

# Disease-related Myotubularins Function in Endocytic Traffic in *Caenorhabditis elegans*<sup>□</sup>

Hope Dang,\* Zhai Li,<sup>†</sup> Edward Y. Skolnik,<sup>†</sup> and Hanna Fares\*<sup>†</sup>

\*Department of Molecular and Cellular Biology, University of Arizona, Tucson, Arizona 85721; and

<sup>†</sup>Departments of Pharmacology and Cell Biology, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, New York 10016

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MTM1, MTMR2, and SBF2 belong to a family of proteins called the myotubularins. X-linked myotubular myopathy, a severe congenital disorder characterized by hypotonia and generalized muscle weakness in newborn males, is caused by mutations in MTM1 (Laporte *et al.*, 1996). Charcot-Marie-Tooth types 4B1 and 4B2 are severe demyelinating neuropathies caused by mutations in MTMR2 (Bolino *et al.*, 2000) and SBF2/MTMR13 (Senderek *et al.*, 2003), respectively. Although several myotubularins are known to regulate phosphoinositide-phosphate levels in cells, little is known about the actual cellular process that is defective in patients with these diseases. Mutations in worm MTM-6 and MTM-9, myotubularins belonging to two subgroups, disorganize phosphoinositide 3-phosphate localization and block endocytosis in the coelomocytes of *Caenorhabditis elegans*. We demonstrate that MTM-6 and MTM-9 function as part of a complex to regulate an endocytic pathway that involves the Arf6 GTPase, and we define protein domains required for MTM-6 activity.

## INTRODUCTION

Myotubularins are a family of phosphatases that can be divided into at least four subgroups based on sequence and phylogenetic analysis (Laporte *et al.*, 2001; Wishart *et al.*, 2001). They have conserved domains that include an N-terminal GRAM domain, which could mediate protein or lipid binding (Doerks *et al.*, 2000), a Rac1-induced localization domain (Laporte *et al.*, 2002a), a phosphatase domain containing the conserved CSDGWRD required for phosphatase activity (Laporte *et al.*, 2001; Wishart *et al.*, 2001), and a SET-interacting domain of unknown function (Laporte *et al.*, 2001). Key residues required for phosphatase activity are absent in the “antiphosphatase” subgroup (which includes SBF2). Furthermore, “substrate-trapping” mutants of catalytically active myotubularins have been made by substituting the conserved nucleophilic cysteine with alanine or serine; these mutants bind more tightly, but not irreversibly, to their substrate (Flint *et al.*, 1997; Blondeau *et al.*, 2000; Laporte *et al.*, 2002a). Finally, some myotubularins contain additional motifs (Laporte *et al.*, 2001), including FYVE domains and plekstrin homology domains, which mediate interactions with specific phosphoinositides (PIs).

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<sup>†</sup> Corresponding author. E-mail address: [fares@email.arizona.edu](mailto:fares@email.arizona.edu).

Abbreviations used: Arf6, adenosine diphosphate-ribosylation factor 6; GFP, green fluorescent protein; mRFP1, monomeric red fluorescent protein; PI, phosphoinositide; PI(3)P, phosphoinositide 3-phosphate; RNAi, RNA-mediated interference; BSA-Rhod, bovine serum albumin conjugated to rhodamine.

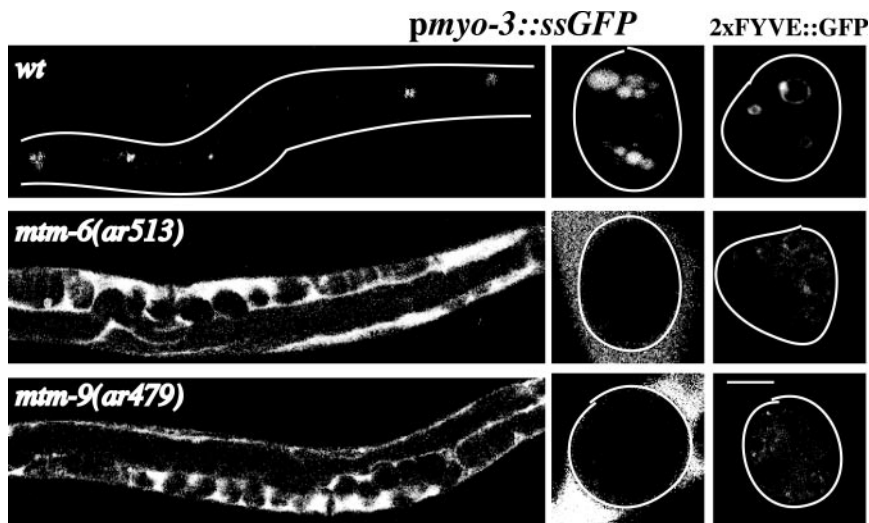
Several myotubularins specifically dephosphorylate the 3'-phosphate of phosphoinositide 3-phosphate [PI(3)P] (Blondeau *et al.*, 2000; Taylor *et al.*, 2000; Walker *et al.*, 2001; Zhao *et al.*, 2001; Berger *et al.*, 2002; Schaletzky *et al.*, 2003), and some have been shown to also act on Phosphatidylinositol 3,5-bisphosphate (Walker *et al.*, 2001; Schaletzky *et al.*, 2003). Although, the overexpression of human MTMR3 results in enlarged vacuoles in *Saccharomyces cerevisiae* (Walker *et al.*, 2001), and the overexpression of human MTM1, MTMR2, or MTMR3 results in enlarged vacuoles in *Schizosaccharomyces pombe* (Blondeau *et al.*, 2000; Laporte *et al.*, 2002b), no actual defect in uptake has been reported in these cells. Furthermore, overexpression of MTM1 in mammalian cells does not noticeably affect vesicular trafficking (Laporte *et al.*, 2002a). Despite this indirect evidence in support of myotubularin function in membrane traffic, no defect in a specific cellular process has so far been identified in cells lacking an endogenous myotubularin.

By screening for mutants unable to internalize green fluorescent protein (GFP) secreted into the body cavity, we previously identified mutations in 14 *cup* (coelomocyte uptake defective) genes that disrupt endocytosis by *Caenorhabditis elegans* coelomocytes, scavenger cells with extremely high endocytic activity (Fares and Greenwald, 2001a). We describe here the molecular identification of CUP-6 and CUP-10 as two of the six myotubularins found in worms (Laporte *et al.*, 2001; Wishart *et al.*, 2001). We also demonstrate a function for these two proteins in an ARF-6- and RME-1-mediated endocytic pathway.

## MATERIALS AND METHODS

### *C. elegans* Strains and Methods

Standard methods were used for genetic analysis (Brenner, 1974). Extrachromosomal arrays were generated by coinjecting restriction digested plasmids and marker DNA, each at 1–10  $\mu$ g/ml along with *EcoRV* digested genomic DNA at 100  $\mu$ g/ml into the germline (Mello and Fire, 1995). Several trans-



**Figure 1.** Endocytosis defects of *mtm* mutant strains. Shown are confocal images of wild-type, *mtm-6(ar513)*, and *mtm-9(ar479)* worms either expressing *pmyo-3::ssGFP* (left two columns) or  $2 \times$  *FYVE::GFP* in their coelomocytes (right column). Worms carrying *pmyo-3::ssGFP* secrete GFP from their muscles into the body cavity. The coelomocytes (bright spots in the wild-type worms) endocytose the GFP from the body cavity. Defects in endocytosis result in GFP accumulating in the body cavity. The left column is a low-magnification image; the middle and last column are higher magnification images showing individual coelomocytes. Outlines of worms and coelomocytes are drawn when needed. Bar, 5  $\mu$ m for the high-magnification images.

genic lines were generated and analyzed from each injection. RNA-mediated interference (RNAi) was carried out as described previously (Xue *et al.*, 2003) by microinjection of double-stranded RNA synthesized in vitro or by feeding bacteria that produce it. Markers used *mtm-6(ar513) III* (Fares and Greenwald, 2001a), *mtm-6(ar515) III* (Fares and Greenwald, 2001a), *mtm-6(ok330) III* (Gene Knockout Consortium), *mtm-9(ar479) V* (Fares and Greenwald, 2001a), and *arls37[pmyo-3::ssGFP] I* (Fares and Greenwald, 2001b). *cdls5 I* is similar to *arls37* but uses the red fluorescent protein DsRed2 (BD Biosciences Clontech, Palo Alto, CA) instead of GFP.

### Molecular Methods

Standard methods were used for the manipulation of recombinant DNA (Sambrook *et al.*, 1989). All enzymes were from New England Biolabs (Beverly, MA), unless otherwise indicated. Site-directed mutagenesis of MTM-6 was done using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA).

### cDNA Sequencing

yk clones (Kohara, personal communication) corresponding to MTM-6 and MTM-9 cDNAs were sequenced in their entirety. The 5' ends of the MTM-6 and MTM-9 mRNAs were determined using 5' rapid amplification of cDNA ends (Roche Diagnostics, Indianapolis, IN).

### Plasmids

All DNA constructs were cloned in front of a coelomocyte-specific promoter (Fares and Greenwald, 2001b). GFP(S65T) (Chalfie *et al.*, 1994) or mRFP1 (Campbell *et al.*, 2002), a monomeric form of DsRed, was used for making all fusion proteins; these markers were introduced at the N terminus of RME-1, or at the C terminus of MTM-6, MTM-9, ARF-6, and  $2 \times$  FYVE(Hrs). Y116A8C.12 is the *C. elegans* ARF-6 (Fares, unpublished data). MTM-6, MTM-9, and RME-1 fusion proteins rescued their corresponding mutants. Endocytosis was normal in coelomocytes expressing ARF-6 (for which no mutation exists) and  $2 \times$  FYVE(Hrs) fusion proteins. Details of plasmid constructions are available upon request.

### Immunoprecipitation

GFP-MTM-6A and Flg-MTM-9 were transfected into human 293 tissue culture cells either alone or together. Total cell lysate (250 mg) was subjected to immunoprecipitation with antibodies against GFP or Flg. The immunoprecipitates were washed three times with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1% Trasylol), separated by SDS gel electrophoresis (10%), and after transfer to nitrocellulose, Western blotted with antibodies to either GFP or Flg. Total cell lysates (50  $\mu$ g) were run as a control to show equal levels of protein expression in the transfected cells.

**Bovine Serum Albumin-Rhodamine (BSA-Rhod) Endocytosis** As described previously (Fares and Greenwald, 2001b; Zhang *et al.*, 2001), BSA-Rhod (Sigma-Aldrich, St. Louis, MO) dissolved at 1 mg/ml in water was injected into the pseudocoelom of young adult hermaphrodites at 20°C. At the indicated times, the worms were mounted in a drop of ice-cold 1% formaldehyde, and slides were kept on ice until viewing.

### Microscopy

Worms were fixed in 1% formaldehyde. Deconvolution images were recorded as digital images (with Z-series containing 0.15- $\mu$ m sections) by using a Series 300 charge-coupled device camera (Photometrics, Tucson, AZ) and deconvolved using DeltaVision software (Applied Precision, Issaquah, WA). Confocal images were taken with a Nikon PCM 2000, by using HeNe 543 excitation for the red dye and argon 488 for the green dye.

### Accession Numbers

Accession numbers were MTM-6A (AY313177), MTM-6B (AY313179), and MTM-9 (AY313178).

### Supplemental Material

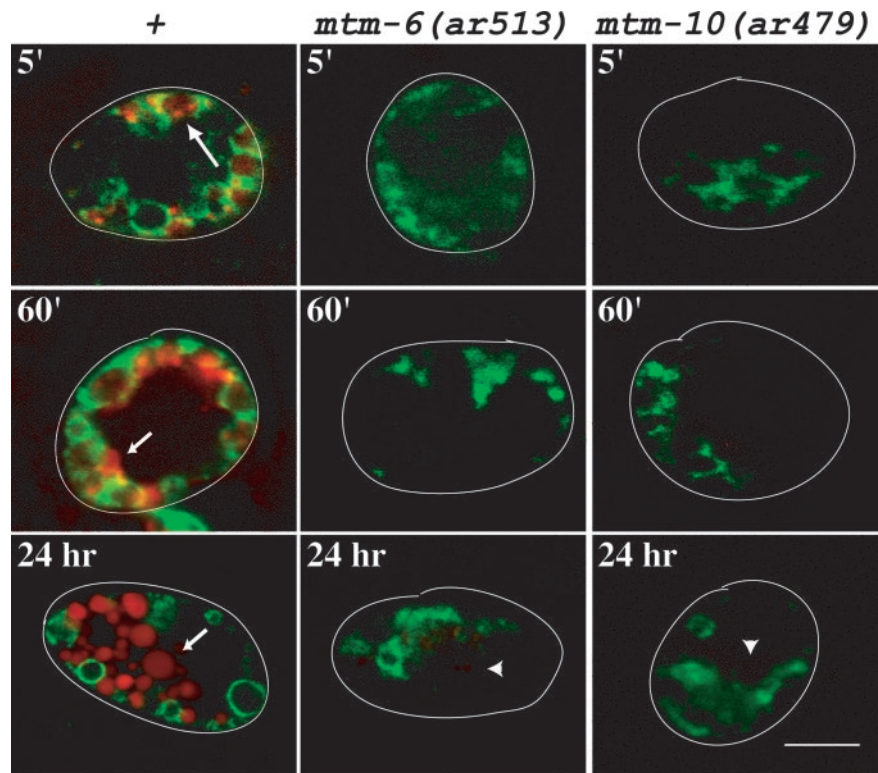
Figures showing the gene structures of *mtm-6* and *mtm-9* are available on the Molecular Biology of the Cell Web site.

## RESULTS

### Identification of CUP-6 and CUP-10 as Myotubularins

Mutations in *cup-6* and in *cup-10* result in decreased coelomocyte endocytosis and hence increased accumulation of GFP in the body cavity of GFP-secreting *pmyo-3::ssGFP* worms (Fares and Greenwald, 2001a; Figure 1). Starting with the map positions of *cup-6* and of *cup-10* (Fares and Greenwald, 2001a), we determined that CUP-6 and CUP-10 correspond to *C. elegans* myotubularins (worm open reading frames F53A2.8 and Y39H10A.3, respectively) based on phenocopy by RNAi (Fire *et al.*, 1998; Xue *et al.*, 2003), transgenic rescue of the mutant phenotypes and sequence analysis of the various alleles (see below). It should be noted that coelomocytes are partially resistant to RNAi, such that the endocytosis defect in coelomocytes after *cup-6* or *cup-10* RNAi is not as severe or as penetrant compared with mutations in the endogenous genes (Xue *et al.*, 2003; Fares, unpublished data).

Specific effects of *cup-6* and *cup-10* mutations on phosphoinositides indicate functional conservation of *C. elegans* and mammalian myotubularins. Direct visualization of intracellular PI(3)P by using a GFP-tagged, PI(3)P reporter  $2 \times$  FYVE(Hrs)::GFP (Gillooly *et al.*, 2000) showed that both genes are required for PI(3)P localization in vivo (Figure 1). Thus, mutations in *cup-6* and in *cup-10*, but not in other members of a large collection of *cup* mutants (Fares and Greenwald, 2001a), caused severe disruption of the PI(3)P staining pattern in vivo (Figure 1; our unpublished data). Given the strong conservation of sequence (Laporte *et al.*,



**Figure 2.** Time course of uptake of BSA-Rhod by coelomocytes expressing RME-8::GFP in wild-type, *mtm-6(ar513)*, or *mtm-9(ar479)* worms. The images are overlays of GFP (green) and rhodamine (red) fluorescence images taken with a confocal microscope. The time the pictures were taken after injection are indicated. The large arrow indicates concentrations of the BSA-Rhod in endosomes labeled with RME-8::GFP. Small arrows indicate lysosomes that are not labeled with RME-8::GFP. The arrowheads indicate extremely faint rhodamine staining in the coelomocytes of the mutant worms after 24 h of uptake. Outlines of worms and are drawn. Bar, 5  $\mu$ m in all images.

2001) and function, we renamed *cup-6* as *mtm-6* and *cup-10* as *mtm-9* to conform to both the mammalian and the worm gene nomenclature.

#### Defective Uptake of Fluid by the Coelomocytes of *mtm-6* and *mtm-9* Mutants

We determined that *mtm-6(ar513)* and *mtm-9(ar479)* coelomocytes were unable to endocytose BSA-Rhod introduced into the body cavity (Figure 2). There are two main advantages of using BSA-Rhod instead of GFP expressed from the *pmyo-3::ssGFP* transgene for a detailed examination of the endocytic pathway in coelomocytes: 1) we can inject the BSA-Rhod at a higher concentration, thus allowing us to monitor early events in endocytosis; and 2) whereas the GFP is degraded in late endosomes and lysosomes, the rhodamine moiety in BSA-Rhod is not affected, thus allowing us to visualize these compartments.

We injected the BSA-Rhod into the body cavity of worms and monitored its uptake by coelomocytes expressing the endosomal marker RME-8::GFP (Zhang *et al.*, 2001). RME-8::GFP labels endosomes, but not lysosomes in coelomocytes. In a wild-type cell, the BSA-Rhod occurs in RME-8::GFP-labeled compartments by 5 min after its injection into the body cavity. At this time, some of the BSA-Rhod occurs as concentrations, presumed to be nascent lysosomes, budding from these RME-8::GFP-labeled compartments (Fares and Greenwald, 2001b; Zhang *et al.*, 2001; Figure 2, large arrow). By 60 min, the BSA-Rhod is concentrated in lysosomes that are not labeled by RME-8::GFP, and remains there for the duration of the experiment (small arrows in Figure 2). In contrast, no BSA-Rhod is seen in *mtm-6(ar513)* and *mtm-9(ar479)* coelomocytes by the 5-min and 60-min time point. Indeed, even after 24 h of incubation in BSA-Rhod, relatively small amounts of the BSA-Rhod are taken up by these cells (Figure 2, arrowhead).

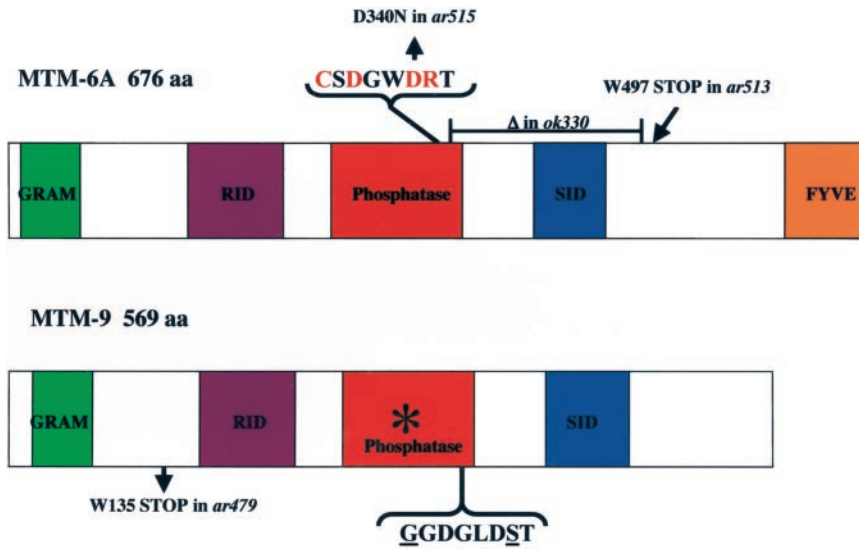
This indicates that the rate of endocytosis in *mtm-6(ar513)* and *mtm-9(ar479)* coelomocytes is sharply reduced, very likely at an early step in the pathway. Also note that the RME-8::GFP staining pattern in *mtm-6* and *mtm-9* worms seems more disorganized than in wild-type cells (Figure 2).

#### Functional Domains of MTM-6

Molecular analysis revealed that *mtm-6* encodes two isoforms, MTM-6A that is sufficient to rescue the endocytic defect of *mtm-6(ar513)* worms (Figure 3), and MTM-6B, which contains additional amino acids at the amino terminus with no obvious motifs (see Supplemental Material). In contrast to MTM-6A::GFP (see below), we could not detect MTM-6B::GFP when overexpressed in coelomocytes, suggesting that it is unstable, nor did it rescue the endocytic defect of *mtm-6(ar513)* worms or disrupt endocytosis in a wild-type cell (our unpublished data). Furthermore, the absence of MTM-6B does not have an obvious effect on coelomocyte endocytosis because simply expressing MTM-6A rescues the coelomocyte endocytosis defect of *mtm-6* mutations that cannot make either form of the protein. Further analysis was therefore done using MTM-6A. *mtm-9* encodes a predicted protein that lacks key residues required for phosphatase activity (Figure 3; Supplemental Material).

The phosphatase domain of MTM-6 is essential for its *in vivo* function. This is supported by two lines of evidence: first, *mtm-6(ar515)*, a mutation with a phenotype as severe as an *mtm-6* null, contains an amino acid substitution of a conserved aspartic acid residue required by the myotubularin to bind to PIs (Wishart *et al.*, 2001; Figure 3). Second, transgenic expression of a substrate-trapping mutant (Flint *et al.*, 1997; Blondeau *et al.*, 2000; Laporte *et al.*, 2002a), MTM-6(C335S), causes dominant inhibition of endocytosis (Figure 4).



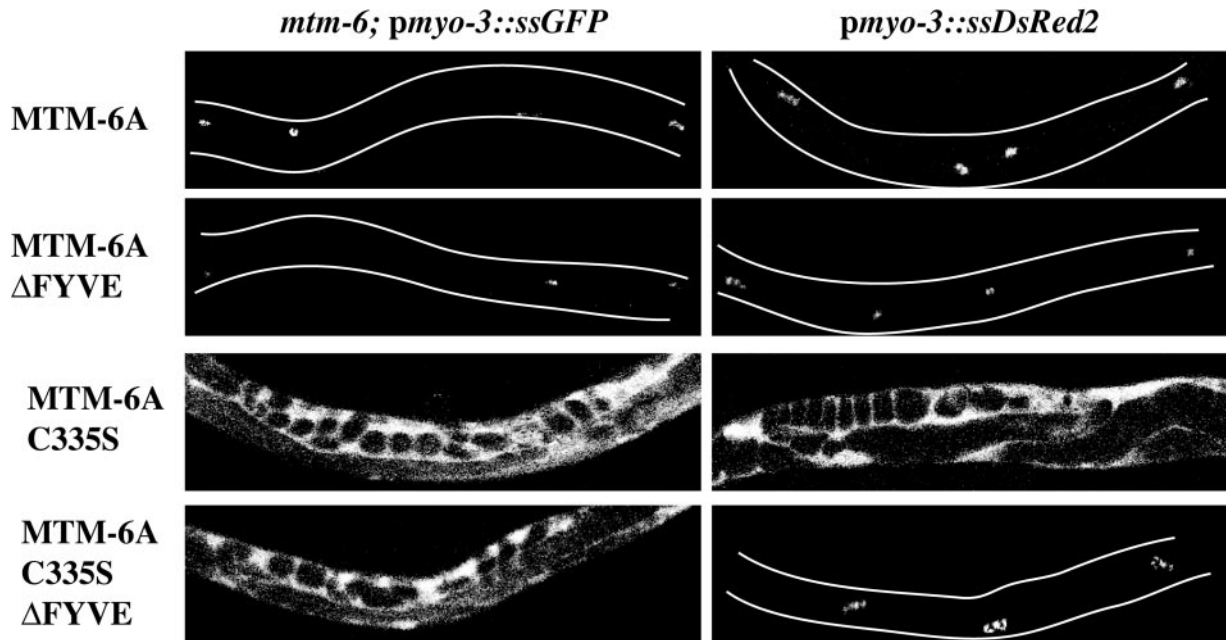


**Figure 3.** MTM-6- and MTM-9-predicted proteins and mutant alleles. Schematic representation of the proteins, and their various domains, predicted from the cDNA sequences. The catalytic amino acids of the phosphatase domain of MTM-6 are indicated in red and the corresponding substituted amino acids of MTM-9 are underlined. The asterisk denotes that the phosphatase domain of MTM-9 lacks residues thought to be required for activity. The predicted changes in the various mutant alleles are shown. *mtm-6(ok330)* and *mtm-9(ar479)* represent the molecular null alleles. All the alleles of *mtm-6* gave identical phenotypes.

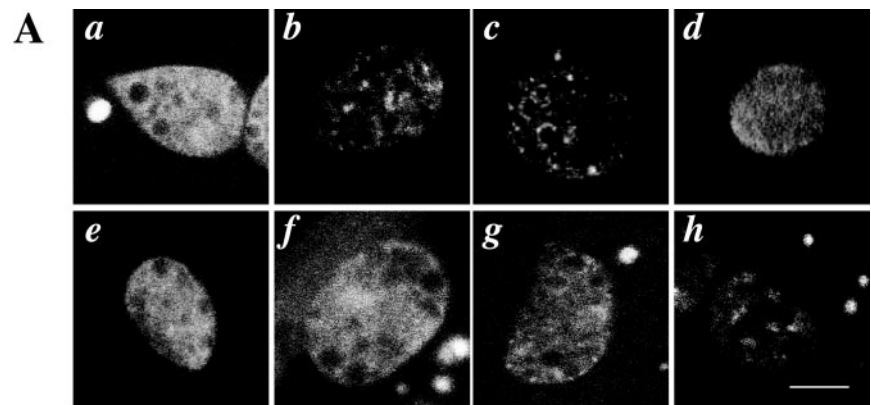
Analysis of specific deletion mutants suggests that the FYVE domain of MTM-6A is required for MTM-6 to maintain its membrane localization. Overexpression of either MTM-6A::GFP or MTM-6AΔFYVE::GFP (fusion protein completely lacking the FYVE domain) rescues the endocytosis defect of *mtm-6*, indicating that, at least under these conditions, the FYVE domain is not required by MTM-6 for its targeting to membranes or for its activity (Figure 4). However, in contrast to MTM-6A(C335S), MTM-6A(C335S)ΔFYVE did not interfere with coelomocyte endocytosis (Figure 4), indicating that the FYVE domain increases the avidity rather than the affinity of MTM-6A to specific membranes.

**Subcellular Localization of MTM-6A and MTM-9**

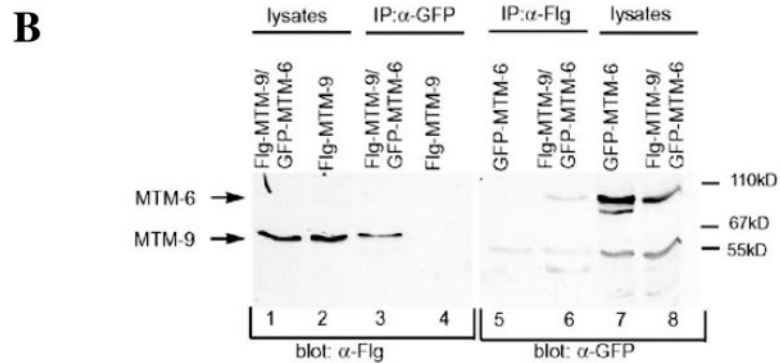
Both MTM-6A::GFP and MTM-6AΔFYVE::GFP are largely cytosolic, with some membrane localization (Figure 5A, *a* and *e*). In agreement with other myotubularin substrate-trapping mutants (Flint *et al.*, 1997; Blondeau *et al.*, 2000; Laporte *et al.*, 2002a), MTM-6A(C335S)::GFP shows a punctate staining pattern and localizes to the plasma membrane and to compartments close to the plasma membrane in coelomocytes (Figure 5A, *b*). This membrane association does not require the presence of MTM-9 because it remains unchanged in *mtm-9(ar479)* worms (Figure 5A, *c*). In contrast, and in support of its inability to give a dominant



**Figure 4.** Effect of MTM-6 alleles on endocytosis by coelomocytes. Shown are low-magnification confocal images of *mtm-6(ar513); pmyo-3::ssGFP* or of *pmyo-3::ssDsRed2* worms carrying transgenes expressing GFP fusions of various forms of MTM-6A. Bright spots in the worms are coelomocytes normally endocytosing the fluid-phase marker. Bright fluorescence in the body cavity indicates absence of endocytosis. Outlines of worms are drawn when needed.



**Figure 5.** Subcellular localization and interaction of MTM-6A and MTM-9. (A) High-magnification confocal images of the GFP fusion protein expressed in the coelomocytes of the following strains (Ex signifies the transgenic array from which the indicated protein is expressed): *a*, +; *Ex[MTM-6A::GFP]*; *b*, +; *Ex[MTM-6A(C335S)::GFP]*; *c*, *mtm-9(ar479)*; *Ex[MTM-6A(C335S)::GFP]*; *d*, *rme-1(b1045)*; *Ex[MTM-6A(C335S)::GFP]*; *e*, +; *Ex[MTM-6AΔFYVE::GFP]*; *f*, +; *Ex[MTM-6A(C335S)ΔFYVE::GFP]*; *g*, +; *Ex[MTM9::GFP]*; and *h*, *Ex[MTM-6A(C335S)]*; *Ex[MTM9::GFP]*. Bar, 5  $\mu$ m in all images. (B) Coimmunoprecipitation of GFP-MTM-6A and Flg-MTM-9 expressed in human 293 cells. Antibodies against GFP or Flg were used to immunoprecipitate the corresponding fusion protein indicated above the gel. Blots were probed for the presence of the other fusion protein by using the antibodies indicated below the gel.



negative phenotype, MTM-6A(C335S)ΔFYVE::GFP is mostly cytoplasmic (Figure 5A, *f*).

Like MTM-6, the phosphatase-inactive MTM-9 protein is also predominantly localized to the coelomocyte cytoplasm, although as for MTM-6, a functional MTM-9::GFP fusion protein reveals some membrane association (Figure 5A, *g*).

#### Physical Association of MTM-6A and MTM-9

Given previous reports of physical interactions between other catalytically active and inactive forms of human and mouse MTM proteins (Kim *et al.*, 2003; Mochizuki and Majerus, 2003; Nandurkar *et al.*, 2003), and the requirement for both MTM-6 and MTM-9 for coelomocyte endocytosis, we thought it possible that MTM-9 could interact with MTM-6A to regulate its activity. Expression of the membrane-associated MTM-6A(C335S) mutant protein increased membrane association of MTM-9, providing *in vivo* evidence that MTM-6A localization or activity regulates MTM-9 localization (Figure 5A, *h*).

This regulation of MTM-9 by MTM-6 may be direct, because we determined that MTM-6A and MTM-9 physically associate and/or are part of the same complex. GFP-MTM-6A and Flg-MTM-9 were expressed in human 293 cells either alone, or together, and the ability of the two proteins to coimmunoprecipitate was determined. We found that MTM-6A and MTM-9 coimmunoprecipitated using antibodies to either GFP or Flg (Figure 5B, lanes 3 and 6). The coimmunoprecipitation was specific because it only occurred when both proteins were expressed in the cells (Figure 5B, lanes 4 and 5).

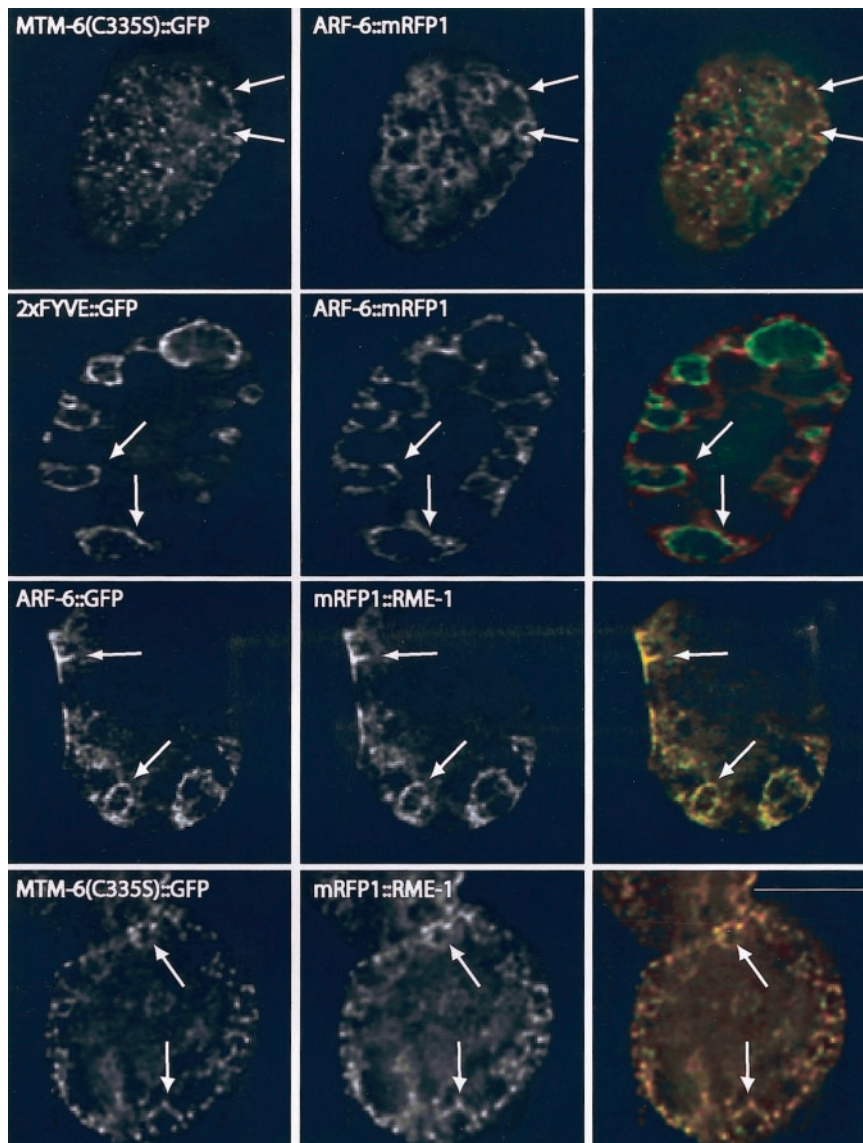
The above-mentioned observations, taken together, provide strong evidence for functional interactions between the

catalytically active and inactive forms of MTM proteins in regulating fluid phase endocytosis by coelomocytes, probably through endocytic organelles visualized using the substrate-trapping MTM-6A(C335S)::GFP fusion protein.

#### Association of MTM-6 with an ARF-6/RME-1 Endocytic Pathway

To better characterize the MTM-6 and MTM-9 endocytic pathway, we further examined the identity of the MTM-6A(C335S)::GFP labeled membranes. Adenosine diphosphate-ribosylation factor 6 (Arf6) is required in some mammalian tissues for nonclathrin-dependent forms of membrane traffic (Naslavsky *et al.*, 2003). *C. elegans* ARF-6 is strongly expressed in coelomocytes (our unpublished data) and gives a pattern of localization similar to MTM-6A(C335S)::GFP (Figure 6). Indeed, MTM-6A(C335S)::GFP and ARF-6::mRFP1 show significant colocalization in coelomocytes (Figure 6). Consistent with a function of MTM-6 in PI(3)P metabolism, 2 × FYVE::GFP and ARF-6::mRFP1 also partially colocalize (Figure 6). Furthermore, using deconvolution microscopy to better visualize membrane-associated protein, we find that MTM-9::GFP and ARF-6::mRFP1 significantly colocalize in coelomocytes (our unpublished data).

These studies indicate that MTM-6/9-proteins function in a specialized endocytic pathway that involves Arf6. We therefore analyzed other components of the Arf6 pathway in *C. elegans*. Rme1/Ehd1/Pincher is an epsin homology domain-containing protein that is required for endocytosis or endocytic trafficking in some mammalian cells (Lin *et al.*, 2001), including the Arf6 pathway in some cell types (Caplan *et al.*, 2002). RME-1 is the *C. elegans* homolog of Ehd1 (Grant *et al.*, 2001) and is required for endocytosis by coelomocytes (Fares and Green-



**Figure 6.** Deconvolution images of wild-type worms expressing the indicated proteins from transgenes. Panels in the right column are overlays of the corresponding GFP (green) and mRFP1 (red) images. Arrows indicate some of the membranes to which both proteins colocalize. Bar, 5  $\mu$ m in all images.

wald, 2001a; Grant *et al.*, 2001). Indeed, like their mammalian counterparts (Caplan *et al.*, 2002), ARF-6::GFP and mRFP1::RME-1 show extensive colocalization in coelomocytes (Figure 6). Consistent with the above results, MTM-6A(C335S)::GFP and mRFP1::RME-1 largely colocalize in coelomocytes (Figure 6). This colocalization is functionally significant because the membrane association of MTM-6A(C335S)::GFP is severely disrupted in *rme-1(b1045)* mutant worms (Figure 5A, d).

## DISCUSSION

We provide in this study our analysis of two biochemically distinct myotubularins and we show, for the first time, essential roles for the two proteins in a newly identified PI(3)P and Arf6/Rme1-associated endocytic pathway.

The identification of mutations in two myotubularins in an unbiased screen for endocytosis mutants strongly argues that a primary role of myotubularins is to regulate endocytosis. Indeed, RNAi analysis suggests that in addition to MTM-6 and MTM-9, worm MTM-1 and MTM-3 are also

required for endocytosis by coelomocytes (Xue *et al.*, 2003). This indicates that the myotubularins have largely nonoverlapping functions in regulating this process, perhaps by regulating PI levels at distinct membranes. This is in agreement with previous studies on human MTM1 and MTMR2 (Laporte *et al.*, 2002b).

At which step(s) of the coelomocyte endocytic pathway are MTM-6 and MTM-9 required? When we monitored endocytosis in coelomocytes after injecting BSA-Rhod into the body cavity of worms, we saw little/no uptake of the dye in *mtm-6* and *mtm-9* coelomocytes. The simplest interpretation of these data is that MTM-6 and MTM-9 are required very early in the endocytic pathway in coelomocytes. We favor this model because it is consistent with the colocalization of the proteins with ARF-6 and RME-1 close to and on the plasma membrane. Indeed, in mammalian cells ARF-6 is required at the plasma membrane and in specialized early endosomes (Naslavsky *et al.*, 2003), whereas a mammalian homolog of RME-1 localizes to cell surface ruffles (Shao *et al.*, 2002). MTM-6 and MTM-9 could also be required in later endocytic compartments by regulating the levels and/or



localization of other essential PI-phosphates directly, or indirectly because PI(3)P is a substrate for making other PI-phosphates. This is consistent with our observation that the RME-8–labeled compartments seem more disorganized in *mtm-6* and *mtm-9* coelomocytes than in wild-type cells. Note that we could detect very little colocalization between RME-8, and ARF-6, RME-1, or CUP-6(C335S) (Fares, unpublished data), indicating that RME-8 localizes to distinct endocytic compartments.

We had previously shown that reducing *mtm-6* levels in the intestine results in the appearance of PI(3)P at the apical membrane rather than on internal vesicles (Xue *et al.*, 2003). This indicates that in this tissue, MTM-6 is required to maintain low levels of PI(3)P at the plasma membrane. Although we do observe a severe disorganization of PI(3)P in coelomocytes, we do not observe PI(3)P at the plasma membrane of coelomocytes in *mtm-6* and *mtm-9* mutants. This is probably due to some functional redundancy among the myotubularins in coelomocytes and/or because of differences in the endocytic pathways used by these two tissues. Previous studies on the role of myotubularins in regulating membrane-associated PI(3)P levels relied on the overexpression of MTM1 and MTMR2 in mammalian COS cells and yielded conflicting results (Kim *et al.*, 2002; Laporte *et al.*, 2002a).

In coelomocytes, PI(3)P levels do not simply increase in the absence of MTM-6 or MTM-9; rather, PI(3)P becomes disorganized. We believe this is due to the involvement of other myotubularins in coelomocytes, such that in the absence of MTM-6 or MTM-9, there is misregulation rather than simple reduction of phosphatase activity. Furthermore, FYVE domains are known to bind to PI(3)P. We could not genetically analyze the requirement for the MTM-6 FYVE domain in the wild-type protein because overexpression of the MTM-6 $\Delta$ FYVE displayed similar cytoplasmic localization and rescue of *mtm-6* mutants as MTM-6. However, although overexpressed MTM-6(C335S) localized to membranes and inhibited endocytosis by coelomocytes, these effects were absent if the FYVE domain was reduced. This suggests that the FYVE domain is required for continued localization to membranes and that its function is probably critical for the function of the endogenous protein, that is, under conditions when the protein is not overexpressed.

MTM-6(C335S) and MTM-9 colocalize with ARF-6 and with RME-1. In the absence of a mutation in *C. elegans* ARF-6, we tried to lower ARF-6 levels in coelomocytes through RNAi. We were unsuccessful because we could not detect any changes in the levels of ARF-6::GFP expressed in these cells after RNAi (Fares, unpublished data). However, RME-1 is required for endocytosis by coelomocytes, and we showed that the colocalization of MTM-6(C335S) with RME-1 is significant because the elimination of RME-1 function in these cells disrupted the membrane association of MTM-6(C335S).

Our conclusion that MTM-6, and probably other myotubularins, functions in ARF-6/RME-1–mediated endocytosis helps explain two previous observations. 1) Human MTM1, MTMR2, MTMR3, and MTMR9 localize, in part, to Rac1-induced membrane ruffles (Laporte *et al.*, 2002a,b; Nandurkar *et al.*, 2003), sites of clathrin-independent endocytosis. Interestingly, Arf6 colocalizes with Rac1 and can influence its ability to effect plasma membrane remodeling (Radhakrishna *et al.*, 1999; Boshans *et al.*, 2000). Furthermore, Rme1/Ehd1/Pincher associates with membrane ruffles in PC12 cells to regulate a clathrin-independent endocytic pathway (Shao *et al.*, 2002). 2) Overexpression of Gas3/PMP22, a component of the compact peripheral myelin,

results in a peripheral neuropathy, Charcot-Marie-Tooth type 1A, which like type 4B, is characterized by myelination abnormalities, although other pathological details differ among the two diseases. It has recently been shown that overexpressed Gas3/PMP22 specifically affects Arf6 membrane trafficking and that some of the endocytosed Gas3/PMP22 localizes to Arf6 compartments (Chies *et al.*, 2003).

Links between Arf6 and phosphoinositides are also known. Arf6 regulates phosphatidylinositol 4,5-bisphosphate levels at the plasma membrane and in endosomes (Brown *et al.*, 2001), and PI(3)P is required to transport endocytosed material from Arf6-endosomes to classic Rab5-labeled early endosomes (Naslavsky *et al.*, 2003). Myotubularins most likely regulate PI(3)P (and probably other forms of PI) in endosomal compartments, including Arf6-endosomes. Furthermore, given the association of myotubularins with the plasma membrane in mammalian cells and in worms, they could also act to reduce PI(3)P levels at the plasma membrane that may interfere with the initial steps of endocytosis.

We propose that similar disruptions in an Arf6 pathway is at least a partial cause for the development of diseases stemming from mutations in human myotubularins. This and future studies will allow us to better probe the molecular basis for myotubular myopathy and Charcot-Marie-Tooth type 4B and will give us a better understanding of the process of endocytosis in general.

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