

The *Neurofibromatosis-2* homologue, *Merlin*, and the tumor suppressor *expanded* function together in *Drosophila* to regulate cell proliferation and differentiation

Brooke M. McCartney*, Rima M. Kulikauskas, Dennis R. LaJeunesse and Richard G. Fehon†

Developmental, Cell and Molecular Biology Group, Duke University, Durham, NC 27708-1000, USA

*Present address: Lineberger Comprehensive Cancer Center, Department of Biology, CB#3280 Coker Hall, University of North Carolina, Chapel Hill, NC 27599-3280, USA

†Author for correspondence (e-mail: rfehon@duke.edu)

Accepted 20 December 1999; published on WWW 21 February 2000

SUMMARY

Neurofibromatosis-2 is an inherited disorder characterized by the development of benign schwannomas and other Schwann-cell-derived tumors associated with the central nervous system. The *Neurofibromatosis-2* tumor suppressor gene encodes Merlin, a member of the Protein 4.1 superfamily most closely related to Ezrin, Radixin and Moesin. This discovery suggested a novel function for Protein 4.1 family members in the regulation of cell proliferation; proteins in this family were previously thought to function primarily to link transmembrane proteins to underlying cortical actin. To understand the basic cellular functions of Merlin, we are investigating a *Drosophila Neurofibromatosis-2* homologue, *Merlin*. Loss of *Merlin* function in *Drosophila* results in hyperplasia of the affected tissue without significant disruptions in differentiation. Similar phenotypes have been observed for mutations in another Protein 4.1 superfamily member in *Drosophila*, *expanded*. Because of the phenotypic and structural similarities between *Merlin* and *expanded*, we asked whether Merlin and Expanded function together to regulate cell proliferation. In this study, we demonstrate

that recessive loss of function of either *Merlin* or *expanded* can dominantly enhance the phenotypes associated with mutations in the other. Consistent with this genetic interaction, we determined that Merlin and Expanded colocalize in *Drosophila* tissues and cells, and physically interact through a conserved N-terminal region of Expanded, characteristic of the Protein 4.1 family, and the C-terminal domain of Merlin. Loss of function of both *Merlin* and *expanded* in clones revealed that these proteins function to regulate differentiation in addition to proliferation in *Drosophila*. Further genetic analyses suggest a role for Merlin and Expanded specifically in Decapentaplegic-mediated differentiation events. These results indicate that Merlin and Expanded function together to regulate proliferation and differentiation, and have implications for understanding the functions of other Protein 4.1 superfamily members.

Key words: Protein 4.1, ERM, FERM domain, Apical membrane, *Neurofibromatosis-2*, *Merlin*, *expanded*, *Drosophila melanogaster*

INTRODUCTION

The regulation of cell proliferation is central to controlling the size and shape of multicellular organisms. Disruption of proliferation control in an organism can have severe consequences; improper activation of cell proliferation can result in the formation of tumors and the development of cancer. Many genes involved directly with the control of the cell cycle, and those involved more indirectly with the control of proliferation, have been identified as oncogenes and tumor suppressors. Among the known tumor suppressor genes, the *Neurofibromatosis 2* (*NF2*) gene is particularly intriguing. *NF2* is an inherited disorder characterized by the formation of benign, Schwann-cell-derived tumors on the eighth cranial nerve, and other benign tumors associated with the central nervous system (Martuza and Eldridge, 1988). The *NF2* gene

encodes a novel member of the Protein 4.1 superfamily, Merlin, that is most highly related to the ERM (Ezrin, Radixin, Moesin) subgroup within the family (Gusella et al., 1999). Members of the Protein 4.1 superfamily all share a conserved region of ~350 amino acids known as the conserved N-terminal region (CNTR) or FERM domain (Chishti et al., 1998; Ward et al., 1998). Some members of the family, including the ERM proteins, interact with transmembrane binding partners through the CNTR and localize them to specific membrane domains via direct interactions between their C-terminal domain and the actin cytoskeleton (Mangeat et al., 1999).

Merlin shares significant sequence identity with ERM proteins, particularly in the CNTR. The C-terminal half of Merlin also contains a predicted coiled-coil domain, but lacks the actin-binding domain found at the C terminus of ERM proteins. Nonetheless, Merlin has been shown to interact with

the actin cytoskeleton through an N-terminal binding region (Xu and Gutmann, 1998). Consistent with this observation, overexpression of Merlin in vertebrate cultured cells causes transient alterations in the F-actin cytoskeleton (Gutmann et al., 1999). Work from several laboratories indicates that Merlin can form homotypic dimers, and heterotypic dimers with ERM proteins, but the functional significance of these interactions is not known (Gronholm et al., 1999). Other Merlin binding partners include NHE3-RF, the Na⁺/H⁺ exchanger regulatory factor that also binds to ERM proteins (Reczek et al., 1997; Murthy et al., 1998), and β II-spectrin (Scoles et al., 1998). Although transmembrane partners for Merlin have not been identified yet, the hyaluronin receptor CD44, the adhesion molecule ICAM-2, the cystic fibrosis transmembrane conductance regulator and the B-adrenergic receptor have been shown to bind ERM proteins directly or indirectly (Tsukita et al., 1994; Hall et al., 1998; Heiska et al., 1998). In addition, recent studies indicate that ERM binding is essential for proper recycling of the B-adrenergic receptor, and thereby regulation of its activity (Cao et al., 1999). Thus, it seems that ERM proteins and perhaps Merlin may bind to and regulate the functions of a number of transmembrane proteins. Nonetheless, it is not yet clear why loss of Merlin function results in misregulation of proliferation.

The discovery that a member of the Protein 4.1 superfamily has a tumor suppressor function was initially surprising because its members traditionally have been considered to play a structural role in linking transmembrane proteins to the cytoskeleton. However, another family member, *Drosophila expanded* (*ex*), has also been shown to have a tumor suppressor function; loss of *ex* function can lead to the development of hyperplastic imaginal discs and overgrown adult structures such as the wing (hence the name expanded; Boedigheimer and Laughon, 1993). Expanded has a divergent CNTR and a C-terminal region that contains a putative coiled-coil domain and three potential SH3-binding domains (Boedigheimer et al., 1993). Like *NF2*, *ex* seems to function in regulating proliferation, and loss of its function does not appear to significantly disrupt patterning of the affected imaginal discs.

We have identified and characterized an *NF2* gene homologue in *Drosophila* called *Merlin* (*Mer*) and shown that it functions similarly to *NF2* in regulating cell proliferation (McCartney and Fehon, 1996; LaJeunesse et al., 1998). To understand its cellular roles, we have begun to investigate the molecular framework in which *Drosophila* Merlin functions by identifying interacting genes and proteins. Given their phenotypic and structural similarities, and previous evidence that other family members can interact heterotypically, we were interested in the possibility that Merlin and Expanded might function together to regulate proliferation during *Drosophila* development. In this study, we show that *Merlin* and *expanded* display dose-sensitive interactions that suggest physical interactions between the proteins that they encode. Cell culture and blot overlay assays indicate that Merlin and Expanded interact directly via the CNTR of Expanded and the C-terminal domain of Merlin. Removal of all Merlin and Expanded function in double mutant clones results in severe differentiation defects that are not observed with either mutation alone. Genetic interactions between *Mer*, *ex* and *decapentaplegic* (*dpp*) are consistent with the notion that

Merlin and Expanded positively regulate DPP function. Taken together, our results indicate that Merlin and Expanded function cooperatively in regulating cell proliferation and differentiation in developing epithelial cells.

METHODS AND MATERIALS

Drosophila stocks, genetic analysis and immunolocalization

All *Drosophila* cultures were maintained on standard corn meal, yeast, molasses and agar medium. The *Mer* alleles used in this study were described previously (Fehon et al., 1997; LaJeunesse et al., 1998). *ex* alleles were a gift from M. Boedigheimer and P. Bryant. K. Wharton provided the *dpp^{hr56}* allele used in this study and the *dpp-lacZ* (B3.0) stock was obtained from the Bloomington *Drosophila* stock center. Whole-mount preparation and analysis of adult eyes, wings and legs were described previously (LaJeunesse et al., 1998). The distance between wing hairs as a measure of cell size was determined using the measure function in NIH Image. Scanning electron microscopy was performed as described previously (Rebay et al., 1993). Somatic mosaic clones of *Mer^f* were generated at 36 hours after egg laying (AEL) using the FRT/FLP system (Xu and Rubin, 1993). *Mer⁻,ex⁻* mutant clones were generated by gamma irradiating (1100 rad) *w¹¹¹⁸ sn³ Mer^f/w¹¹¹⁸ sn³ Mer^f; P{w⁺, UpMer⁺} ex⁺/ex^{e1}* larvae at 72 hours AEL. Immunolocalization was conducted as described (McCartney and Fehon, 1996) with the following antibodies: anti-Merlin (1:7500 dilution; McCartney and Fehon, 1996), anti-Expanded (1:500, provided by M. Boedigheimer and P. Bryant), anti- β -gal (1:1000, Promega), anti-ELAV (1:500, provided by I. Rebay and G. Rubin)

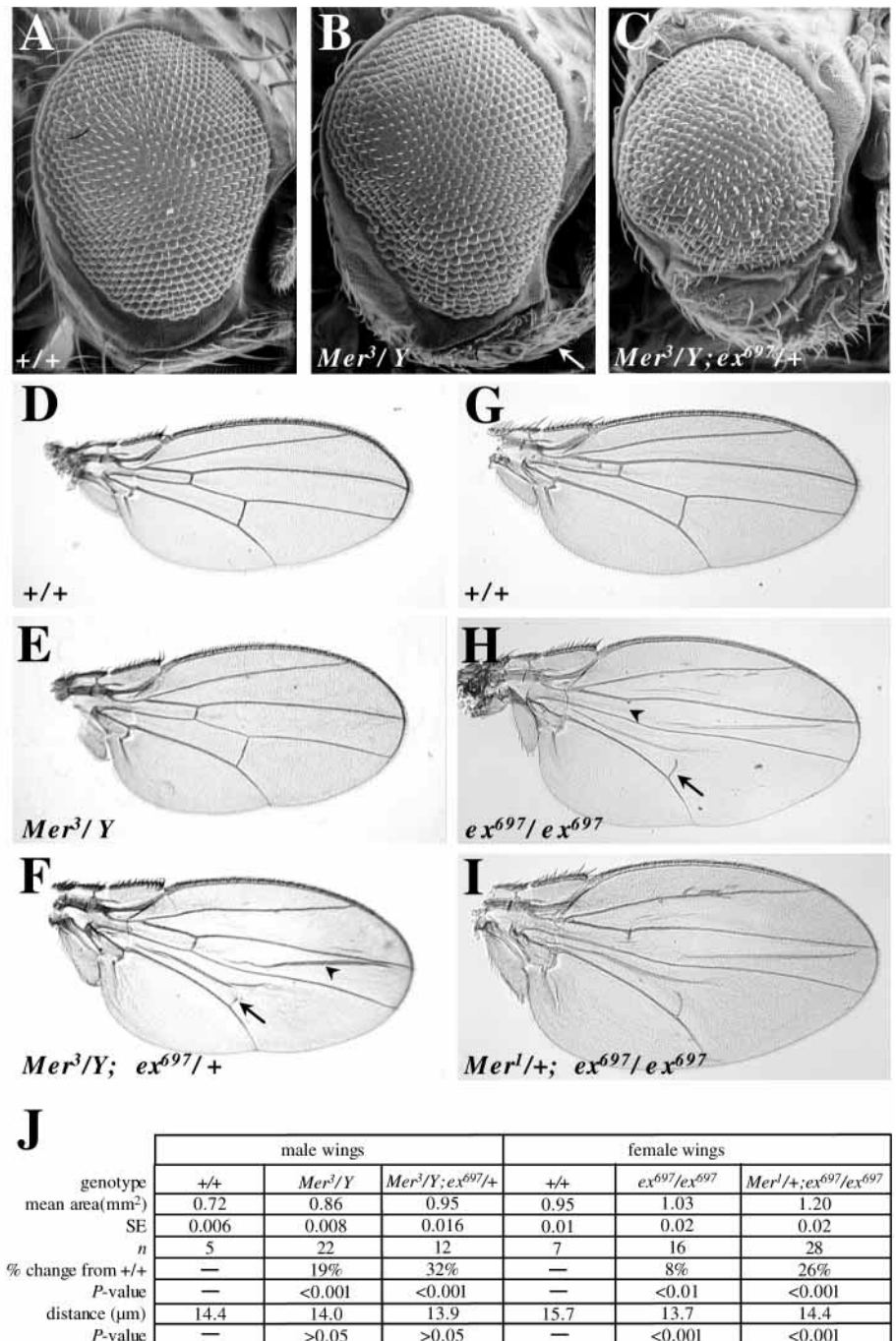
Expression in Schneider-2 cells

Schneider-2 (S2) cell maintenance, transfection and fluorescent staining were performed as described previously (Fehon et al., 1990). Full-length Expanded (EX¹⁻¹⁴²⁹, EX⁺) and an N-terminal Expanded fragment (EX¹⁻³⁹⁹; both provided by M. Boedigheimer and P. Bryant) were subcloned into the *MetallothioneinA* promoter vector pRmHa3 (Bunch et al., 1988). Three additional Expanded C-terminal fragments were PCR amplified, Myc-tagged and subcloned into pRmHa3: EX⁷⁷³⁻¹⁴²⁹, EX⁷⁷³⁻¹⁰⁶⁵ and EX¹⁰⁶⁶⁻¹⁴²⁹. Sequencing confirmed that no PCR errors were introduced. Myc-tagged full-length (MER¹⁻⁶³⁵, MER⁺), N-terminal (MER¹⁻³⁴⁹) and C-terminal (MER³⁴⁵⁻⁶³⁵) Merlin fragments in pCaSpeR-hs were described previously (LaJeunesse et al., 1998). Expression of Expanded constructs was induced by addition of 0.7 mM CuSO₄ for a total of 12–16 hours. Expression of Merlin constructs was induced during this time by a 20 minute incubation at 38°C followed by 6 hours at 25°C. Cells were stained with anti-Expanded (1:500), anti-Merlin (1:2000) or anti-Myc 9c10 (1:100).

Blot overlay experiments

DNA fragments generated by PCR corresponding to MER¹⁻³⁰⁶, MER³⁴⁵⁻⁶³⁶, MER³⁵⁰⁻⁵⁹⁹, MER⁵²²⁻⁶³⁵, EX¹⁻³⁹⁹ and EX⁷⁷³⁻¹⁴²⁹ were subcloned into the GST fusion protein vector pGEX3 (Pharmacia). The MER³⁵⁰⁻⁵⁹⁹ fragment was cloned as an in-frame dimer and therefore contains two tandemly repeated copies of this portion of the protein. The MER¹⁻⁶³⁵ full-length construct was generated by cloning a *MunI* restriction fragment that contains the entire *Mer* coding sequence in frame into pGEX. Fusion proteins were expressed in BL21 cells and purified as described (Frorath et al., 1991). Purified proteins (30–100 μ g/ml in 50 mM NaHCO₃, pH 8) were labeled with the addition of 75 μ g/ml NHS-LC biotin (Pierce) for 2 hours on ice, followed by the addition of 50 mM glycine. Proteins were dialyzed at 4°C for 16 hours in 100 mM sodium phosphate pH 7.0/50% glycerol. Target proteins were also expressed as GST fusions. To

Fig. 1. *Merlin* and *expanded* interact genetically. (A-C) Scanning electron micrographs of adult eyes (anterior is to the right). (A) Wild-type eye. (B) Adults hemizygous for *Mer³* exhibit a weakly roughened, reduced eye with expansion of ventral and posterior head cuticle, and development of ectopic vibrissae, the bristles found ventral and anterior to the eye (arrow). (C) Recessive loss-of-function mutation *ex⁶⁹⁷* dominantly enhanced the small, roughened eye phenotype exhibited by *Mer³*. In addition, numerous interommatidial bristle duplications were observed. (D-F) Wings from adult male flies. (D) Wild type. (E) Hemizygotes for *Mer³* have broadened wing blades. They may also have disruptions of the posterior cross vein (not shown). (F) Recessive loss-of-function mutation *ex⁶⁹⁷* dominantly enhanced the *Mer³/Y* broad wing phenotype producing significantly larger wings that show an increased frequency of posterior cross-vein disruptions (arrow). Due to the size of the wing, the blade often folds during mounting (arrowhead) (G-I) Wings from adult female flies. (G) Wild type. (H) Adults homozygous for *ex⁶⁹⁷* display a broad wing phenotype characterized by disruption of the posterior cross vein (arrow) and the anterior cross vein (arrowhead). (I) Recessive loss-of-function mutation *Mer¹* dominantly enhanced the *ex⁶⁹⁷* broad wing phenotype producing a significantly larger wing. (J) Comparisons of mean wing area and cell size of genotypes shown in D-I. Note that wild-type male wings are smaller than wild-type female wings. By use of the *t*-test, the increases in wing area observed in the mutants were shown to be significant when compared to wild type ($P < 0.01$). Furthermore, the distance between wing hairs in wing compartment 4 was determined as a measure of cell size. These values indicate that the increase in wing area in the mutants is not due to an increase in cell size.



make crude cell lysates, overnight cultures were diluted 1:20 in LB with 100 μg/ml ampicillin and grown for 90 minutes at 37°C. Protein expression was induced with the addition of 2 mM IPTG for 3 hours at 15–18°C. Cells were then pelleted, resuspended in 1/4 volume SDS sample buffer and separated on 3–15% gradient acrylamide gels. Proteins were transferred onto nitrocellulose membranes. Blots were blocked for 30 minutes in 5–10% milk/TBS/0.1% Tween followed by sequential 1 hour incubations in 0.2 mg/ml avidin and 0.1 mg/ml biotin, respectively (the avidin and biotin blocks were omitted in some experiments). Blots were incubated with biotinylated probes at 0.15 to 3.0 μg/ml in 1–5% milk/1× TBS/0.1% Tween-20 for 12–16 hours at 4°C. Blots were then incubated with ExtrAvidin (Sigma) at 1:2000 in 5% milk/TBS/Tween for 1–2 hours at room temperature.

Binding was detected with SuperSignal chemiluminescent reagents (Pierce).

RESULTS

Merlin and *ex* display similar phenotypes and interact genetically

Hypomorphic alleles of either *Mer* (*Mer³*) or *ex* (*ex⁶⁹⁷*) result in very similar adult wing and eye phenotypes. In both cases, mutant adults displayed enlarged wings due to an increase in cell number rather than an increase in cell size (Fig. 1D,E,G,H,I). In fact, the cell size in the mutants appeared to

be slightly decreased (Fig. 1J). Furthermore, this expansion in wing area was often accompanied by the disruption or complete absence of the posterior cross vein (Fig. 1H arrow). In addition, the anterior cross vein was sometimes disrupted in *ex*⁶⁹⁷ adults (Fig. 1H, arrowhead). *Mer* and *ex* mutants have smaller, weakly roughened eyes (Fig. 1B; Boedigheimer and Laughon, 1993). Histological sections of *Mer*³ eyes revealed only minor perturbations in interommatidial organization and no obvious disruptions in ommatidial polarity (data not shown). Concomitant with the reduction in eye size was an apparent expansion of the ventral peripheral head cuticle and the development of ectopic vibrissae (Fig. 1B arrow). Although stronger alleles of both *Mer* (*Mer*¹, *Mer*², *Mer*⁴) and *ex* (*ex*^{el}, a null allele) result in lethality, *Mer* mutant larvae did not develop the hyperplastic discs characteristic of *ex* mutant larvae (data not shown; Boedigheimer and Laughon, 1993).

Dose-sensitive genetic interactions have been shown to be a reliable indicator of functional interactions between genes (Chang et al., 1994). Reduction of *ex* function in the *Mer*³ hemizygous background with either *ex*⁶⁹⁷ or *ex*^{el} resulted in an enhancement of the *Mer*³ head phenotypes (Fig. 1B,C); the adult eye was reduced in size, more ectopic head cuticle and vibrissae were observed, and the bristles normally found between ommatidia were often duplicated and disorganized (Fig. 1C). Those ommatidia that did form contained the normal complement of photoreceptors, however (data not shown). In addition, *Mer*³ wing area and the frequency of posterior cross vein disruptions increased when *ex* function was reduced (Fig. 1E,F,J arrow). Consistent with these observations, reduction of *Mer* function in *ex*⁶⁹⁷ mutants caused an increase in *ex*⁶⁹⁷ wing size (Fig. 1H-J).

Merlin and Expanded colocalize in *Drosophila* tissues and cultured cells

If the observed genetic interactions between *Mer* and *ex* reflect molecular interactions between the proteins that they encode, then the subcellular distribution of these proteins should be similar in expressing tissues. We have previously reported that Merlin is localized in a punctate distribution at the apical plasma membrane in the region of the adherens junction and in the cytoplasm (McCartney and Fehon, 1996), and Expanded has been reported to localize to the apical membrane domain (Boedigheimer and Laughon, 1993). To confirm that they are similarly distributed, we double labeled the two proteins in imaginal disc cells and found that the staining patterns were almost indistinguishable (Fig. 2A,B).

Given their clear colocalization, we asked whether the localization of Merlin in this specific distribution is dependent on the presence of Expanded. We examined Merlin in *ex*^{el} wing imaginal discs and did not find any significant alterations in Merlin localization (not shown). In a complementary experiment, we generated *Mer*⁴ mitotic clones at 36 hours AEL using the FRT/FLP system and examined Expanded localization in the *Mer* mutant tissue. Two notable results were observed. First, we detected no alteration in Expanded localization in the absence of Merlin protein, indicating that Expanded does not depend on Merlin for proper subcellular localization. Second, there was an apparent increase in the level of Expanded autonomously in the *Mer*⁴ tissue as compared with the heterozygous background (Fig. 2C,D arrows indicate

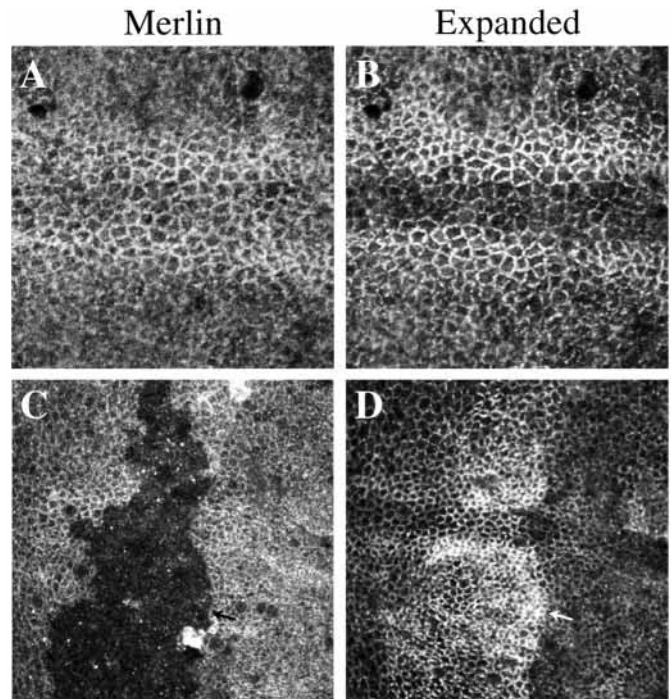


Fig. 2. Merlin and Expanded colocalize at the apical membrane domain in polarized epithelial cells. (A,B) Tangential confocal optical section of a wing imaginal disc from a wild-type third instar larva double labeled for Merlin (A) and Expanded (B) revealed that these proteins colocalize at the apical junctional domain in imaginal disc cells. Expanded appears to be less highly expressed at the presumptive wing margin (B). (C,D) Homozygous *Mer*⁴ mutant clones were induced 36 hours AEL and the wing imaginal discs from third instar larvae were double labeled for Merlin (C) and Expanded (D). A tangential confocal optical section through the apical surface of the disc reveals a clone of *Mer*⁴ mutant tissue identified by loss of Merlin labeling (C, arrow indicates the border of the clone). An apparent increased level of Expanded was observed in the *Mer*⁴ mutant tissue (D, arrow indicates the border of the clone).

a border of the clone). This observation indicates either that loss of Merlin results in increased Expanded expression or that an Expanded epitope is masked in the presence of Merlin protein, perhaps due to direct interactions between these proteins.

Although we did not detect interdependence between Merlin and Expanded for subcellular localization in tissues, it remained possible that these proteins physically interact. For example, each protein could localize to the apical junctional domain of intact tissues via interactions with other proteins in this region of the cell. To examine possible interactions between Merlin and Expanded, we cotransfected constructs expressing these proteins into cultured S2 cells that lack an apical junctional domain to more readily detect possible interactions between them. The localization of Merlin expressed in cultured cells follows a very specific pattern; Merlin is first found at the plasma membrane and is then trafficked into the cytoplasm where it is associated with endocytic compartments (McCartney and Fehon, 1996). Expanded is endogenously expressed at levels virtually undetectable by immunofluorescence in S2 cells (data not shown). When full-length Expanded was expressed in S2 cells,

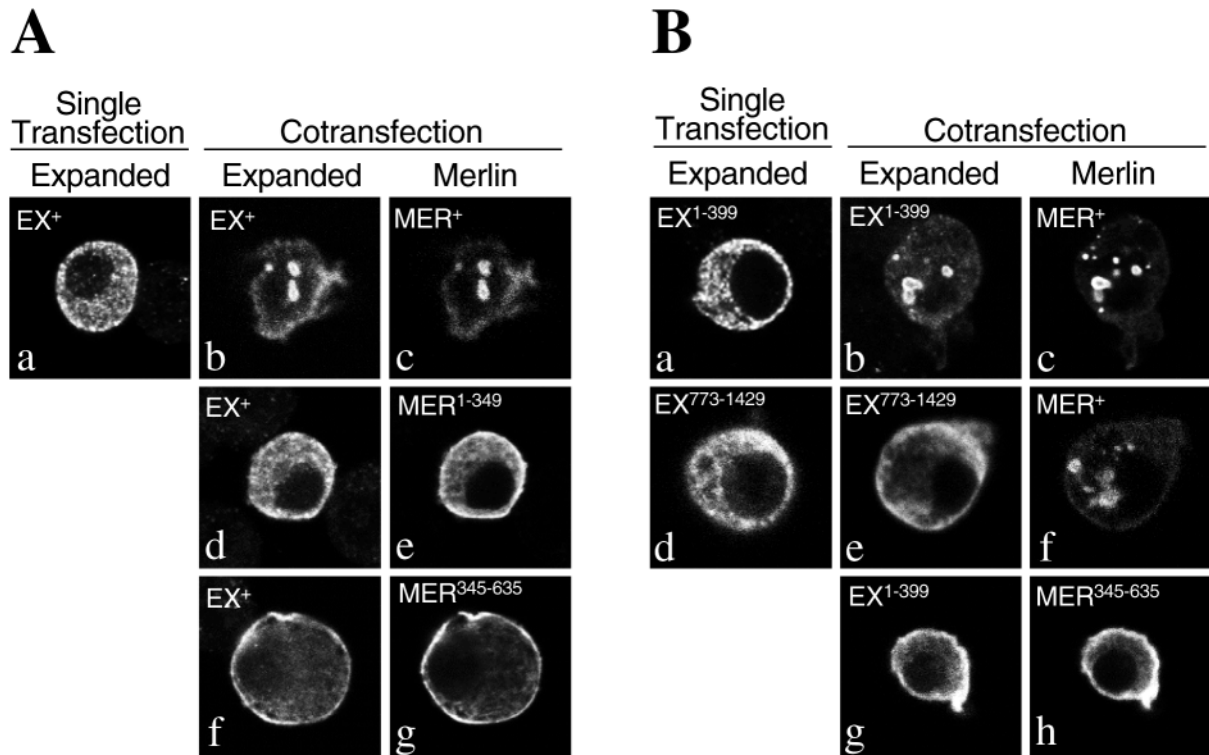


Fig. 3. Expanded is redistributed in response to Merlin in S2 cells. Singly or doubly transfected S2 cells were treated as described in Materials and Methods. EX and MER proteins were stained using specific antibodies for each protein, or anti-Myc as necessary. (A) EX⁺ associates with the C-terminal half of Merlin. EX⁺ is localized in a punctate distribution in the cytoplasm when ectopically expressed in S2 cells (a). When coexpressed with full-length Merlin (MER⁺), EX⁺ colocalizes with MER⁺ at the membrane and in large cytoplasmic compartments (b-c). The CNTR of Merlin is not necessary or sufficient for this interaction as EX⁺ does not colocalize with MER¹⁻³⁴⁹ (d-e), but does colocalize with MER³⁴⁵⁻⁶³⁵ (f,g). (B) Merlin associates with the CNTR of Expanded. The CNTR of Expanded (EX¹⁻³⁹⁹) is sufficient for an interaction with MER⁺ (a-c), whereas a C-terminal fragment of Expanded (EX⁷⁷³⁻¹⁴²⁹) is unable to colocalize with MER⁺ (d-f). Coexpression of EX¹⁻³⁹⁹ with MER³⁴⁵⁻⁶³⁵ (g,h) and indicates that the CNTR of Expanded colocalized with the C-terminal domain of Merlin at the plasma membrane.

it localized in the cytoplasm in a pattern significantly different from that of Merlin alone (Fig. 3A). When these two proteins were coexpressed, the pattern of Merlin localization was unchanged (Fig. 3Ac; McCartney and Fehon, 1996). In contrast, Expanded localization was altered so that it colocalized with Merlin both at the plasma membrane and in cytoplasmic compartments (Fig. 3Aa-c), suggesting a physical interaction between Merlin and Expanded.

We next asked which domains of Merlin and Expanded are responsible for this interaction by coexpressing fragments of these proteins and comparing their localizations. Although coexpression of wild-type Expanded (EX⁺) with MER¹⁻³⁴⁹ had no effect on Expanded localization (Fig. 3Ad,e), coexpression with MER³⁴⁵⁻⁶³⁵ caused a dramatic relocalization of Expanded (Fig. 3Af,g). In complementary experiments, we observed that the localization of EX¹⁻³⁹⁹ was altered by coexpression of full-length Merlin (MER⁺) (Fig. 3Ba-c). The subcellular localization of other, more C-terminal EX fragments (EX⁷⁷³⁻¹⁴²⁹) was not altered by MER⁺ coexpression (Fig. 3Bd-f). Taken together, these results suggest that the CNTR of Expanded (amino acids 1-399) interacts with the C-terminal half of Merlin (amino acids 345-635). To test this hypothesis, we coexpressed EX¹⁻³⁹⁹ with MER³⁴⁵⁻⁶³⁵ and found that these fragments colocalize at the plasma membrane (Fig. 3Bg,h). It is worth noting that these results indicate that a reciprocal interaction between the C-

terminal domain of Expanded and the N-terminal domain of Merlin does not appear to occur.

Merlin and Expanded interact directly

To complement and extend the colocalization experiments, we examined the ability of the Merlin and Expanded proteins to interact directly *in vitro*. Previous blot overlay experiments with ERM proteins have shown that sequences derived from the ERM CNTR bind to the ERM C-terminal tail region (Bretscher et al., 1995). Recent results using the yeast two-hybrid system have confirmed these interactions, and have shown that heterotypic dimers can form between Merlin and ERM proteins (Gronholm et al., 1999). We therefore employed a blot overlay assay to determine if the CNTR of Expanded interacts directly with the C-terminal tail of Merlin. As shown in Fig. 4B, the CNTR of Expanded (EX¹⁻³⁹⁹) binds strongly to full-length Merlin, and to a C-terminal Merlin fragment (MER⁵²²⁻⁶³⁵). These results are consistent with a head-to-tail heterodimeric interaction between Merlin and Expanded, as suggested by experiments with S2 cultured cells. However, we did not detect interactions between Expanded and the larger C-terminal Merlin fragment (MER³⁴⁵⁻⁶³⁵), the same fragment that interacted with Expanded in the cell culture assay. This may indicate that *in vitro* the MER³⁴⁵⁻⁶³⁵ fragment folds into an inaccessible configuration. In addition, EX¹⁻³⁹⁹ bound to a C-terminal Expanded fragment (EX⁷⁷³⁻¹⁴²⁹, Fig. 4B), suggesting

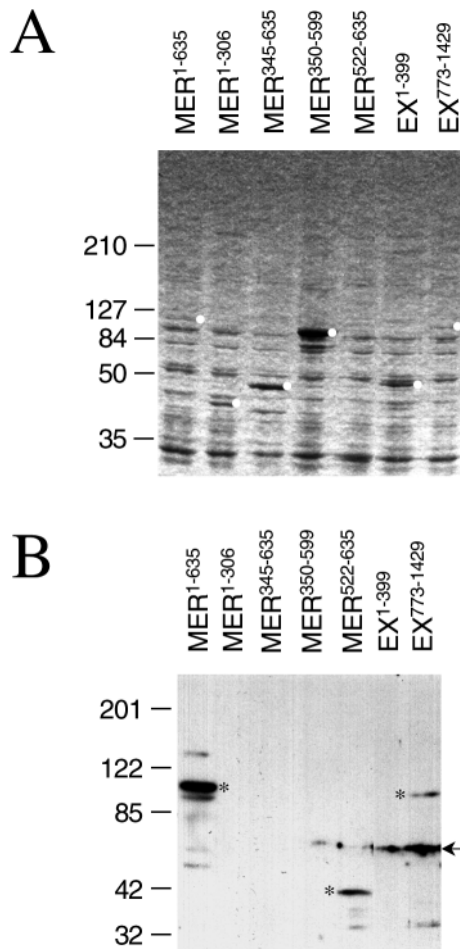


Fig. 4. Merlin and Expanded interact directly in blot overlay experiments. (A) Whole cell lysates from bacterial cells expressing the indicated proteins were separated by SDS-PAGE and detected with coomassie blue staining. Expressed fusion proteins are marked with a white dot. Expression of the MER⁵²²⁻⁶³⁵ protein was below the limit of detection and therefore is not marked. (B) Blot overlay experiment using the protein samples from (A) blotted onto nitrocellulose and probed with biotinylated EX¹⁻³⁹⁹ as described in Materials and Methods. Specific binding is detected between the probe and MER¹⁻⁶³⁵, MER⁵²²⁻⁶³⁵ and EX⁷⁷³⁻¹⁴²⁹ (bands are marked with an asterisk) Binding was not detected with other regions of Merlin and Expanded (or GST alone, data not shown). Arrow to right indicates non-specific band. Numbers at left indicate $M_r \times 10^3$ for standards.

that Expanded interacts with itself in a head-to-tail fashion, as has been shown for the ERM proteins and Merlin (Bretscher et al., 1995; Gronholm et al., 1999). We did not observe interactions between EX¹⁻³⁹⁹ and the C-terminal domain of *Drosophila* Moesin (amino acids 468-578; data not shown), indicating that the observed interaction between Merlin and Expanded is specific to these proteins.

Loss of function of both *Merlin* and *expanded* results in differentiation defects

Previous studies indicate that loss of function of either *Mer* or *ex* in clones results in a 2- to 3-fold overproliferation of the mutant tissue compared with the wild-type twin spot

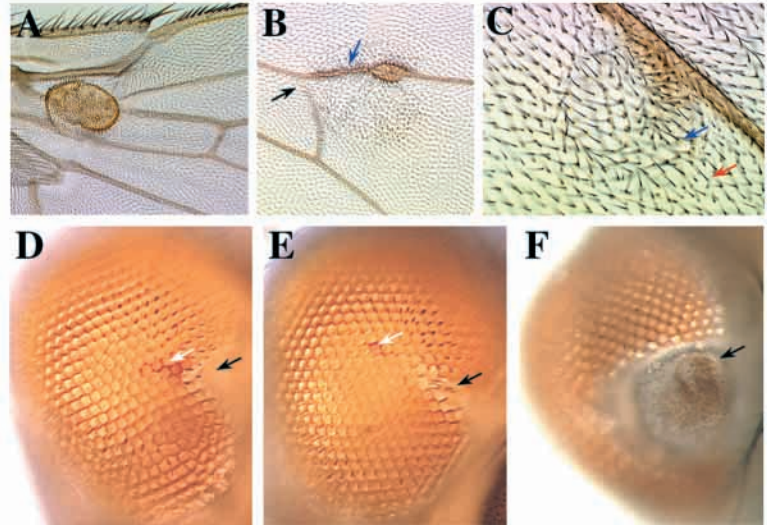
(LaJeunesse et al., 1998; Boedigheimer et al., 1997). In the wing, loss of either *Mer* or *ex* alone in clones has no apparent effect on the differentiation and morphology of the affected tissue (B. M. McC. and R. G. F., unpublished observations; Boedigheimer et al., 1997). Similarly, in the eye, loss of function of *Mer* results in overproliferation without obvious changes in the underlying morphology (LaJeunesse et al., 1998). In contrast, loss of *ex* function in the eye results in defects in planar polarization, in addition to proliferation defects (Blaumueller and Mlodzik, 2000). Because the loss of function of either *Mer* or *ex* results in overproliferation, we examined the consequences of loss of function of both genes using somatic mosaic analysis.

Double mutant clones were generated by gamma-ray induction of mitotic recombination in the $w^{1118} sn^3 Mer^4/w^{1118} sn^3 Mer^4; P\{w^+, UpMer^+\} ex^+/ex^{e1}$ background at 72 hours AEL. The *Mer*⁺ transgene was localized to the distal portion of the left arm of the second chromosome by in situ hybridization (data not shown). We identified double mutant clones in imaginal tissues by staining with antibodies against Merlin and Expanded, providing evidence that clones were generated in this background (data not shown). Clones were marked by the absence of the w^+ minigene in the adult eye, and were unmarked in the adult wings. However, we could identify mutant clones in the wing by phenotypes not observed in non-irradiated controls, or in controls that carried a *Mer*⁺ transgene localized to the right arm of the second chromosome (data not shown).

In the adult wing, we observed that both vein and intervein cells differentiate in mutant tissue (Fig. 5A-C). In contrast, clones that intersected the position of the posterior cross vein disrupted its development (Fig. 5B black arrow), consistent with the variably penetrant disruption of the posterior cross vein observed in hypomorphic alleles of *Mer* or *ex* (Fig. 1H). Clones in the position of the anterior cross vein differentiated normally (data not shown). Within the mutant intervein and vein clones, we observed apparent defects in proliferation control. In the proximal region of the wing, clonal vein tissue was observed to form a raised protrusion (Fig. 5A). In other regions of the wing, bulges in the veins were also observed, although more frequently vein clones were merely broadened when compared with the surrounding vein (Fig. 5B, blue arrow). In the intervein regions, the clonal tissue appeared to bulge and crinkle within the confines of the normal tissue, suggesting overproliferation (Fig. 5B,C). Similar cuticular bulges or protrusions have been reported for mutant clones of genes that have tumor suppressor phenotypes, such as warts (Justice et al., 1995; Xu et al., 1995). Thus we interpret this phenotype to indicate that the *Mer;ex* double mutant clones in the wing proliferate at a greater rate than the single mutant clones, though we were not able to confirm this directly. Based on general morphology, the cells within the clone appeared to differentiate as intervein cells, however, the cuticle deposited at the base of each wing hair within the mutant clone appeared to be thickened (Fig. 5C blue arrow) and was distinct from cuticle produced by either the heterozygous intervein (Fig. 5C red arrow) or vein cells.

Clones that developed within the eye appeared either as small scars with associated clusters of bristles (data not shown), or as elongated scars and associated indentations running from within the eye field toward the anterior margin

Fig. 5. *Merlin*⁻; *expanded*⁻ mutant clones display defects in proliferation and differentiation. (A-F) Mitotic recombination was induced with gamma rays in *w¹¹¹⁸ sn³ Mer⁴/w¹¹¹⁸ sn³ Mer⁴; P{w⁺, UpMer⁺} ex⁺/ex¹* larvae 72 hours AEL. (A-C) *Mer*⁻; *ex*⁻ mutant clones in the adult wing. (A) Mutant clones within the vein protrude from the surface of the wing. (B) In some cases, vein clones do not bulge from the surface, but broaden within the plane of the wing (blue arrow). Mutant clones in intervein tissue that intersect the position of the posterior crossvein disrupt the differentiation of the vein (black arrow). (C) Overproliferation of mutant intervein cells produced clones that buckled within the confines of the heterozygous tissue. The cuticle at the base of each wing hair in the clone (blue arrow) appears thickened compared with that outside the mutant clone (red arrow). (D-F) *Mer*⁻; *ex*⁻ mutant clones in the adult eye. Wild-type twin-spot clones, marking the site of clone induction, are indicated by white arrows. Mutant clones in the center of the eye appear to disrupt progression of the morphogenetic furrow and result in an elongated scar starting within the eye and extending to the anterior margin (black arrows in D,E). The mutant tissue does not differentiate ommatidia and often appears to differentiate as head cuticle (F, black arrow).



(Fig. 5D,E black arrows). Although these clones did not differentiate ommatidia, the position of the twin spot was used to indicate the position of the mutant clone (Fig. 5D,E white arrows). Mutant clones were often associated with overproliferated head cuticle (Fig. 5F, arrow).

Merlin and expanded interact genetically with the *dpp* pathway

Reduction of *dpp* function in the eye imaginal disc (*dpp^{blk}*) results in reduction of the eye along the dorsoventral axis such that the ventral portion of the eye is replaced by head cuticle (Masucci et al., 1990). A similar, yet less severe, phenotype was observed in *Mer*³ hemizygotes (Fig. 1B) and *ex*⁶⁹⁷ homozygotes (Boedigheimer and Laughon, 1993). To ask whether *dpp* expression is disrupted in *Mer* mutants, we expressed the *dpp-lacZ* transgene (B3.0) in the *Mer*³ background. In the wild-type eye-antennal complex, *dpp* is expressed at the lateral margins and in the morphogenetic furrow of the eye disc and in a ventral wedge of tissue in the antennal disc (Fig. 6A; anti-ELAV is labeling the developing photoreceptors). In the *Mer*³ mutants, the ventral portion of the disc is significantly enlarged (Fig. 6B,C). The expression pattern of *dpp* was disrupted such that the cells expressing *dpp* at the margin were displaced to the outer tip of the overproliferated tissue (Fig. 6B arrow). In some cases (<5%), this *dpp* staining was associated with an ectopic furrow and developing photoreceptors (Fig. 6C).

To better understand the functional relationship between *dpp*, *Mer* and *ex*, we next examined genetic interactions between these genes. Reduction of *dpp* dose in *Mer*³ hemizygotes enhanced the severity of the *Mer*³ eye phenotype, resulting in a smaller, more roughened eye and expansion of the head cuticle (Fig. 6D-F). Similar reduction of *dpp* function in *ex*⁶⁹⁷ homozygotes resulted in enhanced eye phenotypes (data not shown) and variably penetrant truncated leg phenotypes (Fig. 6G-I arrows indicate the position of the sex combs), reminiscent of those observed in pharate adults null for *ex* function (Boedigheimer and Laughon, 1993).

DISCUSSION

Loss of Merlin function in humans has been linked to the formation of Neurofibromatosis-2, a genetic disorder characterized by slow growing benign tumors associated with the peripheral and central nervous systems. Previous studies have suggested the existence of second-site modifier loci for *NF2* because the severity and age of onset of the disease does not appear to be completely correlated with specific *NF2* lesions (Baser et al., 1996). In *Drosophila*, we have observed that reduction of *Mer* function alone is characterized by an autonomous overproliferation phenotype without significant alterations in the differentiation of affected tissues. Likewise, inactivating mutations in *ex*, another member of the Protein 4.1 superfamily, produce hyperplastic imaginal discs and a broadened wing phenotype, but do not significantly affect cell differentiation (Boedigheimer and Laughon, 1993). In contrast, we have shown that simultaneous loss of both *Mer* and *ex* function results in an inability to differentiate certain cell types, such as photoreceptors of the adult eye. In addition, even a slight reduction in *ex* function (*ex*^{697/+}) enhances visible *Mer* head and wing phenotypes, and reduction of *Mer* function enhances the similar *ex* phenotypes (Fig. 1). These results indicate that these two structurally related genes function synergistically in developing tissues.

Dose-sensitive genetic interactions of the sort that we have identified between *Mer* and *ex* often reflect physical interactions between the proteins that they encode (Chang et al., 1994). Several additional lines of evidence support the idea that Merlin and Expanded interact directly. First, these proteins are coexpressed in imaginal epithelial cells and colocalize at the subcellular level. Second, when coexpressed in S2 cells these proteins colocalize at the plasma membrane and in the cytoplasm. Deletion mutagenesis experiments indicate that this colocalization requires the CNTR of Expanded and the C-terminal half of Merlin (Fig. 3). Third, studies of Merlin and the related ERM proteins indicate that they form homotypic and heterotypic dimers via an interaction between the CNTR and a domain of ~100 amino acids at the C terminus of the

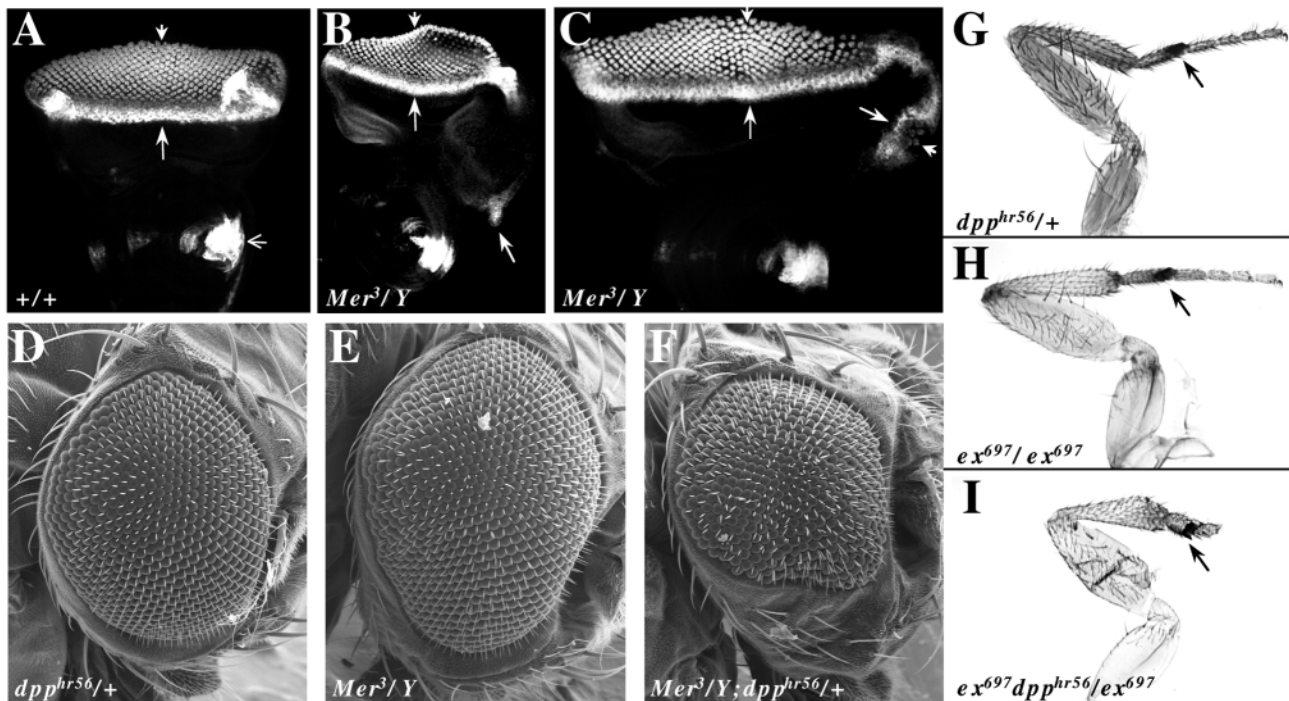


Fig. 6. Merlin and *expanded* interact genetically with *dpp*. (A–C) Eye-antennal discs from third instar larvae stained with ELAV to detect the developing photoreceptors (short arrows) and expressing the *dpp-lacZ* transgene detected with anti- β -galactosidase antibody (long arrows). In all panels ventral is to the right. (A) In the wild-type disc, *dpp* is expressed at the lateral margins and morphogenetic furrow of the eye disc and in a ventral wedge of the antennal disc (line arrow). (B) In a *Mer³/Y* mutant disc, the ventral portion of the eye disc is overgrown and the morphogenetic furrow appears neither to initiate or progress through the ectopic tissue. The *dpp*-expressing cells at the ventrolateral margin are displaced to the anterior tip of the ectopic tissue (arrow). (C) In some *Mer³/Y* eye discs, *dpp* staining indicates that the morphogenetic furrow develops in the ventral portion of the disc forming an ectopic furrow with consequent neuronal differentiation. The three imaginal discs are not depicted at the same scale. (D–F) Reduction of *dpp* function dominantly enhances the *Mer³/Y* head phenotype. (D) *dpp^{hr56/+}*. Flies of this genotype showed a mild reduction in size when compared with wild type. (E) *Mer³/Y*. (F) Reduction of *dpp* function in a *Mer³/Y* background resulted in a smaller, more roughened eye, and increased expansion of peripheral head cuticle. In addition, numerous interommatidial bristle duplications were observed. (G–I) Reduction of *dpp* function in *ex⁶⁹⁷* homozygotes resulted in truncated leg phenotypes. The arrow indicates the position of the sex comb found on the most proximal tarsal segment on the first pair of legs in males. (G–H) Neither *dpp^{hr56/+}* heterozygotes nor *ex⁶⁹⁷* homozygotes displayed significant defects in the adult leg. (I) Reduction of *dpp* function in the *ex⁶⁹⁷/ex⁶⁹⁷* background resulted in legs in which the distal tarsal segments are absent.

protein. It is therefore possible that Merlin can form heterodimers with Expanded via similar interactions. Consistent with this hypothesis, blot overlay and coexpression experiments indicate that Merlin and Expanded interact directly via the CNTR of Expanded and the C-terminal 114 amino acids of Merlin. While technical difficulties prevented us from demonstrating by co-immunoprecipitation that the observed interactions between Merlin and Expanded occur in tissues (Expanded is insoluble in non-denaturing cell lysates – data not shown), the combination of genetic interactions, co-localization and direct *in vitro* interactions strongly suggest that this interaction is biologically relevant.

What is the functional significance of this interaction? Removal by genetic mutation of either protein does not appear to affect the subcellular localization of the other (Fig. 2 and data not shown), indicating that the interaction is not required for proper subcellular localization in tissues. Studies of ERM proteins have shown that they exist in multiple conformations that appear to regulate their ability to interact with transmembrane and other interacting proteins. (Mangeat et al., 1999). Thus, the formation of homotypic and heterotypic dimers or oligomers via interactions between the CNTR and

C-terminal domains may function to maintain ERM proteins in either an active or inactive state. Recent studies of human Merlin indicate that it can form folded monomers, homotypic dimers and heterotypic dimers with ERM proteins (Gronholm et al., 1999). Our results indicate that the CNTR of Expanded interacts directly with the C-terminal domains of Expanded and Merlin, suggesting that Expanded can exist in a similar range of conformations. Based on the results presented here, we propose that Merlin and Expanded form a heterodimeric complex that actively suppresses proliferation (Fig. 7). According to this model, in the heterodimer, the N-terminal domain of Merlin would be free to interact with other proteins, and therefore would be in an activated state. Consistent with this view, we have shown previously that removal of the 35 amino acid C-terminal region of Merlin results in a constitutively active form of the protein that contains all essential Merlin functions (LaJeunesse et al., 1998). In addition, both Merlin and Expanded must possess growth suppression functions that are independent of each other, because the *Mer⁻; ex⁻* double mutant phenotype that we observed was much more severe than either mutation alone (Fig. 5).

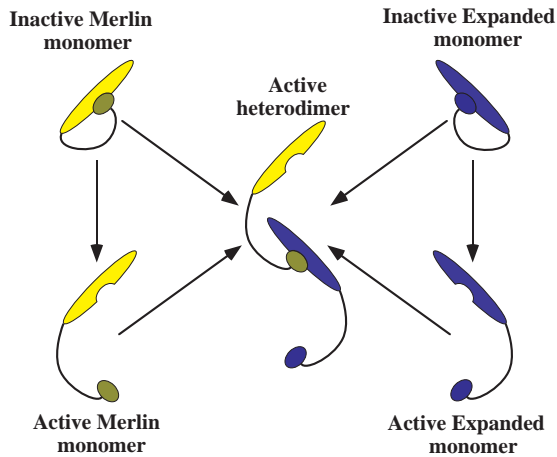


Fig. 7. Proposed model for Merlin/Expanded interactions. Evidence from previous studies indicates that Merlin and ERM proteins have two states, inactive and active, and that the transition between states involves unfolding of the protein. Evidence presented here indicates that Expanded also can form a folded monomer, and that it can bind to Merlin, presumably in an unfolded state. Genetic evidence indicates that each protein retains function in the absence of the other, suggesting that each can function in a monomeric, activated state as well.

Formally, the observed interactions with Expanded could function to positively or negatively regulate Merlin function. Our previous results indicate that overexpression of an activated Merlin allele has no dominant phenotype, indicating that beyond a minimum threshold, tight regulation of the activated form of *Drosophila* Merlin is not important in vivo. However, Merlin is expressed at low levels in cells, suggesting that specific mechanisms may be required to activate and/or stabilize Merlin function. Thus, Expanded may function by stabilizing the activated conformation, thereby increasing the overall level of Merlin function. Although a complete elucidation of the functions of interactions between Merlin and Expanded will require an in depth understanding of the biochemical properties and interactions of both proteins, this model is consistent with our genetic and biochemical results, and is consistent with known functions of other family members.

Merlin and Expanded in patterning events

Our phenotypic analyses of *Mer* and *ex* mutants suggest a relationship between Merlin and Expanded and the DPP signal transduction pathway. We investigated this potential relationship by examining mutant phenotypes and genetic interactions associated with the eye and leg. In developing eye imaginal discs from *Mer*³ hemizygotes, we found that often the ventral portion of the eye disc was overgrown and DPP expression was disrupted so that the morphogenetic furrow did not initiate or failed to progress through the ectopic tissue (Fig. 6A-C). A similar effect has been seen with mutant clones of *Mad*, a downstream effector of DPP signaling, in the posterior portion of the eye (Wiersdorff et al., 1996). Such clones inhibit furrow initiation and can result in the formation of an ectopic furrow ventral to the clone. In addition, hypomorphic mutant phenotypes of either *Mer* or *ex* are enhanced by heterozygosity for the *dpp*^{hr56} allele (Fig. 6D-I). In the eye, the reduction of

dpp function enhances the *Mer*³ hemizygous phenotype, resulting in an eye that is greatly reduced in size, especially ventrally. Because *dpp* is required for ommatidial recruitment, this phenotype is consistent with a positive role for Merlin in DPP function. Similarly, in the leg imaginal disc, we observed that reduction of *dpp* function enhances hypomorphic *ex* phenotypes so that the distal elements of the leg are reduced significantly. In the developing leg, DPP and the ligand Wingless (WG) are expressed in distinct domains that meet in the center of the disc, the region that corresponds to the most distal segment of the adult leg (Serrano and O'Farrell, 1997). The combined action of DPP and WG activates expression of *distal-less*, which in turn specifies the formation of the proximal-distal axis. Loss of distal structures in the leg is characteristic of *dpp* hypomorphs (Spencer et al., 1982). Thus, the truncated leg phenotype produced as the result of reduction of *dpp* function in *ex*⁶⁹⁷ homozygotes indicates that Expanded also has a positive role in the DPP signaling pathway.

This and other genetic studies of *Mer* and *ex* mutant phenotypes have revealed at least two functions for these genes. First, these two genes are required for normal cell proliferation in developing imaginal tissues. Second, the data presented here implicate Merlin and Expanded in DPP-mediated patterning events in several *Drosophila* tissues. Previous studies of *dpp* and related genes have demonstrated that this pathway has functions in both proliferation and patterning during development of the wing imaginal disc (Burke and Basler, 1996). Although it is appealing to think that the effects of *Mer* and *ex* on patterning and proliferation are both mediated through the DPP pathway, this seems unlikely given that loss of either gene seems to negatively affect DPP patterning functions, but simultaneously causes overproliferation of mutant cells. It therefore seems more likely that the proliferation phenotypes of *Mer* and *ex* loss-of-function mutations are mediated through effects on one or more other pathways that regulate proliferative events. Recent studies of Merlin and the related ERM proteins indicate that they may have multiple binding partners (Mangeat et al., 1999), and therefore may function to regulate more than one signaling pathway simultaneously in developing tissues.

Conclusion

We have identified Expanded as a novel binding partner for the *Drosophila* NF-2 tumor suppressor homologue Merlin. Although a vertebrate homologue of *ex* has not yet been identified, the interaction between Merlin and Expanded and their shared role in the regulation of cell proliferation make *ex* an intriguing candidate for a second-site-modifier of the NF-2 phenotype. Thus, further study of Expanded and its relationship to Merlin function in developing tissues should provide insights both into the mechanisms by which cell proliferation is regulated, and into the mechanisms of and possible therapeutic approaches toward the NF-2 disease.

We would like to thank M. Boedigheimer, P. Bryant, K. Wharton, G. Rubin and the Bloomington stock center for providing valuable reagents, S. Jong for technical assistance, our colleagues in the Fehon laboratory for suggestions and discussions, and M. Peifer (UNC) for his patience. This work was supported by NIH grant NS34783 to R. G. F., a National Neurofibromatosis Foundation Young Investigator Award to B. M. M. and a National Research Service Award (F32-NS10224) to D. R. L.

REFERENCES

- Baser, M. E., Ragge, N. K., Riccardi, V. M., Janus, T., Gantz, B. and Pulst, S. M. (1996). Phenotypic variability in monozygotic twins with neurofibromatosis 2. *Am. J. Med. Genet.* **64**, 563-567.
- Blaumueller, C. M. and Mlodzik, M. (2000). The *Drosophila* tumor suppressor *expanded* regulates growth, apoptosis, and patterning during development. *Mech. Dev.* **90**, in press.
- Boedigheimer, M., Bryant, P. and Laughon, A. (1993). *Expanded*, a negative regulator of cell proliferation in *Drosophila*, shows homology to the NF2 tumor suppressor. *Mech. Dev.* **44**, 83-84.
- Boedigheimer, M. and Laughon, A. (1993). *expanded*: a gene involved in the control of cell proliferation in imaginal discs. *Development* **118**, 1291-1301.
- Boedigheimer, M. J., Nguyen, K. P. and Bryant, P. J. (1997). Expanded functions in the apical cell domain to regulate the growth rate of imaginal discs. *Dev. Genet.* **20**, 103-110.
- Bretscher, A., Gary, R. and Berryman, M. (1995). Soluble ezrin purified from placenta exists as stable monomers and elongated dimers with masked C-terminal ezrin-radixin-moesin association domains. *Biochemistry* **34**, 16830-16837.
- Bunch, T. A., Grinblat, Y. and Goldstein, L. S. B. (1988). Characterization and use of the *Drosophila* metallothionein promoter in cultured *Drosophila melanogaster* cells. *Nuc. Acids Res.* **16**, 1043-1061.
- Burke, R. and Basler, K. (1996). Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. *Development* **122**, 2261-2269.
- Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A. and von Zastrow, M. (1999). A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor. *Nature* **401**, 286-290.
- Chang, H. C., Karim, F. D., O'Neill, E. M., Rebay, I., Solomon, N. M., Therrien, M., Wassarman, D. A., Wolff, T. and Rubin, G. M. (1994). Ras signal transduction pathway in *Drosophila* eye development. *Cold Spring Harb. Symp. Quant. Biol.* **59**, 147-153.
- Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N., et al. (1998). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends Biochem. Sci.* **23**, 281-282.
- Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A. T. and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologous genes in *Drosophila*. *Cell* **61**, 523-534.
- Fehon, R. G., Oren, T., LaJeunesse, D. R., Melby, T. E. and McCartney, B. M. (1997). Isolation of mutations in the *Drosophila* homologues of the human *Neurofibromatosis 2* and yeast *CDC42* genes using a simple and efficient reverse-genetic method. *Genetics* **146**, 245-252.
- Frorath, B., Scanarini, M., Netter, H. J., Abney, C. C., Liedvogel, B., Lakomek, H. J. and Northemann, W. (1991). Cloning and expression of antigenic epitopes of the human 68-kDa (U1) ribonucleoprotein antigen in *Escherichia coli*. *Biotechniques* **11**, 364-371.
- Gronholm, M., Sainio, M., Zhao, F., Heiska, L., Vaheri, A. and Carpen, O. (1999). Homotypic and heterotypic interaction of the neurofibromatosis 2 tumor suppressor protein merlin and the ERM protein ezrin. *J. Cell Sci.* **112**, 895-904.
- Gusella, J. F., Ramesh, V., MacCollin, M. and Jacoby, L. B. (1999). Merlin: the neurofibromatosis 2 tumor suppressor. *Biochim. Biophys. Acta* **1423**, M29-36.
- Gutmann, D. H., Sherman, L., Seftor, L., Haiepek, C., Hoang Lu, K. and Hendrix, M. (1999). Increased expression of the NF2 tumor suppressor gene product, merlin, impairs cell motility, adhesion and spreading. *Hum. Mol. Genet.* **8**, 267-275.
- Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J. and Lefkowitz, R. J. (1998). A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na⁺/H⁺ exchanger regulatory factor family of PDZ proteins. *Proc. Natl. Acad. Sci. USA* **95**, 8496-8501.
- Heiska, L., Alftan, K., Gronholm, M., Vilja, P., Vaheri, A. and Carpen, O. (1998). Association of ezrin with intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2). Regulation by phosphatidylinositol 4, 5-bisphosphate. *J. Biol. Chem.* **273**, 21893-21900.
- Justice, R. W., Zilian, O., Woods, D. F., Noll, M. and Bryant, P. J. (1995). The *Drosophila* tumor suppressor gene *warts* encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. *Genes Dev.* **9**, 534-546.
- LaJeunesse, D. R., McCartney, B. M. and Fehon, R. G. (1998). Structural analysis of *Drosophila* Merlin reveals functional domains important for growth control and subcellular localization. *J. Cell Biol.* **141**, 1589-1599.
- Mangeat, P., Roy, C. and Martin, M. (1999). ERM proteins in cell adhesion and membrane dynamics. *Trends Cell Biol.* **9**, 187-192.
- Martuza, R. L. and Eldridge, R. (1988). Neurofibromatosis 2 (bilateral acoustic neurofibromatosis). *N. Engl. J. Med.* **318**, 684-688.
- Masucci, J. D., Miltenberger, R. J. and Hoffmann, F. M. (1990). Pattern-specific expression of the *Drosophila* decapentaplegic gene in imaginal disks is regulated by 3' cis-regulatory elements. *Genes Dev.* **4**, 2011-2023.
- McCartney, B. M. and Fehon, R. G. (1996). Distinct cellular and subcellular patterns of expression imply distinct functions for the *Drosophila* homologues of moesin and the neurofibromatosis 2 tumor suppressor, merlin. *J. Cell Biol.* **133**, 843-852.
- Murthy, A., Gonzalez-Agosti, C., Cordero, E., Pinney, D., Candia, C., Solomon, F., Gusella, J. and Ramesh, V. (1998). NHE-RF, a regulatory cofactor for Na⁽⁺⁾-H⁺ exchange, is a common interactor for merlin and ERM (MERM) proteins. *J. Biol. Chem.* **273**, 1273-1276.
- Rebay, I., Fehon, R. G. and Artavanis-Tsakonas, S. (1993). Specific truncations of *Drosophila* Notch define dominant activated and dominant negative forms of the receptor. *Cell* **74**, 319-329.
- Reczek, D., Berryman, M. and Bretscher, A. (1997). Identification of EBP50: A PDZ-containing phosphoprotein that associates with members of the Ezrin-Radixin-Moesin family. *J. Cell Biol.* **139**, 169-179.
- Scoles, D. R., Huynh, D. P., Morcos, P. A., Coulsell, E. R., Robinson, N. G., Tamanoi, F. and Pulst, S. M. (1998). Neurofibromatosis 2 tumour suppressor schwannomin interacts with betaII-spectrin. *Nat. Genet.* **18**, 354-359.
- Serrano, N. and O'Farrell, P. H. (1997). Limb morphogenesis: connections between patterning and growth. *Curr. Biol.* **7**, 186-195.
- Spencer, F. A., Hoffmann, F. M. and Gelbart, W. M. (1982). Decapentaplegic: A gene complex affecting morphogenesis in *Drosophila melanogaster*. *Cell* **28**, 451-461.
- Tsukita, S., Oishi, K., Sato, N., Sagara, J., Kawai, A. and Tsukita, S. (1994). ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J. Cell Biol.* **126**, 391-401.
- Ward, R. E. IV, Lamb, R. S. and Fehon, R. G. (1998). A conserved functional domain of *Drosophila* Coracle is required for localization at the septate junction and has membrane-organizing activity. *J. Cell Biol.* **140**, 1463-1473.
- Wiersdorff, V., Lecuit, T., Cohen, S. M. and Mlodzik, M. (1996). Mad acts downstream of Dpp receptors, revealing a differential requirement for dpp signaling in initiation and propagation of morphogenesis in the *Drosophila* eye. *Development* **122**, 2153-2162.
- Xu, H. M. and Gutmann, D. H. (1998). Merlin differentially associates with the microtubule and actin cytoskeleton. *J. Neurosci. Res.* **51**, 403-415.
- Xu, T. and Rubin, G. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.
- Xu, T., Wang, W., Zhang, S., Stewart, R. A. and Yu, W. (1995). Identifying tumor suppressors in genetic mosaics: the *Drosophila* *lats* gene encodes a putative protein kinase. *Development* **121**, 1053-1063.