

Distinct Cellular and Subcellular Patterns of Expression Imply Distinct Functions for the *Drosophila* Homologues of Moesin and the Neurofibromatosis 2 Tumor Suppressor, Merlin

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Abstract. Interest in members of the protein 4.1 superfamily, which includes the ezrin-radixin-moesin (ERM) group, has been stimulated recently by the discovery that the human neurofibromatosis 2 (NF2) tumor suppressor gene encodes an ERM-like protein, merlin. Although many proteins in this family are thought to act by linking the actin-based cytoskeleton to transmembrane proteins, the cellular functions of merlin have not been defined. To investigate the cellular and developmental functions of these proteins, we have identified and characterized *Drosophila* homologues of moesin (Dmoesin) and of the NF2 tumor suppressor merlin

(Dmerlin). Using specific antibodies, we show that although these proteins are frequently coexpressed in developing tissues, they display distinct subcellular localizations. While Dmoesin is observed in continuous association with the plasma membrane, as is typical for an ERM family protein, Dmerlin is found in punctate structures at the membrane and in the cytoplasm. Investigation of Dmerlin in cultured cells demonstrates that it is associated with endocytic compartments. As a result of these studies, we propose that the merlin protein has unique functions in the cell which differ from those of other ERM family members.

RECENT studies have identified a number of cytoplasmic proteins that interact with and regulate the functions of transmembrane proteins, including transmembrane receptors. Examples include proteins that function in signal transduction (Greenwald and Rubin, 1992), proteins that regulate membrane trafficking to and from the cell surface (Mays et al., 1994), and those that mediate interactions with underlying cytoskeletal components (Bennett, 1990). In the latter category are members of the protein 4.1 superfamily, which include protein 4.1, its *Drosophila* homologue Coracle (Fehon et al., 1994), talin (Rees et al., 1990), and members of the ezrin-radixin-moesin (ERM)¹ family (Gould et al., 1989; Funayama et al., 1991; Lankes and Furthmayr, 1991). Erythrocyte protein 4.1 is known to associate with the transmembrane protein glycophorin C as well as with the spectrin/actin cytoskeleton, thereby providing structural integrity to the membrane cytoskeleton (Bennett, 1990). On the other hand,

a *Drosophila* protein 4.1 homologue, Coracle, has been shown to lack the spectrin/actin interaction domain identified in its human and *Xenopus* counterparts, and shows a strikingly different subcellular pattern from either cytoskeletal actin or spectrin (Fehon, et al., 1994). These results, together with the discovery of family members with divergent structures, such as protein tyrosine phosphatases (Gu et al., 1991; Yang and Tonks, 1991; Banville et al., 1994), indicate that the members of the 4.1 superfamily may perform a wide range of cellular functions.

Like protein 4.1, members of the ERM family are thought to act primarily by linking transmembrane proteins to the cortical actin cytoskeleton. Studies of ezrin have shown that the NH₂-terminal 300 amino acids associate with the plasma membrane (Algrain et al., 1993), while the most COOH-terminal 35 amino acids comprise a binding domain for F-actin (Turunen et al., 1994). This region is highly conserved in radixin and moesin, although their ability to bind F-actin has not yet been directly established. Proteins in this family have been found to colocalize in areas of rich actin content, such as microvilli and membrane ruffles (Bretscher, 1989; Sato et al., 1992; Franck et al., 1993). In addition, several of the ERM proteins localize to the junctional regions of vertebrate cells and tissues, although precise identification of the junctionally associated ERM proteins has been hampered by antibody cross-reactivity (Sato et al., 1992). Because of their

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1. Abbreviations used in this paper: Dmerlin, *Drosophila* merlin; Dmoesin, *Drosophila* moesin; ERM, ezrin-radixin-moesin; NF2, neurofibromatosis 2; S2 cells, Schneider 2 cells.

similarity in structure and extensive colocalization, the specific function of each of these highly homologous proteins has been difficult to distinguish. A recent study in cultured cells indicates, however, that these proteins may have both unique and redundant functions in the establishment and maintenance of microvilli, cell-cell adhesion, and cell-substrate adhesion (Takeuchi et al., 1994).

Interest in the ERM family has been stimulated by the discovery that human neurofibromatosis 2 (NF2) is caused by mutations in merlin (moesin-ezrin-radixin-like protein), a novel member of the ERM family (Rouleau et al., 1993; Trofatter et al., 1993). The hallmark of NF2 is the presence of bilateral acoustic schwannomas (acoustic neuromas) affecting the eighth cranial nerve, in addition to other neurally associated tumors, such as meningiomas and ependymomas (Martuza and Eldridge, 1988). Because NF2 is difficult to detect, the identification of this disease gene has been crucial in developing an effective screen for patients at risk (MacCollin et al., 1993). Based on its structural similarity to the ERM family, previous studies have proposed that merlin acts similarly to other ERM proteins by linking the plasma membrane to the underlying cytoskeleton (Rouleau et al., 1993; Trofatter et al., 1993). However, this observation alone has led to neither a specific hypothesis concerning its role in tumor suppression nor to an understanding of merlin's cellular functions.

To investigate the functions of ERM proteins in a developmental system that is amenable to experimental manipulation, we have identified a merlin homologue (*Dmerlin*) and a moesin homologue (*Dmoesin*) in *Drosophila melanogaster*. A partial *Dmoesin* clone was previously identified in a screen for *Drosophila* cDNAs, which alter cell shape in yeast (Edwards et al., 1994). Using specific antibodies, we describe and compare the tissue and subcellular localizations of these ERM family members during *Drosophila* development and in Schneider 2 (S2) cultured cells. Our results indicate that while *Dmerlin* and *Dmoesin* are frequently coexpressed in developing tissues, they display strikingly distinct subcellular localizations. In contrast to *Dmoesin*, which shows the continuous plasma membrane-associated localization typical of ERM family proteins, *Dmerlin* is localized in punctate structures that are associated with both the plasma membrane and the cytoplasm. We further demonstrate that *Dmerlin* is associated with endocytic compartments in cultured cells. Together, these results lead us to propose that merlin has unique functions, possibly related to endocytic processes, that differ from those of all other known ERM family members.

Materials and Methods

Cloning and Sequencing

Degenerate oligonucleotides were designed using an amino acid sequence alignment between mouse radixin (GenBank/EMBL/DBJ accession No. X60672) and the *Echinococcus multilocularis* major tegument protein (GenBank/EMBL/DBJ accession No. M61186). Primers of the following sequences were generated against regions indicated by the numbered arrows above the sequence (Fig. 1).

Primer 1, 5'-AT(A/C/T) GCI CA(A/G) GA(C/T) (C/T)TI GA(A/G) ATG TA(C/T) GG-3';

Primer 2, 5'-C(G/T)G AT(A/G) TAI A(A/G)(C/T) TC(A/G) TG(A/G) TTI CC-3';

Primer 3, 5'-GC(C/T) TG(A/G/T) AT(C/T) TTC AT(C/T) TG(C/T) TGI AC(C/T) TC-3'.

PCR amplifications were performed using *Drosophila* genomic DNA as a template and the following cycle conditions: 94°C, 15 s; 45°C, 15 s; 72°C, 45 s for 30 cycles. Amplification products were purified by electrophoresis and subcloned for sequencing as described previously (Fehon et al., 1994). Clones of *Dmerlin* and *Dmoesin* were obtained from a 0–4-h pNB40 plasmid embryonic library (Brown and Kafatos, 1988) and a 2–14-h lambda-dzaph phage embryonic library (Stratagene, La Jolla, CA), respectively, using the subcloned degenerate PCR products as probes. The inserts were subcloned into Bluescript for sequencing. Both strands of double-stranded DNA from the subclones were sequenced as described previously (Fehon et al., 1994) and analyzed using the Geneworks DNA analysis program (IntelliGenetics, Inc., Mountain View, CA). The alignments obtained from this analysis were modified based on alignments that were generated by the BLAST server in the National Center for Biotechnology Information GenBank database (Altschul et al., 1990).

Antibody Preparation and Immunolocalization

The *Dmoesin* fusion protein for raising antibodies was constructed by cloning the Sall fragment, comprising amino acids 23–463 (bp 225–1555) into pGEX-2 (Smith and Johnson, 1988). This fusion was purified as described (Frorath et al., 1991), except that the sample was treated with 6 M urea and dialyzed against PBS. For *Dmerlin*, a tandem repeat of the coding region for amino acids 445–561 (bp 1433–02201) was inserted into pGEX-3. This fusion was expressed and purified as previously described (Smith and Johnson, 1988), with the exception that cultures were grown at 25°C to improve solubility of the fusion protein. The boundaries of the fusion proteins are indicated by arrows above the sequence (Fig. 1).

Polyclonal antisera were raised in mice and guinea pigs against the *Dmerlin* and *Dmoesin* fusion proteins. In addition, one of these mice was used to generate *Dmoesin* mAbs (Harlow and Lane, 1988). Affinity-purified *Dmerlin* guinea pig polyclonal antisera were prepared using the Amino-Link immobilization kit (Pierce Chemical Co., Rockford, IL) following the instructions provided, except that the coupling reaction was performed for 16 h at 4°C. In addition, the serum was applied to the column beads by batch mixing for 1 h at room temperature. The eluted antibody was further purified by absorption against glutathione S-transferase bound to glutathione agarose beads. Immunostaining of embryos and imaginal discs was performed as described previously (Fehon et al., 1994), with the exception that embryos were hand devitelized for phalloidin staining.

Immunoblotting

Single embryos were stage selected and individually lysed in 20 µl of lysis buffer, pH 7.6, containing 10 mM Tris, pH 7.5, 50 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholic acid, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 µM sodium orthovanadate, and 0.01% BSA. Samples were subject to immunoblot analysis as previously described (Fehon et al., 1994), probed with either the whole guinea pig α-*Dmerlin* polyclonal antisera (1:5,000) or the mouse α-*Dmoesin* polyclonal antisera (1:10,000), and incubated overnight at 4°C. The washed blots were incubated with an HRP secondary antibody (1:10,000) at room temperature for 3 h and developed using the ECL kit (Amersham Life Science Inc., Arlington Heights, IL).

Transfection of Schneider 2 Cells and the Labeling of Endocytic Compartments

For the expression of *Dmerlin* in cultured cells, a 2,198-bp MunI fragment (bp 333–2531) that encodes the predicted full-length *Dmerlin* protein was cloned into the EcoRI site of either pRmHa-3 (metallothionein promoter vector; Bunch et al., 1988) or pCaSpeR-hs (heat shock protein-70 promoter). For expression of *Dmoesin*, the entire cDNA was excised from Bluescript as an EcoRI/XhoI (partial) fragment and cloned into the corresponding sites of pHSX (Rebay, 1993). This fragment was then excised using flanking NotI sites and cloned into pCaSpeR-hs. The use of different promoters allowed for either continuous or pulsed expression of induced proteins.

The maintenance, transfection, induction, and immunofluorescent analysis of Schneider 2 (S2) cells was performed as previously described (Fehon et al., 1990). In the dextran labeling experiment, S2 cells were transfected with the *Dmerlin* pCaSpeR-hs construct and heat shocked at 38°C for 1.5 h. After 1 h of recovery at 25°C, lysine-fixable Texas red dextran

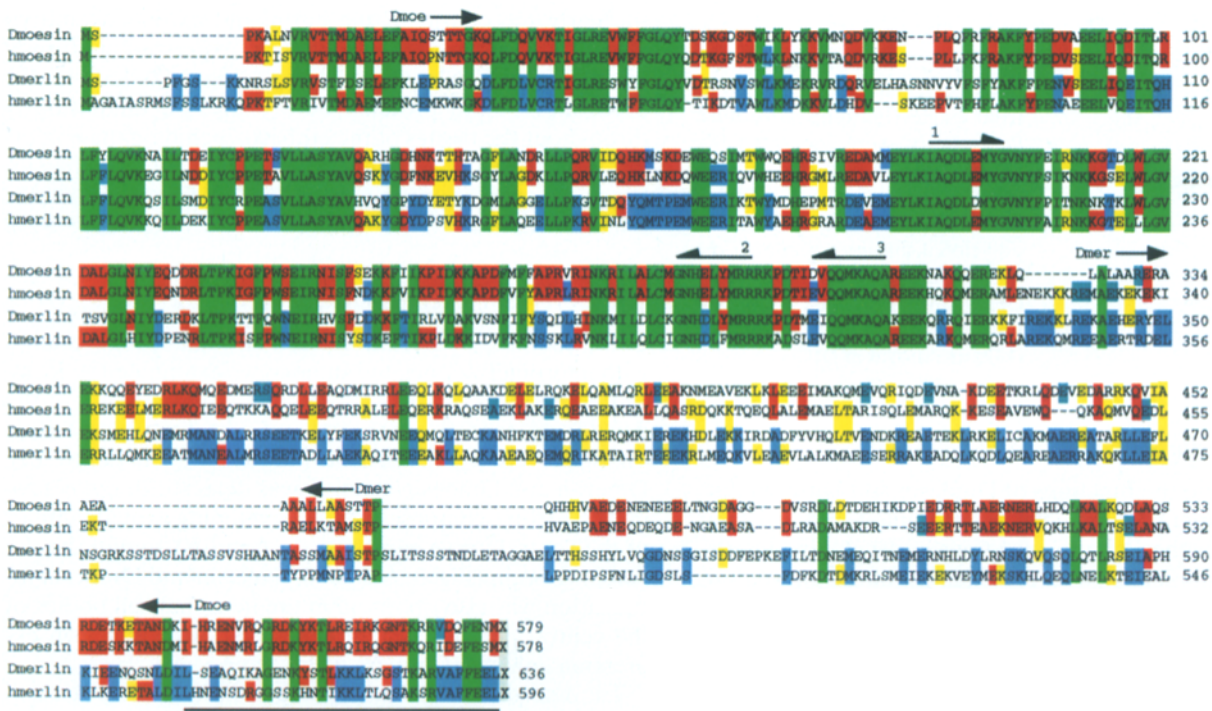


Figure 1. Deduced protein sequence alignment of Dmoesin, human moesin, Dmerlin, and human merlin. These four sequences are 38% identical (*shaded in green*). Amino acid residues shaded red indicate identities between the moesin proteins, those shaded in blue are identical between the merlin proteins, and yellow shading indicates amino acids shared by one moesin sequence and one merlin sequence. Sequences corresponding to the PCR primers used in the cloning of Dmerlin and Dmoesin are indicated by numbered arrows. Arrows labeled Dmer and Dmoesin define the boundaries of the Dmerlin and Dmoesin fusion proteins constructed for use in generating antibodies. Sequences corresponding to the actin-binding domain in ezrin are underlined. These sequence data are available from GenBank/EMBL/DBJ under accession Nos. U49724 (*Dmerlin*) and L38909 (*Dmoesin*).

(mol wt = 10,000; Molecular Probes, Eugene, OR) was added to an aliquot of cells to a final concentration of 0.5 mg/ml. The cells were then incubated for an additional 0.5 h before they were fixed and stained with the Dmerlin mouse polyclonal antibody.

Results

Identification of a Merlin and a Moesin Homologue in *Drosophila*

Two predominant products (one of these sequences contained two small introns, and thereby gave rise to a larger PCR product) were isolated after PCR using degenerate oligonucleotide primers and *Drosophila* genomic DNA as a template (see Fig. 1 and Materials and Methods for design of primers). Sequence analysis of each subcloned PCR product determined that while not identical, both sequences shared extensive similarity with the vertebrate ERM genes. Analysis of more than 30 independent subclones from these amplification products failed to reveal any other ERM-related sequences in *Drosophila*. Using the subcloned PCR products as probes, *Drosophila* cDNA libraries were screened (see Materials and Methods), and the complete sequences were then determined from these cDNA clones (Fig. 1).

Based on sequence analysis, both of these genes are clearly members of the ERM gene family. One sequence, denoted Dmerlin, is 55% identical to human merlin, while only 45% identical to human ezrin and mouse radixin, and 39%

identical to human moesin. The similarity between *Drosophila* and human merlin extends over the entire length of the two sequences, and is greatest in the NH₂ terminus, where all ERM proteins share high identity. In this region, however, areas can be identified where the merlin sequences, while similar to each other, diverge from the moesin sequences (Fig. 1, *blue shading*). The similarity between Dmerlin and human merlin is particularly notable at the COOH terminus, where the merlin proteins diverge from the other ERM family members. Comparisons of the merlin sequences using the UPGMA method (unweighted pair group method with arithmetic mean; Nei, 1987) place Dmerlin and human merlin together in a distinct group apart from the rest of the ERM family (data not shown).

Sequences from the second *Drosophila* ERM family member were found to share 58% overall identity with both human moesin and human ezrin, 57% identity with mouse radixin, and only 41% identity with human merlin. Designation of this gene as *Dmoesin*, rather than as ezrin or radixin, was based primarily on its lack of the polyproline tract characteristic of the latter two proteins (Sato et al., 1992). Furthermore, Dmoesin shares a greater degree of identity in the COOH-terminal divergent region with human moesin (26%) than with human ezrin (25%) or mouse radixin (22%). Finally, comparisons of these sequences using UPGMA place *Dmoesin* closer to human moesin than to either ezrin or radixin in the ERM subfamily (data not shown).

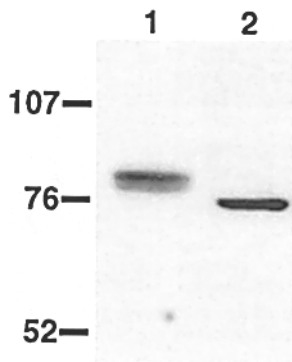


Figure 2. Specificity of the α -Dmerlin and α -Dmoesin antibodies. Protein samples derived from single Canton-S *Drosophila* embryos were separated by SDS-PAGE, transferred to nitrocellulose, and probed with either the guinea pig α -Dmerlin polyclonal antibody (lane 1) or the mouse α -Dmoesin polyclonal antibody (lane 2). Positions of the protein standards (kD) are shown at left.

The Expression of Dmerlin and Dmoesin

To investigate the functions of Dmoesin and Dmerlin during *Drosophila* development, we examined in detail their cellular and subcellular localizations in developing tissues. Before examining their expression, immunoblot analysis was used to test the specificity of the antibodies that were generated against the two proteins (Fig. 2). The Dmerlin antibody recognized a doublet of ~ 81 and 85 kD, and the Dmoesin antibody recognized a single band of ~ 72 kD. Even when lane 1 in Fig. 2 was reprobbed with the Dmoesin antibody, and the blot was overexposed, the Dmerlin protein was not recognized (data not shown). Thus, the antisera do not cross-react on immunoblots, and subsequent immunostaining of tissues confirmed this (see Figs. 3–6). Specificity of the Dmerlin antiserum was ensured by selecting the unique COOH-terminal half of the protein to use as an immunogen, and is supported by immunoblot analysis of nontransfected versus transfected S2 cells (data not shown).

Using these specific antisera, we then performed immunolocalization experiments on stages that represented ovarian, embryonic, and imaginal development. During *Drosophila* oogenesis, Dmerlin and Dmoesin displayed strikingly different tissue distributions; this distinction was clearly observed as early as the germarium, the location of the germline stem cells (Fig. 3, A and B insets). Dmerlin was expressed predominantly in the germline, while Dmoesin was expressed at greater levels in the follicle cells (Fig. 3, A and B). In addition, Dmerlin expression became enhanced in the developing oocyte at approximately stage 6 of development and persisted until the end of oogenesis. Lower levels of Dmerlin expression were also detected at the apical ends of the follicle cells at stage 10 late in oogenesis (data not shown). In contrast, Dmoesin expression was found at the apical and basolateral ends of the follicular epithelium, although some expression was detected in the germline of early egg chambers (Fig. 3 B, S2–4) and in the nurse cells at stage 10 (data not shown). Fully developed oocytes (stage 14) clearly displayed membrane associated Dmerlin, while no Dmoesin expression was detected at this stage (data not shown).

In contrast to the largely complementary pattern observed during oogenesis, Dmerlin and Dmoesin appeared to be coexpressed in most cells during embryogenesis. Dmerlin and Dmoesin were present from cellularization (Fig. 5, A and B) throughout embryonic development (Fig. 3, C–F). Dmerlin expression was found to be enhanced in

the early mesoderm of the germband extended embryo (Fig. 3 C), whereas Dmoesin was expressed uniformly throughout the embryo at this stage (Fig. 3 D). Late in embryogenesis, both proteins were expressed ubiquitously throughout the tissues of the embryo, including the epidermis, salivary glands, foregut, midgut, hindgut, and the embryonic nervous system (Fig. 3, E and F). At this stage, Dmerlin expression was enhanced in the midgut (Fig. 3 E).

The Developing Nervous System

Given that loss of merlin function in humans is correlated with neuronally associated tumors, we examined the localization patterns of Dmerlin and Dmoesin in tissues of the developing central and peripheral nervous systems (Fig. 4). In the embryonic central nervous system (stage 15), Dmerlin and Dmoesin staining was detected in the neuropil, a structure composed of the developing axonal bundles of the ventral nerve cord (Fig. 4, A and B), and in the developing brain (Fig. 3, E and F). Dmerlin and Dmoesin localization was also observed in the neuronal cell bodies of the central nervous system (Fig. 4 B), with Dmoesin expression enhanced in these cells.

The neurons of the embryonic peripheral nervous system develop from specialized regions within the epidermis, the proneural clusters, during stage 11 of embryogenesis. The cell bodies of the differentiated bipolar sensory neurons can be observed in a regular pattern within the epidermis of a stage 17 embryo (Fig. 4, C and D). Dmoesin was enriched at the membranes of these cells at this stage (Fig. 4 D), whereas Dmerlin was found to localize to an intensely staining spot within the cell body (Fig. 4 C).

Differentiation of the photoreceptor neurons in the developing eye takes place within the eye imaginal disc of the third instar larva. In this tissue, Dmerlin staining was enhanced at the region of the morphogenetic furrow, the location within the eye disc where differentiation of the photoreceptors is initiated (Fig. 4 E), and was uniform throughout the rest of the eye epithelium. In contrast, Dmoesin was found to be primarily associated with the membranes of the developing photoreceptors posterior to the furrow (data not shown).

By 24 h after pupariation, the photoreceptors of the developing eye have differentiated and are covered apically by four cone cells and two primary pigment cells (Fig. 4, G and H). These structures are surrounded by secondary and tertiary pigment cells and together compose one ommatidium, the *Drosophila* unit eye (Wolff and Ready, 1993). Interspersed at regular intervals between the outer pigment cells are the bristle precursor cells, which will give rise to the mechanosensory bristles of the adult eye. Apically in this tissue, Dmoesin was localized at the membranes of the cone cells, secondary and tertiary pigment cells, and in the bristle precursor cells (Fig. 4 H). In contrast, Dmerlin was localized primarily in the cytoplasm of the secondary and tertiary pigment cells, and was greatly enhanced in the bristle precursor cells (Fig. 4 G). Little staining was detected in the cone cells. At a more basal focal plane within the disc, both Dmerlin and Dmoesin were more intensely expressed in the center of each ommatidium. This corresponds to the region of the rhabdomeres, the photosensitive microvilli of the photoreceptors, which project into

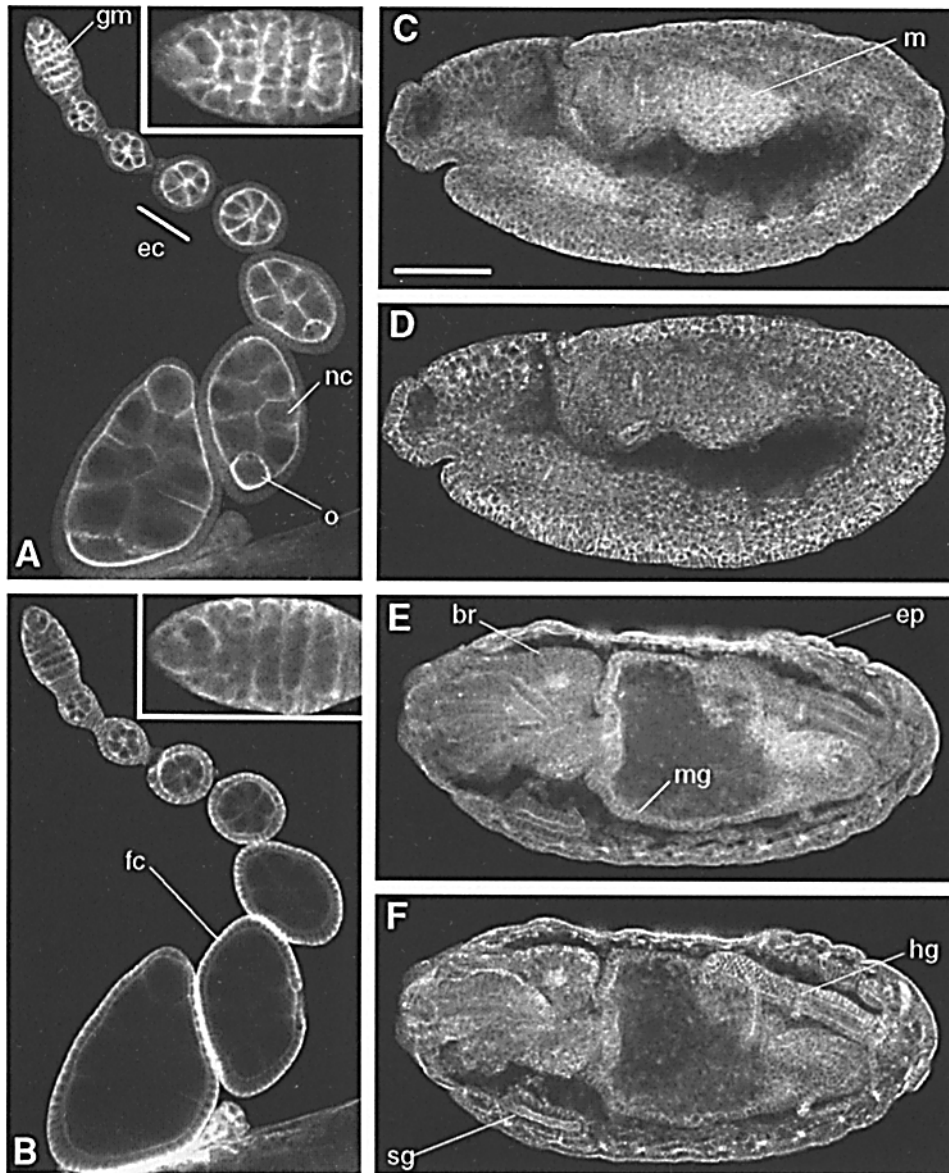


Figure 3. Expression of Dmerlin and Dmoesin during oogenesis and embryogenesis. Developing ovarioles and embryos were costained with the following antibodies: *A*, *C*, and *E*, guinea pig α -Dmerlin pAb; *B*, mouse α -Dmoesin mAb; *D* and *F*, mouse α -Dmoesin pAb. In *A* and *B*, anterior is at the top, and in *C*–*F*, anterior is to the left. During *Drosophila* oogenesis, egg chambers (*ec*), consisting of 15 germline-derived nurse cells (*nc*) and one presumptive oocyte (*o*), surrounded by the somatic follicular epithelium (*fc*), are pinched off from the germarium (*gm*) and move posteriorly as they develop. Therefore, as shown in *A* and *B*, the developmental stages of the oocyte can be simultaneously observed in a single ovariole. Dmerlin is localized primarily in the germ line of each ovariole as observed in the germarium, and in the nurse cells and the developing oocyte of later egg chambers (*A*). In contrast, Dmoesin is expressed primarily in the somatically derived follicle cells surrounding each egg chamber (*B*). Both proteins are expressed ubiquitously during embryogenesis (*C*–*F*), although Dmerlin expression is enhanced in the mesoderm (*m*) of the germband-extended embryo (*C*) and in the midgut epithelium (*mg*) of the stage 15 embryo (*E*). Dmerlin and Dmoesin are localized in late embryonic tissues such as the brain (*br*), epidermis (*ep*), hindgut (*hg*), and salivary glands (*sg*). Bar, 40 μ m (*A* and *B*), 70 μ m (*C*–*F*).

the center of each ommatidium (Fig. 4, *I* and *J*). At this focal plane, the contrast between the punctate Dmerlin staining and the membrane-associated staining of Dmoesin in the pigment cells was also apparent.

The Subcellular Localization of Dmerlin and Dmoesin

Although the tissues that expressed these ERM proteins were largely overlapping, the subcellular localizations of Dmerlin and Dmoesin within embryonic and imaginal tissues were distinct (Figs. 4, *G*–*J*, and 5). Generally, Dmoesin appeared to be associated with the plasma membranes in a continuous pattern (Fig. 5, *B*, *D*, and *F*). In contrast, Dmerlin was localized in punctate structures associated with the plasma membrane and within the cytoplasm (Fig. 5, *A*, *C*, and *E*). As a control for fixation artifact, embryos

were fixed using the heat/methanol method (Miller et al., 1989) before immunolocalization. These embryos displayed the same pattern of Dmerlin and Dmoesin subcellular localization as did embryos that were fixed with paraformaldehyde (data not shown).

In polarized epithelia, such as the embryonic hindgut, salivary gland, or the imaginal disc, both Dmoesin and Dmerlin were found in the highest concentration in the most apical part of the cell. Colocalization experiments in wing imaginal discs with *Drosophila* Armadillo (Peifer, 1993), a β -catenin homologue, demonstrated that at least part of the detected Dmerlin and Dmoesin protein was associated with the adherens junction (data not shown). In addition, Dmoesin was localized to an apical cap in imaginal disc cells (Fig. 5 *F*), a region known to contain abundant microvilli (Poody and Schneiderman, 1970). Exami-

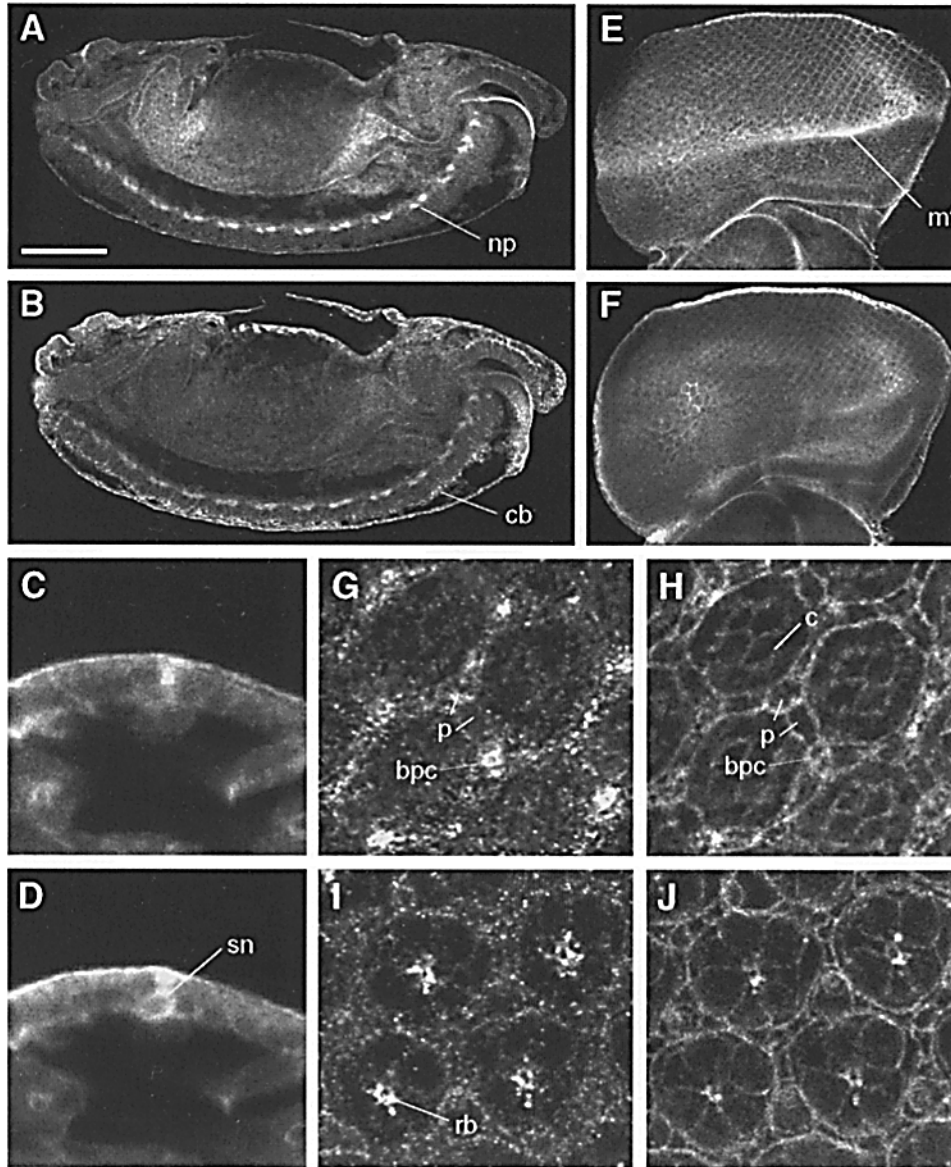


Figure 4. Dmerlin and Dmoesin are expressed in the developing central and peripheral nervous systems. Embryonic and imaginal tissues were double labeled using the guinea pig α -Dmerlin pAb (A, C, E, G, and I) and mouse α -Dmoesin pAb (B, D, F, H, and J). In A and B, both Dmerlin and Dmoesin antibodies stain the neuropil (np), the developing axonal bundles of the ventral nerve cord, in stage 16 embryos. Dmoesin expression is enhanced in the cell bodies (cb) of the developing central nervous system. In the embryonic peripheral nervous system of the stage 17 embryo, Dmerlin and Dmoesin are found associated with the bipolar sensory neurons (sn) (C and D). In the third instar eye imaginal disc (E and F), Dmerlin localization is enhanced in the morphogenetic furrow (mf). A tangential section of the apical-most plane of the pupal eye imaginal disc, ~ 24 h after pupariation, demonstrates that Dmerlin is localized in the secondary and tertiary pigment cells (p) and the bristle precursor cells (bpc) (G). At this focal plane, Dmoesin is found in the secondary and tertiary pigment cells and the cone cells (c) (H). At a focal plane deeper within the imaginal disc, both the α -Dmerlin and the α -Dmoesin antibodies (I and J) stain the center of each ommatidium, in the region of the photoreceptor rhabdomeres (rb). Bar, 70 μ m (A and B), 10 μ m (C and D), 50 μ m (E and F), or 6 μ m (G–J).

nation of the microvilli present during cellularization in the preblastoderm embryo (Foe et al., 1993) revealed that both Dmoesin and filamentous actin were localized in these structures (Fig. 5, G and H). Their patterns of localization were overlapping but not entirely identical; Dmoesin staining represented a subset of actin-staining structures. In contrast, Dmerlin did not colocalize with actin or Dmoesin in the apical buds (Fig. 5 I).

Dmerlin Is Associated with Endocytic Compartments in S2 Cells

The distinct subcellular distributions of Dmerlin and Dmoesin in embryonic and imaginal tissues prompted us

to examine their distributions in S2 cultured cells, an experimentally manipulable system. In these experiments, cells were transfected with inducible cDNA constructs bearing the full-length coding regions of either Dmerlin or Dmoesin.

1 h after continuous Dmerlin expression was induced under the metallothionein promoter, we observed that the protein was diffusely distributed throughout the cytoplasm and localized to the plasma membrane (Fig. 6 A). After 3 h of expression, however, Dmerlin was found to be associated with intracellular punctate structures in addition to its plasma membrane localization (Fig. 6 B). Subsequently, within the cell, we observed association with large, cytoplasmic structures that had a distinctly vesicular appear-

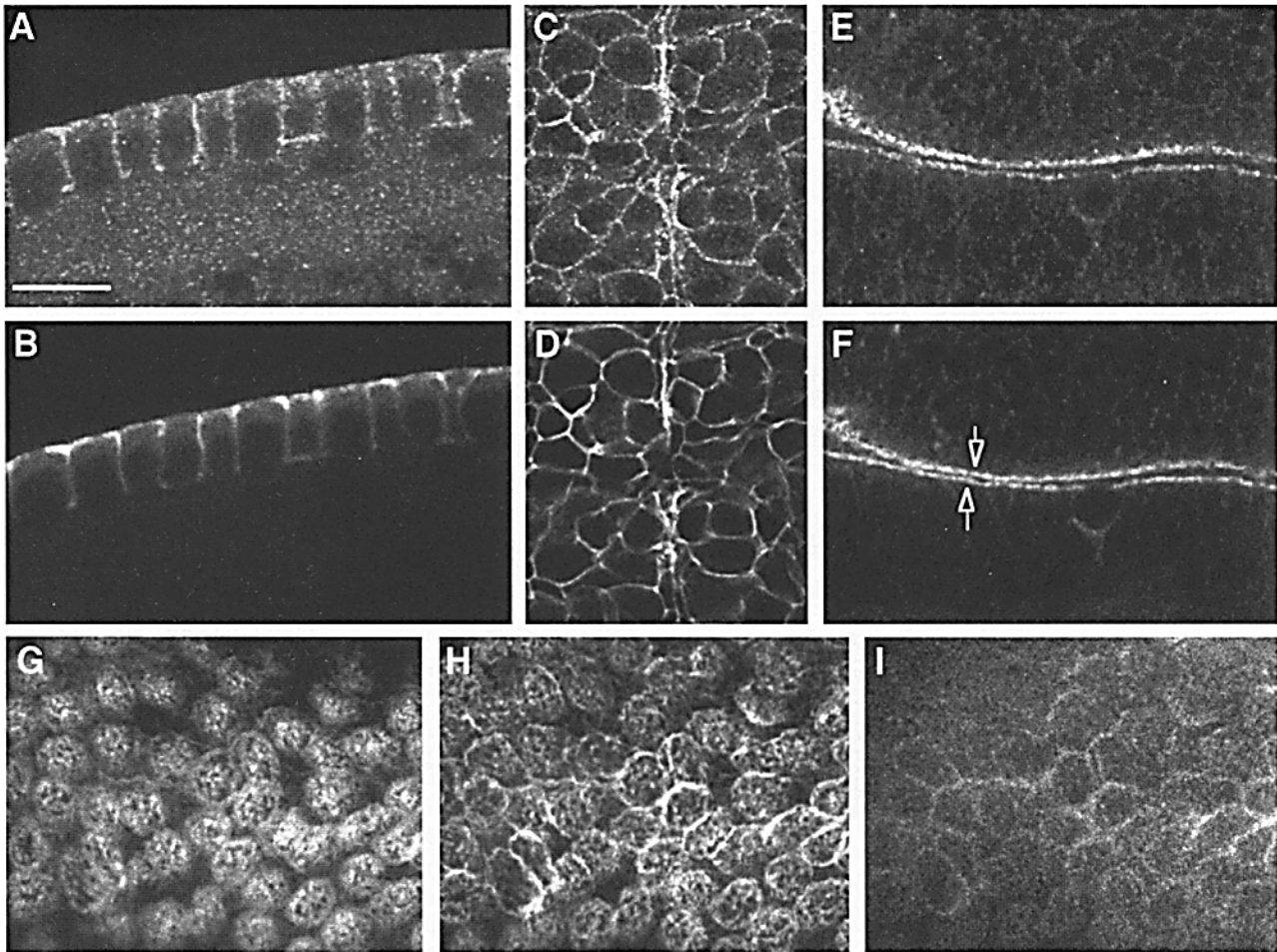


Figure 5. Dmerlin and Dmoesin display distinct subcellular distributions in embryonic and imaginal cells. In *A–F*, embryos were double labeled with guinea pig α -Dmerlin pAb (*A*, *C*, and *E*) and either mouse α -Dmoesin mAb (*B* and *F*) or pAb (*D*). In *G–I*, cellularizing embryos were triple labeled with rhodamine phalloidin (*G*) to label F-actin, the mouse α -Dmoesin mAb (*H*), and the guinea pig α -Dmerlin pAb (*I*). In all tissues, Dmoesin is uniformly distributed along the plasma membrane, while Dmerlin shows a much more punctate appearance. Confocal optical sections are presented through an embryo undergoing cellularization (*A* and *B*) and along the ventral midline of a germband-extended embryo (*C* and *D*). Oblique sections through the wing imaginal epithelium are shown in *E* and *F*. The arrows in *F* mark the apical membranes of these cells, which are apposed in this highly folded epithelial tissue. In *G–I*, a tangential section through the surface of a cellularizing embryo displays the microvilli in the apical buds. Bar, 12 μ m (*A* and *B*), 16 μ m (*C* and *D*), 10 μ m (*E* and *F*), and 15 μ m (*G–I*).

ance (Fig. 6, *C* and *D*). Cells induced to express lower levels of Dmerlin protein displayed punctate cytoplasmic staining over a similar time course (data not shown), indicating that the observed changes in Dmerlin localization were not a consequence of exceeding a threshold concentration of protein. If that were the case, then we would expect that cells expressing lower levels of Dmerlin protein would display changes in its localization over a delayed time course.

Dmoesin is endogenously expressed at high levels at the plasma membrane of S2 cells (Fig. 6 *E*). When cells were induced to overexpress Dmoesin (Fig. 6 *F*), the protein was found to localize primarily to the membrane and was also observed in the cytoplasm. Expression of Dmoesin at high levels, however, never produced the punctate cytoplasmic localization or the association with large vesicular structures, which are characteristic of Dmerlin's distribution. The cytoplasmic staining we observed for Dmoesin

can likely be attributed to a combination of synthesis, and a saturation of the plasma membrane for this protein.

The punctate, vesicular appearance of Dmerlin in tissues and cells (Figs. 5 and 6), together with its apparent progression from the cell membrane into the cytoplasm in S2 cells (Fig. 6, *A–D*), suggest that Dmerlin may be associated with membrane internalized from the cell surface. To test this hypothesis, we asked if Dmerlin expressed in S2 cells is associated with endocytic structures labeled by fluorescent dextran uptake (Swanson, 1989). We observed a striking similarity in the patterns of fluorescent dextran-filled endocytic compartments and Dmerlin punctate structures (Fig. 6, *G–L*). Most often, Dmerlin protein appeared to partially surround dextran-labeled structures (Fig. 6, *I* and *L*). Such a localization would be expected for a cytoplasmic protein that is closely associated with a membrane-bound endocytic compartment. Similar results were obtained with cells that were transfected with a metal-

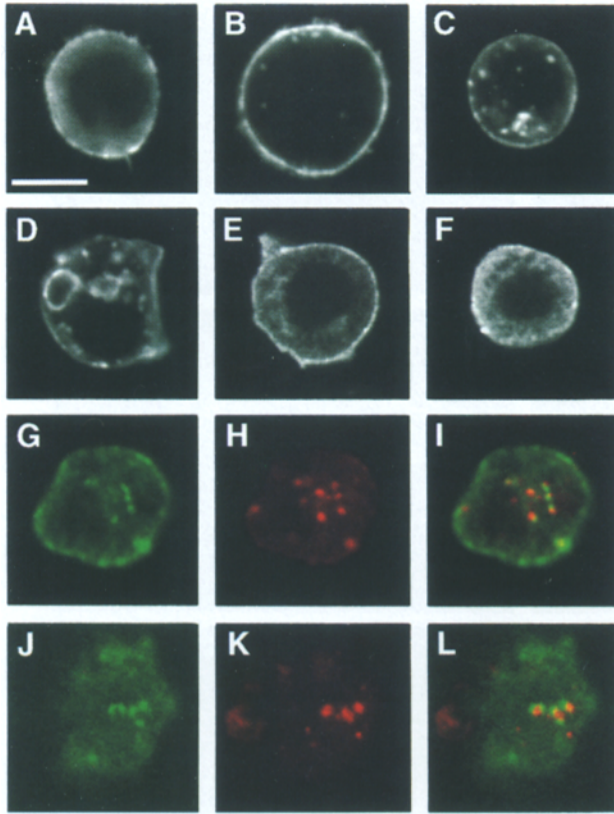


Figure 6. Expressed Dmerlin is associated with endocytic structures in cultured cells. S2 cells were stained with either the mouse α -Dmerlin pAb (A–D and G–L) or the mouse α -Dmoesin pAb (E and F). Cells were fixed and stained 1 h (A), 3 h (B), 5 h (C), or 7.5 h (D) after induction of expression of the Dmerlin cDNA under the control of the metallothionein promoter with 0.7 mM copper sulfate. The endogenous distribution of Dmoesin in S2 cells is displayed in E, and the pattern of localization after expression of the hs-Dmoesin cDNA construct in F. In G–J, the localization of Dmerlin was examined in S2 cells after the induction of the hs-Dmerlin cDNA construct and labeling with a dextran marker for fluid phase endocytosis. Dmerlin protein is displayed in G and J, while the endocytic compartments labeled with dextran are shown in H and K. These images are merged together to examine the relative localization of Dmerlin to endocytic structures in I and L. Bar, 5 μ m.

lothionein promoter construct, indicating that the observed colocalization was not a result of heat shock (data not shown).

Discussion

To understand more about the functions of ERM family members, particularly in developing tissues, we have made use of specific antibodies together with the confocal microscope to study cellular and subcellular expression patterns of two *Drosophila* members of this complex gene family, Dmerlin and Dmoesin. In particular, we wanted to determine the extent to which the NF2 tumor suppressor merlin functions analogously to the other family members, ezrin, radixin, and moesin. If they do function analogously, then we would expect them to display similar tissue and subcellular localizations in expressing cells. Instead, in

cells that express both proteins, we find that Dmoesin and Dmerlin display strikingly different subcellular localizations (Fig. 5). In particular, while both proteins are found in the apical domain of polarized epithelial cells, Dmoesin displays a uniform, membrane-associated distribution, while Dmerlin is localized in a punctate pattern. Similarly distinct subcellular patterns are observed in the cellular blastoderm, the embryonic epidermis, and the pupal eye. Furthermore, Dmoesin, but not Dmerlin, colocalizes with actin in microvilli in the cellularizing embryo (Fig. 5, G–I). While most tissues express both proteins, in some cases, such as the ovary and in some tissues of the nervous system, we find a complementary pattern of expression (Figs. 3 and 4). Taken together, these observations of distinct expression patterns support the notion that Dmerlin and Dmoesin serve distinct cellular functions.

The cellular functions of the ERM family in vertebrates are thought to be performed in two specific cellular domains: the microvilli, where ezrin is a component (Bretscher, 1983), and the adherens junction, from which radixin was first isolated (Tsukita et al., 1989). Vertebrate moesin has been observed in both the microvilli and the adherens junction (Sato et al., 1992; Takeuchi et al., 1994), although its presence in the adherens junction has been questioned (Berryman et al., 1993; Franck et al., 1993). In subsequent studies, it has been shown that ezrin and radixin, with moesin, are necessary for the formation and/or maintenance of microvilli in cultured cells (Takeuchi et al., 1994). Similarly, all three seem to play largely redundant roles in cell–cell and cell–substrate adhesion (Takeuchi et al., 1994). The expression pattern that we observe for Dmoesin in microvilli and in the region of the adherens junction suggests that it may function in both of these cellular domains. Interestingly, so far, we have detected only a single ERM family member (excluding Dmerlin) in *Drosophila*, despite sequencing multiple independent subclones of the degenerate PCR product, suggesting that Dmoesin is the only ERM family member in *Drosophila*. Dmoesin may therefore serve all of the functions that are provided by the combination of ezrin, radixin, and moesin in vertebrate tissues. Thus, it may be possible to study ERM function in *Drosophila* in the absence of the functional redundancy and antibody cross-reactivity that have plagued previous studies.

Several observations regarding the Dmoesin sequence and localization suggest that, like other ERM proteins, it may bind to filamentous actin. Of particular note is a region of ~ 35 amino acids at the very COOH terminus of Dmoesin (Fig. 1, *underlined sequence*), a sequence that is highly conserved in vertebrate moesin, radixin, and ezrin. In ezrin, this region has been shown to bind filamentous actin (Turunen et al., 1994), suggesting that the COOH terminus of Dmoesin may serve a similar function. In addition, we find that there is a striking colocalization of F-actin and Dmoesin in the apical buds of the precellularized embryo (Fig. 5, G and H). Interestingly, the other localizations that we have noted for Dmoesin are also similar to those of filamentous actin, namely in the region of the adherens junctions, the apical membrane, and associated with the basolateral membrane (Fehon et al., 1994). Finally, a previous study provides evidence that the COOH-terminal half of Dmoesin interacts with cytoskeletal actin

when expressed in yeast (Edwards et al., 1994). Taken together, these results suggest that Dmoesin binds cytoskeletal actin and functions similarly to the vertebrate ERM proteins.

The question of functional redundancy between members of the ERM family has been further complicated by the discovery of merlin, the NF2 tumor suppressor, as a fourth member of this group. While its tumor suppressor phenotype clearly suggests that it performs functions distinct from the other family members, its functional similarities with and differences from the other ERM proteins remain unclear. The identification of two ERM family members, Dmoesin and Dmerlin in *Drosophila*, together with the production of specific antibody reagents for each, has allowed us to compare the cellular and subcellular distributions of Dmerlin to those of Dmoesin in some detail. Unlike Dmoesin or any other ERM family member, we find that Dmerlin localizes to punctate structures that are found adjacent to the apical membrane and within the cytoplasm. The punctate pattern of Dmerlin staining we have observed has not been noted for filamentous actin, nor have double labeling experiments revealed any strong correlation between these proteins (Fig. 5 D). In addition, the COOH termini of both human and *Drosophila* merlin are divergent from the other ERM proteins in the region of the putative actin-binding domain (Fig. 1, blue shading in the COOH-terminal region). This suggests that merlin does not interact with cytoskeletal actin in the same way that has been proposed for other members of the ERM family.

We have shown that when Dmerlin is expressed in cultured cells, it appears to traffic from the plasma membrane to the cytoplasm, and is associated with endocytic compartments marked by fluorescent dextran taken up from the surrounding medium (Fig. 6). In addition, the developing oocyte, which is more actively involved in endocytosis (DiMario and Mahowald, 1987), expresses increased levels of Dmerlin relative to the adjacent nurse cells (Fig. 3 A). However, this accumulation (stage 6) occurs before the increase in endocytosis corresponding with yolk protein uptake in the oocyte (stage 8; DiMario and Mahowald, 1986). While additional experiments, including a genetic analysis of *Dmerlin* function, will be required to rigorously define the cellular functions of Dmerlin, these results, taken together with the unique punctate cytoplasmic localization that we have observed in a variety of tissues, suggest a role for Dmerlin in endocytosis. Unfortunately, similar studies have not yet been performed for human merlin, so it is not known whether its behavior is similar to that of Dmerlin. However, a role in endocytosis may be consistent with the role in signal transduction processes suggested by human merlin's tumor suppressor function (Rouleau et al., 1993; Trofatter et al., 1993). For example, crippled EGF receptors, incapable of internalization via endocytosis, have been shown to produce a transformed phenotype because activated receptor-ligand complexes cannot be cleared from the cell surface (Wells et al., 1990). In addition, *Drosophila shibire*, a homologue of vertebrate dynamin that is required for normal endocytosis (van der Bliek and Meyerowitz, 1991), displays phenotypes that are similar to those of mutations in the Notch-receptor signaling pathway (Poodry, 1990). Given this connection, it is intriguing

to note that many of the transmembrane receptors involved in cell-cell interactions are localized to the same apical membrane domain where we find Dmerlin (Banerjee et al., 1987; Fehon et al., 1991; Zak and Shilo, 1992).

While the exact cellular functions of Dmerlin and Dmoesin remain to be elucidated, our results to date strongly suggest that Dmoesin is functionally analogous to vertebrate ERM proteins, while Dmerlin is a functionally distinct ERM family member. In particular, taking together all the available evidence, we propose that merlin plays a unique role in the regulation of cellular growth and signaling, possibly by functioning in endocytic processes. The molecular genetic tools available in *Drosophila*, such as mutational analysis, should allow us to define more precisely the different functions of these ERM family members. In addition, such studies may also allow for the development of a *Drosophila* model for the NF2 disease. By examining primary phenotypes and genetic interactions between *Dmerlin* mutations and mutations in members of a variety of signaling pathways also present in vertebrates, we hope to identify the cellular pathways in which merlin functions.

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