol. Cell.* 10, 2787–2802.

Hirst, J., and Robinson, M.S. (1998). Clathrin and adaptors. *Biochim. Biophys. Acta.* 1404, 173–193.

Hofmann, M.W., Honing, S., Rodionov, D., Dobberstein, B., von Figura, K., and Bakke, O. (1999). The leucine-based sorting motifs in the cytoplasmic domain of the invariant chain are recognized by the clathrin adaptors AP-1 and AP-2 and their medium chains. *J. Biol. Chem.* 274, 36153–36158.

Keen, J.H. (1990). Clathrin and associated assembly and disassembly proteins. *Annu. Rev. Biochem.* 59, 415–438.

Kirchhausen, T., Davis, A.C., Frucht, S., Greco, B.O., Payne, G.S., and Tubb, B. (1991). AP17 and AP19, the mammalian small chains of the clathrin-associated protein complexes show homology to Yap17p, their putative homolog in yeast. *J. Biol. Chem.* 266, 11153–11157.

Kirchhausen, T., Nathanson, K.L., Matsui, W., Vaisberg, A., Chow, E.P., Burne, C., Keen, J.H., and Davis, A.E. (1989). Structural and functional division into two domains of the large (100- to 115-kDa) chains of the clathrin-associated protein complex AP-2. *Proc. Natl. Acad. Sci. U. S. A.* 86, 2612–2616.

Lee, J., Jongeward, G.D., and Sternberg, P.W. (1994). *unc-101*, a gene required for many aspects of *Caenorhabditis elegans* development and behavior, encodes a clathrin-associated protein. *Genes. Dev.* 8, 60–73.

Mello, C.C., Kramer, J.M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *Embo. J.* 10, 3959–3970.

Nakai, M., Takada, T., and Endo, T. (1993). Cloning of the YAP19 gene encoding a putative yeast homolog of AP19, the mammalian small chain of the clathrin-assembly proteins. *Biochim. Biophys. Acta.* 1174, 282–284.

Nakayama, Y., Goebel, M., O'Brine Greco, B., Lemmon, S., Pingchang Chow, E., and Kirchhausen, T. (1991). The medium chains of the mammalian clathrin-associated proteins have a homolog in yeast. *Eur. J. Biochem.* 202, 569–574.

Ohno, H., Stewart, J., Fournier, M.C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J.S. (1995). Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* 269, 1872–1875.

Ohno, H., Tomemori, T., Nakatsu, F., Okazaki, Y., Aguilar, R.C., Foelsch, H., Mellman, I., Saito, T., Shirasawa, T., and Bonifacino, J.S. (1999).  $\mu 1B$ , a novel adaptor medium chain expressed in polarized epithelial cells. *FEBS. Lett.* 449, 215–220.

Page, R.D. (1998). Gene tree: comparing gene and species phylogenies using reconciled trees. *Bioinformatics* 14, 819–820.

Pevsner, J., Volkandt, W., Wong, B.R., and Scheller, R.H. (1994). Two rat homologs of clathrin-associated adaptor proteins. *Gene* 146, 279–283.

Phan, H.L., Finlay, J.A., Chu, D.S., Tan, P.K., Kirchhausen, T., and Payne, G.S. (1994). The *Saccharomyces cerevisiae* APS1 gene encodes a homolog of the small subunit of the mammalian clathrin AP-1 complex: evidence for functional interaction with clathrin at the Golgi complex. *Embo. J.* 13, 1706–17.

- Robinson, M.S. (1989). Cloning of cDNAs encoding two related 100-kD coated vesicle proteins (alpha-adaptins). *J. Cell. Biol.* 108, 833–842.
- Robinson, M.S. (1994). The role of clathrin, adaptors and dynamin in endocytosis. *Curr. Opin. Cell. Biol.* 6, 538–544.
- Rodionov, D.G., and Bakke, O. (1998). Medium chains of adaptor complexes AP-1 and AP-2 recognize leucine- based sorting signals from the invariant chain. *J. Biol. Chem.* 273, 6005–6008.
- Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shim, J., and Lee, J. (2000). Molecular genetic analysis of *apm-2* and *aps-2*, genes encoding medium and small chains of AP-2 clathrin associated-protein complex in *C. elegans*. *Mol Cells* 10, 309–316.
- Simpson, F., Peden, A.A., Christopoulou, L., and Robinson, M.S. (1997). Characterization of the adaptor-related protein complex, AP-3. *J. Cell. Biol.* 137, 835–845.
- Stepp, J.D., Huang, K., and Lemmon, S.K. (1997). The yeast adaptor protein complex, AP-3, is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole. *J. Cell. Biol.* 139, 1761–1774.
- Stepp, J.D., Pellicena-Palle, A., Hamilton, S., Kirchhausen, T., and Lemmon, S.K. (1995). A late Golgi sorting function for *Saccharomyces cerevisiae* Apm1p, but not for Apm2p, a second yeast clathrin AP medium chain-related protein. *Mol. Biol. Cell.* 6, 41–58.
- Sternberg, P.W., Yoon, C., Lee, J., Jongeward, G., Kayne, P.S., Katz, W.S., Lesa, G., Liu, J., Golden, A., Huang, L.S., and Chamberlin, H.M. (1994). Molecular genetics of proto-oncogenes and candidate tumor suppressors in *Caenorhabditis elegans*. Cold Spring Harb. Sym. Quant. Biol. 59, 155–163.
- Travernarakis, N., Wang, S.L., Dorovkov, M., Ryazanov, A., and Driscoll, M. (2000). Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nat. Genet.* 24, 180–183.
- Thomas, J. (1990). Genetic analysis of defecation in *Caenorhabditis elegans*. *Genetics* 124, 855–872.
- Thurieu, C., Brosius, J., Burne, C., Jolles, P., Keen, J.H., Mattaliano, R.J., Chow, E.P., Ramachandran, K.L., and Kirchhausen, T. (1988). Molecular cloning and complete amino acid sequence of AP50, an assembly protein associated with clathrin-coated vesicles. *DNA* 7, 663–669.
- Yoon, C.H., Lee, J., Jongeward, G.D., and Sternberg, P.W. (1995). Similarity of *sli-1*, a regulator of vulval development in *C. elegans*, to the mammalian proto-oncogene *c-cbl*. *Science* 269, 1102–1105.
- Zizioli, D., Meyer, C., Saftig, P., von Figura, K., and Schu, P. (1999). Early embryonic death of mice deficient in gamma-adaptin. *J. Biol. Chem.* 274, 5385–5390.