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A Systematic Screen for Dominant Second-Site Modifiers of *Merlin/NF2* Phenotypes Reveals an Interaction With *blistered/DSRF* and *scribbler*

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ABSTRACT

Merlin, the Drosophila homologue of the human tumor suppressor gene *Neurofibromatosis 2* (*NF2*), is required for the regulation of cell proliferation and differentiation. To better understand the cellular functions of the *NF2* gene product, Merlin, recent work has concentrated on identifying proteins with which it interacts either physically or functionally. In this article, we describe genetic screens designed to isolate second-site modifiers of *Merlin* phenotypes from which we have identified five multiallelic complementation groups that modify both loss-of-function and dominant-negative *Merlin* phenotypes. Three of these groups, *Group IIa/scribbler* (also known as *brakeless*), *Group IIc/blistered*, and *Group IId/net*, are known genes, while two appear to be novel. In addition, two genes, *Group IIa/scribbler* and *Group IIc/blistered,* alter Merlin subcellular localization in epithelial and neuronal tissues, suggesting that they regulate Merlin trafficking or function. Furthermore, we show that mutations in *scribbler* and *blistered* display second-site noncomplementation with one another. These results suggest that *Merlin*, *blistered*, and *scribbler* function together in a common pathway to regulate Drosophila wing epithelial development.

NEUROFIBROMATOSIS type 2 (NF2) is a dominant autosomal disorder characterized by benign slow growing tumors associated with the glial cells of the eighth cranial nerve and other glial cells throughout the central nervous system (Martuza and Eldridge 1988). The product of the NF2 tumor-suppressor gene is a protein called Merlin (Rouleau *et al.* 1993; Trofatter *et al.* 1993). Merlin is a novel member of the 4.1 superfamily and has the greatest similarity to the Ezrin, Radixin, and Moesin (ERM) proteins. ERM proteins are membrane/cytoskeletal adapters that link a variety of transmembrane proteins to the underlying actin cytoskeleton (Algrain *et al.* 1993). ERM proteins have two functional domains: the N-terminal FERM domain (Chishti *et al.* 1998) interacts with transmembrane proteins, and the C-terminal domain binds filamentous actin. ERM proteins are believed to regulate processes such as signal transduction by organizing the plasma membrane into distinct functional domains (Helander *et al.* 1996). Consistent with this notion, ERM proteins have been shown to directly or indirectly regulate the localization and/or activity of several transmembrane proteins such as CD44, ICAM-2, and the β-adrenergic receptor (Tsukita *et al.* 1994; Helander *et al.* 1996; Heiska *et al.* 1998). Interestingly, Merlin has been demonstrated to interact physically with CD44 (Sainio *et al.* 1997) and a recent study indicates that this interaction may play an important role in growth regulation (Herrlich *et al.* 2000).

The activity of ERM proteins is tightly regulated by binding PIP_2 and via a C-terminal phosphorylation event (Matsui *et al.* 1999). Likewise, Merlin exists at the plasma membrane in at least two forms, transiting from an inactive to an active state by an unknown mechanism (LaJeunesse *et al.* 1998). Merlin expression level and phosphorylation are responsive to changes in cell adhesion, cell confluency, and growth factor stimulation, suggesting that Merlin activity is precisely regulated, perhaps through intercellular signaling mechanisms (Shaw *et al.* 1998). Merlin has been shown to form homotypic dimers and heterotypic dimers with other ERM proteins (Gronholm *et al.* 1999). In addition, it has been shown to bind actin filaments (Xu and Guttman 1998), βII spectrin (Scoles *et al.* 1998), and the EBP50/ Na^+/H^+ exchanger regulatory factor that also binds ERM proteins (Reczek *et al.* 1997; Murthy *et al.* 1998). Recently, another Drosophila protein 4.1 family member, *expanded*, was shown to interact genetically and physically with *Merlin* to regulate cellular proliferation and differentiation of imaginal disc tissue (McCartney *et al.* 2000). However, the significance of these interactions is unclear and the mechanisms by which Merlin function is regulated remain unknown.

To identify genes involved in Merlin function, we performed a genetic screen designed to identify dominant second-site modifiers of Drosophila *Merlin* phenotypes. Second-site modifier screens are powerful tools

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for dissecting pathways associated with specific cellular and developmental processes, and have been successful in identifying genes that interact functionally as well as physically with the target (Simon *et al.* 1991; Rebay *et al.* 2000). Furthermore, the identification of genetic modifiers should be useful for understanding the NF2 disorder, as several studies have suggested a role for second-site genetic modifiers in the expressivity and penetrance of *NF2*-related phenotypes (McClatchey *et al.* 1998; Bruder *et al.* 1999a,b). Through our genetic screens, we identified 29 modifying mutations out of 100,000 progeny derived from mutagenized parents, 23 of which fall into five multiallelic complementation groups. Three of the complementation groups contain new alleles of previously characterized loci: *blistered,* the Drosophila homologue of serum response factor (Guillemin *et al.* 1996; Montagne *et al.* 1996), the extra vein gene *net*, and a newly identified gene called *scribbler* (*sbb*) or *brakeless*, which encodes a novel nuclear protein of unknown function (Rao *et al.* 2000; Senti *et al.* 2000; Yang *et al.* 2000). The remaining two multiallelic groups and the five single-allele groups are novel. Mutations in all five groups dominantly modified phenotypes caused by ectopic expression of a dominant negative *Merlin* allele and phenotypes associated with a recessive hypomorphic *Merlin* allele. In addition, alteration of Merlin subcellular localization was observed in tissues from larvae homozygous for two of the modifying loci. These results together with observations of second-site noncomplementation between some of the groups suggest that all function together with *Merlin* to regulate cell proliferation. Thus, characterization of all of these genes will provide further insight into mechanisms of Merlin function and the NF2 disorder.

MATERIALS AND METHODS

Drosophila cultures and stocks used: All Drosophila cultures were maintained on standard cornmeal, yeast, molasses, agar medium and all crosses were performed at 25° unless otherwise specified. Meiotic mapping of the complementation groups was based on lethality and used the following *P*-element insertion stocks: second chromosome mapping stocks, *P{w*$^{+mC}$ *= lacW} l(2)10424*^{k06801} at [2-18]/26B1-2; *P{w*$^{+mC}$ *= lacW} Pen*^{k14401} at [2-40.5]/31A; *P{w*$^{+mW.hs}$ *= GawB} ap*^{md544} at [2-55.2]/41F9-10; *P{w*$^{+mW.hs}$ *= GawB} 559.1* at [2-59]/44D2-5; *P{w*$^{+mC}$*lacW} AA48* at [2-87]/56A1-2; and *P{w*$^{+mW.hs}$ *= GawB} Dll*^{md23} at [2-107]/60E1-2; third chromosome mapping stocks, *P{w*$^{+mC}$ *= lacW} l(3)L1170*^{L1170} at [3-0]/61C7-8; *P{w*$^{+mC}$ *= lacW} l(3)j2B9*^{j2B9} at [3-28]/67B4-5; *P{w*$^{+mC}$ *= lacW} Trl*^{s2325} at [3-36]/70F1-4; 5*P{w*$^{+mC}$ *= lacW} l(3)j1E6*^{j1E6} at [3-46]/82A3-5; and *P{w*$^{+mC}$ *= lacW} dco*^{j3B9} at [3-102]/100B2-4. The Gal4/UAS system (Brand and Perrimon 1993) was used to overexpress Mer$^{\Delta BB}$. A second chromosome insert of *UASMer*$^{\Delta BB}$ (LaJeunesse *et al.* 1998) was recombined with either *engrailed::Gal4* or *apterous::Gal4* enhancer traps lines to generate the *en*Δ*BB* and *ap*Δ*BB* chromosomes, respectively. *Mer*^{3} was previously described (Fehon *et al.* 1997; LaJeunesse *et al.* 1998; McCartney *et al.* 2000) All deficiencies were from the Bloomington deficiency kit collection (FlyBase).

Screen protocol: Three-day-old white-eyed males from an isogenized second and third chromosome (marked with *ebony*, *e*^{4}) stock were treated either with 25 mm EMS or 4000 rads of γ-ray radiation and mated to virgin *en*Δ*BB/CyO w*$^{+}$ females. In the F_1 generation, male flies having a modified phenotype were collected and backcrossed to *en*Δ*BB/CyO w*$^{+}$ females for two further generations to eliminate any somatic mosaics that arise during mutagenesis. In the F_3 generation mutations that still modified *en*Δ*BB* phenotypes were mapped to a chromosome via the segregation of modification. Stocks were generated at this step by crossing mutations that mapped to the second chromosome to *Sco/SM6a* and those that mapped to the third to *MRKS/TM6a*. We established complementation groups by taking mutations that mapped to the same chromosome and testing for lethal noncomplementation.

Wing measurements: All crosses for flies used in wing measurements were maintained in the same incubator at 25° and collected simultaneously to eliminate phenotypic variation due to environmental factors. Flies of the appropriate genotype were incubated in 70% ethanol for at least 24 hr. We have found that this makes the cuticles easier to manipulate. Wings were removed in a drop of water on a siliconized slide and mounted in a drop of Aquamount on a glass slide. Only wings that had been flattened during the mounting process were used for further analysis. Images were captured using a Zeiss (Thornwood, NY) Axioplan microscope equipped with a Sony DCX-760MD camera and imported into Adobe Photoshop. Using the free draw tool the area to be calculated was outlined, filled in, and analyzed using the MEASURE tool of NIH Image. For the *en*Δ*BB* experiments, the area between wing vein II and the posterior margin was calculated, and for the *Mer*^{3} experiments the area of the entire wing was measured.

Sequencing and molecular characterization of *brakeless* alleles: Homozygous third instar larvae from *Group IIa/sbb*^{256} and *Group IIa/sbb*^{324} were collected and their genomic DNA were extracted using a standard protocol (Berkeley Drosophila Genome Project). The genomic region containing the entire *sbb* coding sequence (~8 kb) was amplified using intronic primers in four separate PCR reactions. These reaction products were sequenced using nested internal primers and the ABI-Prism Big Dye protocol. Sequences were then assembled and analyzed using the Sequencher program (Gene Codes Corporation, Ann Arbor, MI).

RESULTS

Deficiency kit screen results: To approximate the number of loci that modify *Merlin* phenotypes in a dose-sensitive fashion we initially screened a collection of deficiencies, the so-called "deficiency kit" (FlyBase 1999), that represent ~70% of the genome in haploids. Deficiencies were tested for dominant effects on phenotypes displayed by a hypomorphic allele of *Merlin*, *Mer*^{3}, and by ectopic expression of a dominant-negative form of *Merlin*, *Mer*$^{\Delta BB}$ (LaJeunesse *et al.* 1998; McCartney *et al.* 2000).

Mer^{3} flies are semiviable and display a variety of phenotypes in the head, eye, wings, and legs (McCartney *et al.* 2000). Wings from *Mer*^{3} hemizygous male flies are broadened and have a low penetrance of disruptions of the posterior cross vein (data not shown). In the head, *Mer*^{3} flies express slightly rough, smaller eyes (Figure 1B) and ~10% have ectopic growths and vibrissae al-

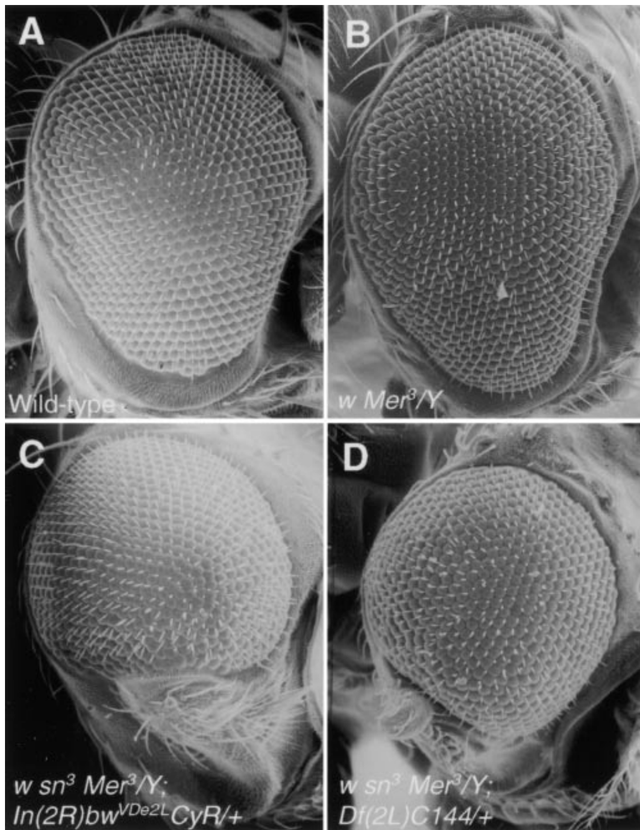

FIGURE 1.—Deficiencies that modify *Mer*^{3} phenotypes. (A) Scanning electron micrograph (SEM) of a wild-type adult male eye. (B) SEM of a *Mer*^{3}*/Y* eye. Note the slightly smaller and rougher appearance compared to the wild-type eye. (C and D) Adult eyes from *Mer*^{3}*/Y; In(2R)bw*^{VDe2L} *CyR/+* and *Mer*^{3}*/Y; Df(2L)C144/+* flies, respectively. In both cases, the presence of the deficiency results in an enhancement of *Mer*^{3} phenotypes including reduction in the size of the eye and formation of aberrant head cuticle, bristles, and outgrowths. Histological examination of sections taken through these eyes reveals very minor perturbation of ommatidial organization similar to those seen in Mer^{3}/Y eyes alone (data not shown).

most exclusively in the anterior ventral portion of the eyes. Twenty-three deficiencies were found to modify *Mer*^{3} phenotypes (Table 1). Two of these deficiencies were strong interactors, *Df(2L)C144* and *In(2R)bw*^{VDe2L}. *Mer*^{3} flies heterozygous for *Df(2L)C144* or *In(2R)bw*^{VDe2L} had head defects and small rough eyes (Figure 1,C and D). The chromosomal region encompassing *Df(2L)C144* has been saturated for lethal mutations (LITTLETON and BELLEN 1994) but none of the lethal complementation groups uncovered by this deficiency modify the *Mer*^{3} phenotype (data not shown). This result suggests that more than a single mutation within this deficiency may be responsible for the dose-sensitive modification phenotype. Alternatively, the gene responsible for this effect is not mutable to lethality, or the *Df(2L)C144* chromosome carries an independent mutation that interacts genetically with *Mer*^{3}.

Expression of dominant-negative *Mer*$^{\Delta BB}$ in the developing wing results in overproliferation of the wing blade (LAJEUNESSE *et al.* 1998). This dominant-negative form of Merlin has seven conserved amino acids within the FERM domain removed, and it interferes with the activation of wild-type Merlin (LAJEUNESSE *et al.* 1998). A chromosome carrying both the *UAS::Mer*$^{\Delta BB}$ transgene and the *engrailed::Gal4* driver (denoted *en*Δ*BB*) displays a phenotype that is sensitive to gene dose (Figure 2), an essential feature for screens designed to identify extragenic dose-sensitive modifiers (SIMON *et al.* 1991; REBAY *et al.* 2000). Flies heterozygous for *en*Δ*BB* have moderately overgrown posterior wing compartments with no disruption in venation (Figure 2B). Homozygosity for *en*Δ*BB* results in enlargement of the posterior wing compartment with disruptions in venation, particularly along vein V and the posterior cross vein (Figure 2C). Flies homozygous for *en*Δ*BB* also hold their wings out from the body axis (data not shown).

Using the deficiency kit, we identified 20 interacting deficiencies that enhance the phenotype caused by heterozygosity for the *en*Δ*BB* chromosome (Table 1). The degree of enhancement ranged from slight (30–50% of wings showing ectopic vein material) to very strong (100% of wings showing ectopic vein material with some wing blistering). The strongest interacting deficiency, *Df(2R)Px2*, expressed a dominant extra vein phenotype in a wild-type *Merlin* background. However, heterozygosity for both *Df(2R)Px2* and *en*Δ*BB* resulted in a blistered wing phenotype not observed with either alone, suggesting the presence of an interaction. For all deficiencies except *Df(3R)Antp17* and *Df(3R)awd*^{krb}, the observed interacting cytological regions were substantiated and defined by overlapping deficiencies that displayed the same enhancement phenotype. However, this does not exclude the possibility of multiple interacting genes within these regions or the presence of additional modifying loci on deficiency-bearing chromosomes.

In summary, between the two screens we identified eight deficiencies that modified both *Mer*^{3} and *Mer*$^{\Delta BB}$. However, neither analysis of lethal *P*-element insertions nor analysis of previously characterized genes uncovered by these deficiencies revealed the individual loci responsible for the modification of either *Merlin* phenotype (data not shown).

F$_1$ second-site modifier screen results: As a complement to the deficiency kit screen, we performed a genetic screen looking for dose-sensitive modification of the phenotypes expressed by flies carrying the *en*Δ*BB* chromosome. The design of this screen is shown in Figure 2D. To identify modifiers of the *en*Δ*BB* phenotype, ~100,000 F$_1$ male flies expressing the enΔBB transgene (~75,000 from EMS-mutagenized flies and ~25,000 from X-ray-mutagenized flies) and carrying potential modifiers were examined for ectopic venation along the vein V/posterior cross vein intersection and/or the presence of outheld wings. From this screen, we identified 29 enhancer mutations and no suppressor muta-

TABLE 1

Summary of deficiency kit screens

Deficiency	Breakpoints	Interaction with $Mer^{\Delta BB}$	Interaction with Mer^{3}	Genes
Df(2L)net-PMF	21A1;21B8	***	—	*Group IId/net*
Df(2L)al	21B8;21D1	*	Yes	*expanded*
Df(2L)ast2		—	Yes	
Df(2L)C144	23A1;23C5	*	Yes, strong	
Df(2L)JS32		—	Yes	
Df(2L)c1-h3		—	Yes	
Df(3L)30A-C		—	Yes	
Df(2L)Mdh	30D;31F	**	—	*Group IIb*
Df(2L)J39		—	Yes	
Df(2L)cact-256	35F;36D	*	—	
Df(2L)H20	36A8;36E2	*	—	
DF(2R)M41A	41A	***	Yes	
In(2R)bw^{VDe2L}	41A;42A	**	Yes, strong	
Df(2R)nap1	41D1;42D2	*	Yes	
Df(2R)pk78S	42C1;43F8	*	Yes	
Df(2R)44CE		—	Yes	
Df(2R)cn88b		**	—	
Df(2R)en-A	47D3;48B5	*	Yes	
Df(2R)en-30	48A3;48C8	*	—	
Df(2R)PC4	55A;55F	*	Lethal	*Group IIa/scribbler*
Df(2R)or-BR6		—	Yes	
Df(2R)Px2	60C5;60D10	****[a]	ND	*Group IIc/blistered*
Df(3L)HR119	63C2;63F7	*	Yes	
DF(3L)ZN47		—	Yes	
Df(3L)AC1	67A5;67D13	*		
DF(3L)brm11		—	Yes	
Df(3L)vw3		—	Yes	
Df(3R)Antp17	84B1;84D12	*	—	
Df(3R)TE32	86E2;87C7	—	Yes	
Df(3R)ry615		—	Yes	
Df(3R)P14		—	Yes	
Df(3R)Cha7		—	Yes	
Df(3R)e-N19		—	Yes	
Df(3R)Tl-P	97A;98A2	*	—	
Df(3R)awd^{krb}	100C;100D	*	—	

—, no interaction observed; ND, not determined; *, 30–50% of wings have ectopic material along posterior cross vein (PCV) and vein V; **, 50–74% of wings have ectopic material along PCV and vein V; ***, 75–100% of wings have ectopic vein material and defects in anterior cross vein (ACV) and PCV; ****, 100% ectopic wing material and >10% of wings blistered.

[a] Deficiency has a dominant Plexate phenotype—wing blistering observed.

tions. Twenty-three of the enhancer mutations fell into five allelic complementation groups on the basis of lethality (Table 2). Four of these complementation groups were on the second chromosome and one was on the third chromosome.

Examples of the modified *en*Δ*BB* phenotypes that we observed in the screen are shown in Figure 3 (middle column). In all cases, an increase in ectopic venation at the vein V/posterior cross vein intersection was observed, although the amount of material varied between groups and within each group depending on allele strength. We kept only those mutations in which at least 50% of the wings expressed a modification of *en*Δ*BB* phenotypes. Mutations that displayed dominant phenotypes in the absence of the *en*Δ*BB* chromosome were discarded. To show that these mutations also affect overgrowth, we compared the area in the posterior compartment (between vein III and the posterior margin) of *en*Δ*BB*/modifier wings to *en*Δ*BB*/+ wings (Table 3). Several members of each complementation group were analyzed and in each case there was an increase in size of *en*Δ*BB* wings with the presence of a modifying mutation when compared to outcrossed *en*Δ*BB* wings.

Genetic tests to identify relevant modifiers: To further characterize these modifying mutations, several tests were designed to distinguish between mutations relevant for understanding the mechanisms of Merlin function and those mutations that are uninformative or

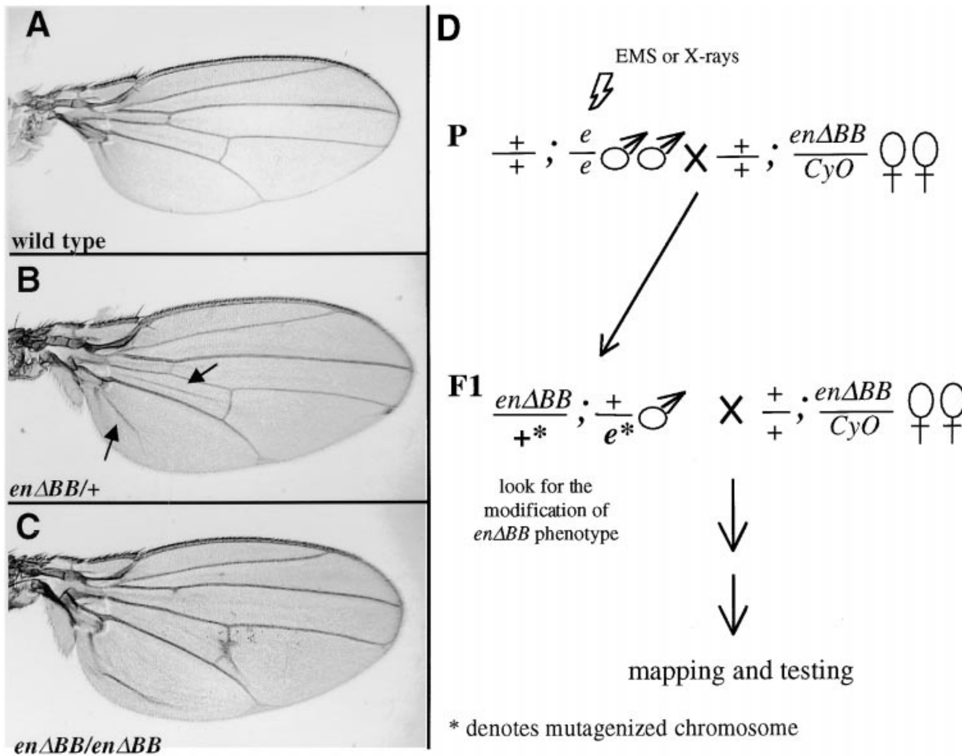

FIGURE 2.—The *en*Δ*BB* phenotype is dose sensitive. (A) Wild-type female wing; (B) wing from female heterozygous for *en*Δ*BB*/+. Note the folds in the posterior compartment (arrows) and the slight disruption of the anterior cross vein. (C) Wing from a female homozygous for *en*Δ*BB*. Note the increase in posterior wing compartment in comparison to the heterozygous *en*Δ*BB* flies in B, the disruption of the posterior cross vein, and the ectopic material along vein V and the loss of anterior cross vein. (D) Screen protocol: males carrying isogenized second and third chromosomes were treated with mutagen (either EMS or γ rays) and mated to virgin *en*Δ*BB*/*CyO* w^+ females. In the F_1 generation, male flies having a modified phenotype were collected and backcrossed to *en*Δ*BB*/*CyO* w^+ females for two further generations to eliminate any somatic mosaics that arise during mutagenesis. Flies that displayed modification phenotypes in the F_3 generation were established as stocks and the chromosomal location of the modifying mutation was mapped.

misleading. For instance, mutations that affected the expression of the *engrailed::GAL4* driver would indirectly influence the dominant $Mer^{\Delta BB}$ phenotype and would be of little interest. To eliminate mutations that have dominant transcriptional effect on *engrailed* expression and to demonstrate the direct effect of a modifier on $Mer^{\Delta BB}$ activity, we tested each candidate's ability to modify phenotypes displayed by *ap*Δ*BB* flies. In *ap*Δ*BB* flies, the *apterous::Gal4 driver* expresses *UAS::*$Mer^{\Delta BB}$ at high levels throughout the dorsal surface of the developing wing blade (Figure 3C), resulting in phenotypes that are more severe than those expressed by *en*Δ*BB*. Wings from *ap*Δ*BB* flies are outheld, overgrown, and have ectopic venation primarily along veins II and V (LaJeunesse *et al.* 1998). *Group IIc* and *Group IIa* mutations produced a significant blistered wing phenotype in combination with *apterous* $Mer^{\Delta BB}$ (data not shown). Mutations in all five complementation groups display modification of the *ap*Δ*BB* phenotype (Figure 3, third column) indicating that the modification is due to an effect on $Mer^{\Delta BB}$ activity and not the *engrailed::Gal4* driver.

Modification of Mer^3 phenotypes: The second genetic test avoided overexpression of $Mer^{\Delta BB}$ altogether and instead examined the ability of the interactor to modify phenotypes expressed by a hypomorphic *Merlin* mutant allele, Mer^3. Mer^3 hemizygous males are semiviable with visible phenotypes expressed in the wings, legs, and head already described in results (McCartney *et al.* 2000). Under normal conditions ~50% of the expected number of Mer^3 male flies eclose. Mutations in three complementation groups, *Group IIa*, *Group IIb*, and *Group IIIa*, dominantly enhanced Mer^3 to lethality or reduced eclosion of Mer^3 males to <1% (Table 2).

The other two groups, *Group IIc* and *Group IId*, also modified Mer^3 phenotypes, but in a qualitatively different manner. The modification was restricted to the wing and we observed neither alteration of viability nor head or leg defects (Table 2). The Mer^3 wing phenotype is characterized by increase in the size of the wing blade with mild disruptions in venation, particularly the posterior cross vein (Figure 4B, Table 4). Hemizygous Mer^3 flies that are also heterozygous for a mutation in *Group IIc* had significantly smaller wings when compared to Mer^3 wings alone, but had a significant increase in the number of posterior cross vein disruptions (Figure 4C, Table 4). Mutations in *Group IId* also significantly reduced the size of the wing. Unlike the *Group IIc* modification, however, *Group IId* dominantly reduced the disruptions in venation (Figure 4D, Table 4).

Disruption of Merlin subcellular localization: As a further test of the relevance of the interacting complementation groups, we examined the subcellular localization of Merlin in cells that were homozygous for mutations in each group. In previous work, we showed that

TABLE 2

Summary of F_1 second-site modifier screen

Complementation groups	Mutagen	Chromosomal location	Interactions with $Mer^{\Delta BB}$	Interactions with Mer^3	Merlin distribution	Lethal period
Group IIa/sbb^{94}	EMS	55C1–2/2-82.2	***	<1%	Altered	Viable[a]
IIa/sbb^{151}	EMS		***	lethal	—	Pupal
IIa/sbb^{216}	EMS		**	<1%	—	Viable[a]
IIa/sbb^{256}	EMS		***	lethal	Altered	Pupal
IIia/sbb^{259}	EMS		**	lethal	Altered	Pupal
IIa/sbb^{270}	EMS		**	<1%	Altered	Pupal
IIa/sbb^{324}	EMS		**	<1%	—	Pupal
Group IIb^{182}	EMS	30A1–F/2-33	**	<1%	Normal	Viable[b]
IIb^{187}	EMS		***	<1%	Normal	Viable
IIb^{209}	EMS		***	<1%	Normal	Viable
Group IIc/bs^{237}	EMS	60C1–2	**	—	Altered	Viable[a]
bs^{211}	EMS		**	—	—	Viable
bs^{253}	EMS		*	MWP	—	Pupal/viable
bs^{242}	EMS		***	MWP	Altered	Larval
bs^{246}	EMS		**	—	Altered	Viable
bs^{364}	X ray		****	—	—	Larval
bs^{221}	EMS		***	—	—	Late larval
Group IId/net^{107}	EMS	21B8	**	MWP	Normal	Viable[a]
net^{383}	X ray		***	MWP	Normal	Viable
Group IIIa^{202}	EMS	66E–F/3-26	*	—	Normal[c]	Pupal[c]
IIIa^{239}	EMS		***	<1%	Normal[c]	Embryonic/larval
IIIa^{278}	EMS		**	<1%	Normal[c]	Larval/pupal
IIIa^{320}	EMS		*	—	Normal[c]	Larval/pupal

MWP, modified wing phenotype; —, not tested; *, 30–50% of wings have ectopic material along PCV and vein V; **, 50–74% of wings have ectopic material along PCV and vein V; ***, 75–100% of wings have ectopic material and defects in ACV and PCV; ****, 100% ectopic wing material and >10% of wings blistered.

[a] Determined as hemizygous over deficiency.

[b] Viable over deficiency but lethal *in trans* with one another.

[c] Determined as transheterozygotes.

the proper subcellular distribution of Merlin is important for its function (LaJeunesse *et al.* 1998). Mutations in two of the modifying loci resulted in altered Merlin subcellular distribution. Within the cells of the imaginal epithelium, Merlin is found associated with the apical plasma membrane in the region of the adherens junction and throughout the apical cytoplasm associated with discrete punctate structures (McCartney and Fehon 1996). In *Group IIa* or *Group IIc* mutant backgrounds, Merlin is mislocalized to large vesicular bodies basal to the adherens junction (Figure 5). These vesicular bodies are not present in every cell within the imaginal epithelium and can be found to a greater extent within the central nervous system, particularly the ventral ganglion (data not shown). We have tested for the presence of actin, α-spectrin, two adherens junctions components (Armadillo and Moesin), Notch, and the septate junction protein Coracle, and none co-localized with Merlin within these bodies (data not shown). The identity of these structures is currently unknown. Regardless, the observed alteration of the subcellular localization of Merlin is consistent with the idea that these modifiers function with Merlin to regulate proliferation and differentiation.

Characterization of the complementation groups: *Group IIb:* Group IIb mapped to the left arm of the second chromosome at 2-[33], between *P{w^{+mC} = lacW} l(2)10424^{k06801}* at 2-[18]/26B1-2 and *P{w^{+mC} = lacW} Pen^{k14401}* at 2-[36]/31A and has been placed in the cytological region 30D;31F on the basis of noncomplementation of a recessive wing phenotype with *Df(2L)Mdh* (Figure 6B). All *Group IIb* mutations complement all previously described mutations (see materials and methods) that map to this region. All transallelic combinations of the three *Group IIb* alleles identified in this screen have an early larval lethality with no distinct phenotypes. The allele strengths of the three alleles based on interaction with *en*Δ*BB* are as follows: *IIb^{187}*, *IIb^{182}* > *IIb^{209}*.

Group IIc/blistered: Group IIc mapped to the end of the right arm of the second chromosome and failed to complement the lethality of *Df(2R)Px2,* a deletion of

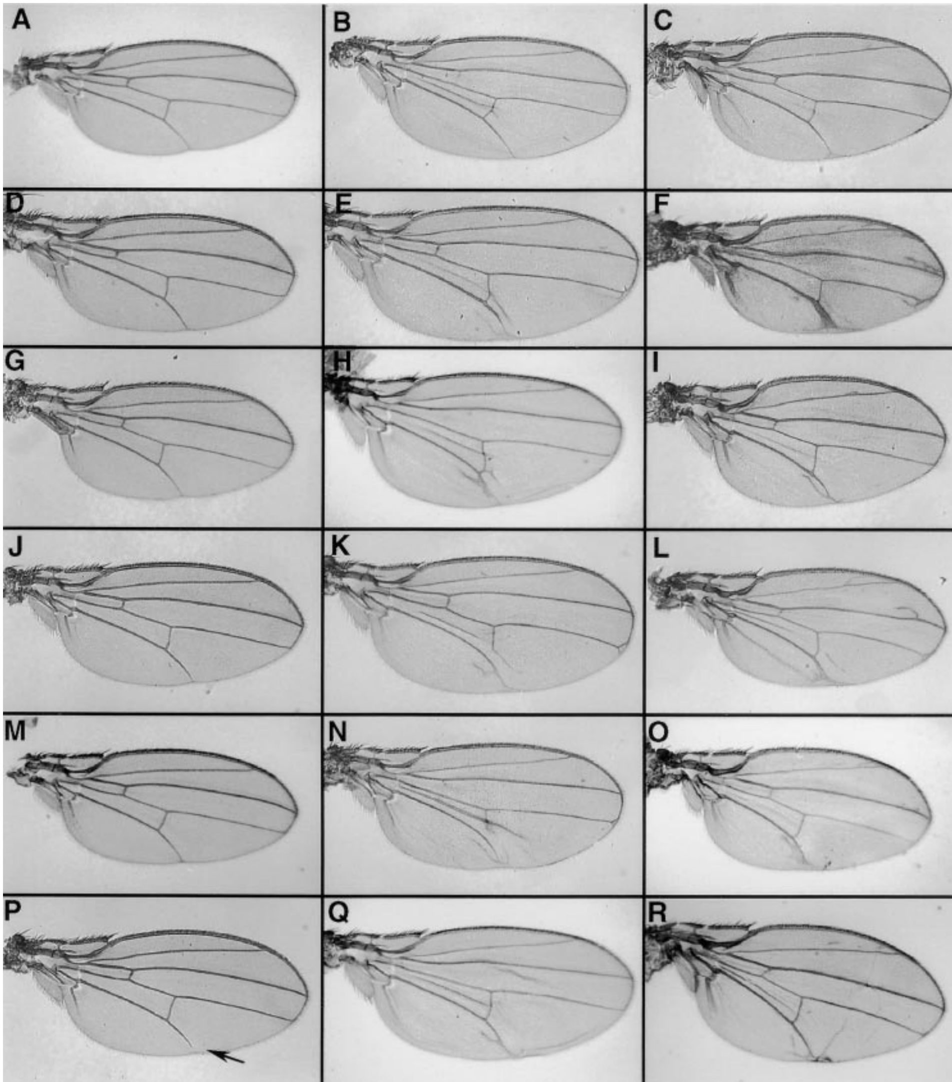

FIGURE 3.—Examples of modifier wing phenotypes. (A) Wild-type wing. First column (D, G, J, M, and P), female wings heterozygous for mutations identified in the screen. Note that all mutations are recessive except for P, which has an abrupt vein V (arrow). Second column, modification of *en*Δ*BB* heterozygous phenotype (B) by modifiers (E, H, K, N, and Q). Third column, modification of *ap*Δ*BB* (C) phenotypes showing that the modifications are not promoter specific. All wings are from female flies. The genotypes are as follows: (A) wild type; (B) *en*Δ*BB*/+; (C) *ap*Δ*BB*/+; (D) *Group* $IIc\text{-}bs^{242}$/+; (E) *en*Δ*BB*/ *Group* $IIc\text{-}bs^{242}$; (F) *ap*Δ*BB*/*Group* $IIc\text{-}bs^{242}$; (G) *Group* IIa^{256}/+; (H) *en*Δ*BB*/*Group* IIa^{256}; (I) *ap*Δ*BB*/*Group* IIa^{256}; (J) *Group* $IId\text{-}net^{383}$/+; (K) *en*Δ*BB*/*Group* $IId\text{-}net^{383}$; (L) *ap*Δ*BB*/ *Group* $IId\text{-}net^{383}$; (M) *Group* IIb^{187}/+; (N) *en*Δ*BB*/*Group* IIb^{187}; (O) *ap*Δ*BB*/ *Group* IIb^{187}; (P) *Group* $IIIa^{239}$/+; (Q) *en*Δ*BB Group* $IIIa^{239}$; and (R) *ap*Δ*BB*/ *Group* $IIIa^{239}$.

60C6;60D9. *Group IIc* mutations were shown to be allelic to *blistered* (*bs*), a gene located within this interval, by failure to complement bs^{03267}, a null mutation. *blistered* encodes the Drosophila homologue of serum response factor (GUILLEMIN *et al.* 1996; MONTAGNE *et al.* 1996). Seven new alleles of *blistered* were identified. All are recessive and appear to be hypomorphs except for the X-ray allele, *Group IIc*/bs^{364}. This allele is semidominant with ectopic vein material, which are characteristic properties of null or strong hypomorphic *blistered* alleles (FRISTROM *et al.* 1994). Moreover, all lethal allelic combinations showed the abbreviated larval tracheal phenotype, another characteristic *blistered* phenotype (data not shown; GUILLEMIN *et al.* 1996). The allelic series of *blistered* as determined by lethal period is identical to the allelic series established by the interaction with *en*Δ*BB* with the order as follows: $bs^{364} > bs^{242} > bs^{221} > bs^{246} > bs^{253} > bs^{237} > bs^{217} > bs^{211}$. This result suggests that the interaction of *blistered* with *Merlin* is a direct function of gene dosage. Transheterozygous combinations of all *blistered* alleles (except for transallelic combinations of

TABLE 3

Enhancement of $Mer^{\Delta BB}$ overproliferation wing phenotype

Genotype	*n*	Mean area of posterior compartment (mm²)	SE	% deviation from *en*Δ*BB*/+
+/+	30	0.510	0.007	−31.0
*en*Δ*BB*/+	20	0.735	0.009	—
bs^{242}/*en*Δ*BB*	25	0.773	0.008	+5.5
IIa^{256}/*en*Δ*BB*	30	0.746	0.005	+2.7
IIa^{270}/*en*Δ*BB*	40	0.776	0.008	+6.8
IIa^{94}/*en*Δ*BB*	35	0.832	0.006	+13.7
net^{383}/*en*Δ*BB*	20	0.798	0.009	+8.2
IIb^{187}/*en*Δ*BB*	20	0.772	0.011	+5.5
IIb^{209}/*en*Δ*BB*	27	0.790	0.010	+6.8
$IIIa^{320}$/+; *en*Δ*BB*/+	26	0.759	0.014	+2.7

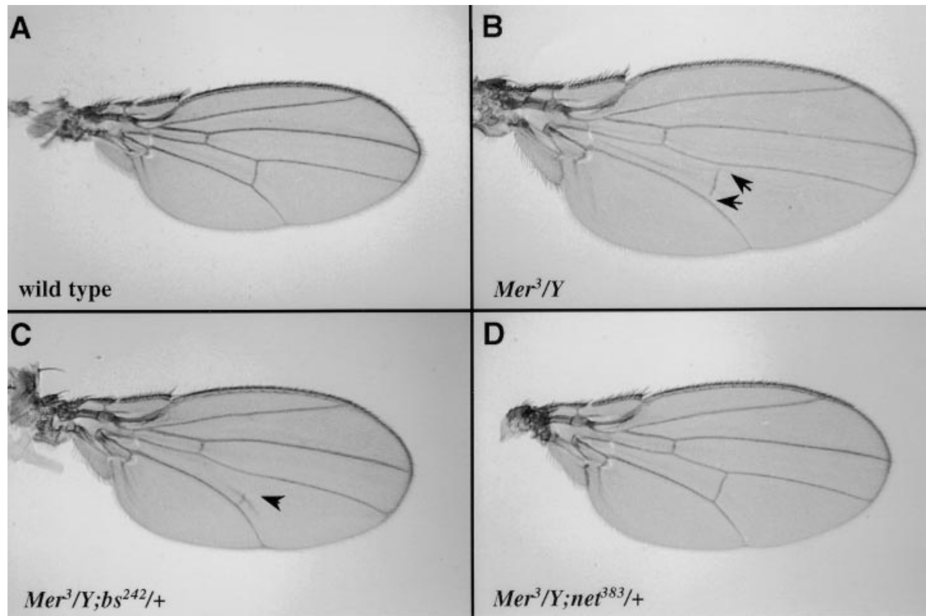

FIGURE 4.—Modification of the *Mer³* wing phenotype by *blistered* and *net*. (A) Wing from a wild-type male fly; (B) wing from a *Mer³* hemizygous male fly. Note the overall increase in size and slight disruption of the posterior cross vein (between the two arrows). (C) Wing from a *Mer³* hemizygous male also heterozygous for *Group IIc/bs²⁴²*. Note the reduced size compared to *Mer³* wing and disrupted posterior cross vein (arrowhead). (D) Wing from a *Mer³* hemizygous male heterozygous for *Group IId/net³⁸³*. Note the reduced size and suppression of any defects in venation.

bs$^{364, 242, 221}$, which are larval lethal) produce adult escapers that have a tube wing phenotype due to conversion of all intervein tissue to vein (FRISTROM *et al.* 1994; MONTAGNE *et al.* 1996; Figure 6C).

Group IId/net: Deletion mapping localized Group IId to the tip of the left arm of the second chromosome. Complementation analysis using *Group IId*^{107} suggested that it was a terminal deficiency at the tip of 2L, because it failed to complement *Df(2L)PMF47c*, *Df(2L)net62*, and three mutations located within these deficiencies: *lethal (2) giant larvae*, *broad head*, and *net*. Of these genes, only mutations in *net* modified *Merlin* phenotypes, suggesting that *net* is the modifying locus within this region. *net* belongs to the *plexus* phenotypic group within the "excess-of-vein" mutant class (DIAZ-BENJUMEA and GARCIA-BELLIDO 1990). Two new alleles of *net* were recovered. *net*^{383} is a viable X-ray allele that has the characteristic ectopic venation phenotype (Figure 6D).

Group IIIa: Group IIIa is a novel group that maps by meiotic recombination to the left arm of the third chromosome at 3-[26] between $P\{w^{+mC} = lacW\}l(3)L1170^{L1170}$ and $P\{w^{+mC} = lacW\}l(3)j2B9^{j2B9}$, falling roughly within cytological interval 66A;66D. Four alleles were identified. No deficiency was identified that uncovers this complementation group. The allele strength based on the lethality of heteroallelic combinations is as follows: $IIIa^{239} > IIIa^{278} > IIIa^{320} > IIIa^{202}$. The strongest allelic combination, *Group IIIa*^{239}/*Group IIIa*^{278}, displays late embryonic/early larval lethality with no distinct phenotypes. Weaker allelic combinations, such as *Group IIIa*^{320}/*Group IIIa*^{202} and *Group IIIa*^{278}/*Group IIIa*^{202}, die as early pupae with little development past the white prepupa stage (data not shown). *Group IIIa*^{239} heterozygous flies have a weakly penetrant (28%) abrupt vein V phenotype, resulting in a gap between the margin and the end of the vein (Figure 3P, small arrow).

TABLE 4

Modification of *Mer³* wing phenotypes

Genotype	*n*	Mean area (mm²)	% deviation from wild type	SE	% deviation from *Mer³/Y*	% of wings with ectopic or disrupted	
						V2	V5/PCV
+/Y; +/+	32	0.622	0	0.007	−40	0	0
Mer³/Y; +/+	24	1.039	+67	0.009	0	3	25
Mer³/Y; bs²⁴²/+	21	0.762	+22.5	0.010	−27	100	100
Mer³/Y; bs²⁵³/+	40	0.808	+30	0.007	−22	50	50
Mer³/Y; bs³⁶⁴/+	27	0.786	+26	0.008	−24	96	96
Mer³/Y; bs⁰³²⁶⁷/+	28	0.772	+24	0.0011	−26	100	100
Mer³/Y; net³⁸³/+	23	0.717	+15	0.008	−31	4	29
Mer³/Y; net¹⁰⁷/+	28	0.799	+29	0.012	−23	7	7

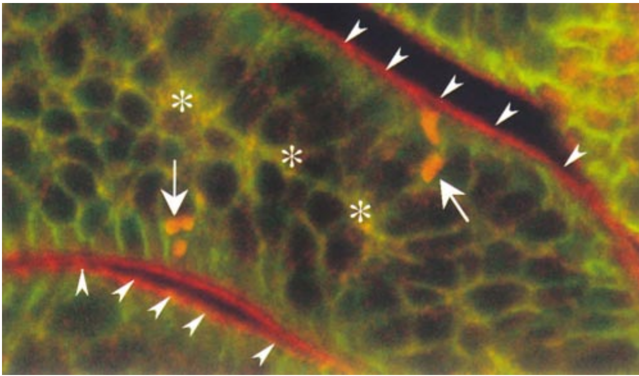

Figure 5.—Mislocalization of Merlin in *Group IIa/scribbler* genetic backgrounds. Optical cross section of the wing imaginal epithelia from a sbb^{324}/sbb^{324} mutant third instar larva. The majority of Merlin (in red) is found in the apical regions of the cell (small arrowheads), as would be found in a wild-type genetic background. However, in some cells, basal aggregations of Merlin protein (large arrows) are seen. Asterisks mark the basal portions of the cells. The septate junction and cell membrane are labeled in green using anti-Coracle antibody.

Group IIa/scribbler: Group IIa mutations mapped to 2-[83] between *P{w + mc + lacW}AA48* and $P\{w^{+mW.hs} =$ *GawB}559.1.* A test of the available deficiencies in the 54D–55F region showed that *Group IIa* mutations mapped to cytological region 55C2;55F on the basis of failure to complement the lethality of *Df(2R)PC4.* In addition, two lethal *P*-element insertions in this cytological interval (*l(2)04440* and *l(2)k00702*) failed to complement visible wing phenotypes of *Group IIa* mutations (data not shown). However, neither *P*-element mutation genetically interacted with either $Mer^{\Delta BB}$ or Mer^{3}.

The most severe *Group IIa* alleles were hemizygous pupal lethal, although rare escapers can be found with wing defects including reduced size and ectopic vein material (Figure 6E). On the basis of the hemizygous lethal period the following allelic series of *Group IIa* mutations was constructed: IIa^{270}, IIa^{256}, $IIa^{151} > IIa^{324}$, IIa^{259}. Two alleles, IIa^{94} and IIa^{216}, are homozygous/hemizygous viable and express a wing phenotype *in trans* with the lethal *Group IIa* alleles and the deficiency that is similar to the wing phenotypes displayed by the rare escapers from the lethal *Group IIa* alleles. Both mutations were placed into *Group IIa* on the basis of meiotic mapping. There is no direct correlation of the allelic series based on lethal period with that based on interaction with *Merlin* phenotypes, suggesting that the *Merlin* interactions involve something other than simple loss of function.

During our investigation, three other laboratories identified the same *P*-element insertions (*l(2)04440* and *l(2)k00702*) as mutations in a gene called *scribbler* or *brakeless* (Rao *et al.* 2000; Senti *et al.* 2000; Yang *et al.* 2000). Two groups demonstrated that, in *scribbler* mutants, axons from photoreceptors R1–R6 failed to stop properly upon reaching their targets in the optic lobe of the pupal brain during Drosophila eye development (Rao *et al.* 2000; Senti *et al.* 2000). A third group studying Drosophila foraging behavior named this gene *scribbler* because homozygous mutant larvae display aberrant crawling patterns (Yang *et al.* 2000). Although the

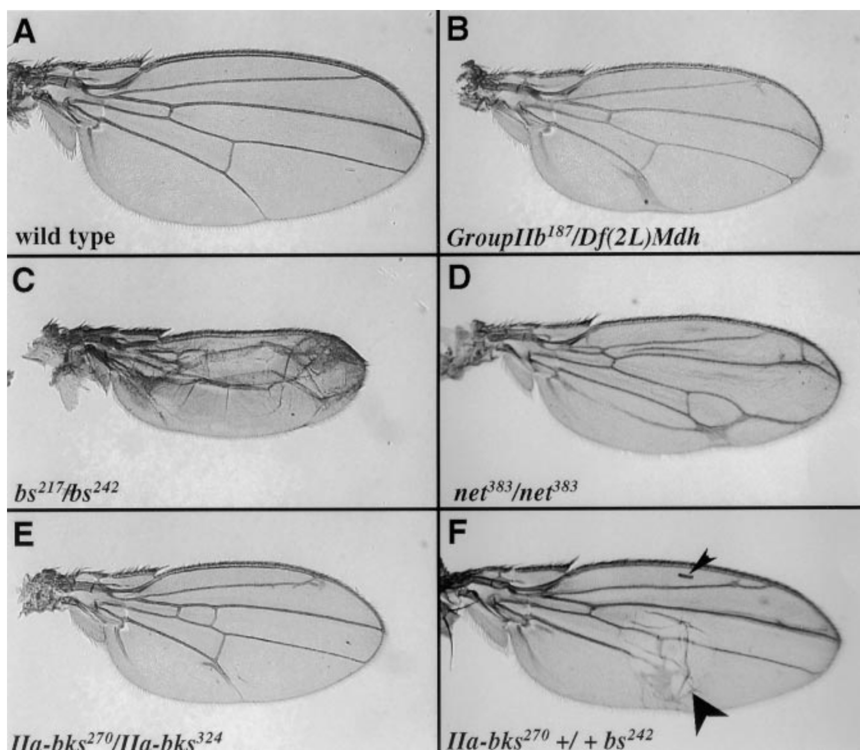

Figure 6.—Wing phenotypes of Merlin modifiers. (A) Wild-type female wing. (B) Wing from $IIb^{187}/Df(2L)Mdh$, which displays extra wing vein. (C) Wing from bs^{217}/bs^{242} female fly. All intervein cells have been transformed into vein tissue giving the wing a tube-like appearance. (D) Wing from net^{383} homozygous female fly with disturbed normal vein patterning and extra veins. (E) Wing from IIa^{270}/IIa^{324} escaper. Note ectopic wing vein material along veins II and V. (F) Wing from IIa^{270} +/ + bs^{242} transheterozygous female fly demonstrating second-site noncomplementation between two recessive mutations (compare to Figure 2, D and G). The broad arrowhead indicates a large blister in the wing. Note the alteration in the shape and size when compared to A and the presence of ectopic vein material (thin arrowhead).

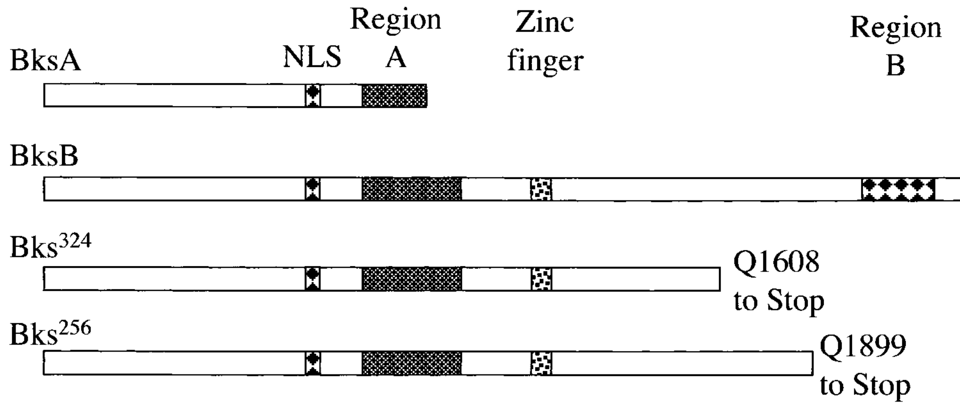

Figure 7.—Two of the *Group IIA/sbb* alleles identified in this screen are missense mutations that affect only the SBBB isoform. *Group IIA/sbb*^{324} correlates with a nonsense mutation at arginine 1608 and *Group IIA/sbb*^{256} with a nonsense mutation at arginine 1899. Both mutations may produce truncated SBBB proteins missing Region B, a novel conserved C-terminal domain.

sbb alleles isolated in our screen do not express either phenotype, *Group IIa* alleles (*Group IIa*^{256} and *Group IIa*^{324}) fail to complement the lethality of a strongly hypomorphic *scribbler* allele *sbb*^{4}, which correlates with a 436-bp deletion in the third exon of *sbb* (Rao *et al.* 2000). In addition, sequence analysis of the same two *Group IIa* alleles revealed nonsense mutations within the *scribbler* coding region (Figure 7). Together these results indicate that *Group IIa* corresponds to the *scribbler* gene, and is henceforth called by this name.

Second-site noncomplementation between *scribbler* and *blistered*: In the course of our complementation analysis, we observed second-site noncomplementation between the *scribbler* alleles identified in this screen and mutations in *blistered*. Such interactions are relatively uncommon and when observed are usually a good predictor of strong functional relationships between the interacting genes (Shearn 1989; Tripoulas *et al.* 1996; Halsell and Kiehart 1998). Interactions were observed using both those *blistered* alleles identified in our screen and the null *sbb*^{03627} allele. Transheterozygous combinations of strong alleles of *blistered* and *scribbler* produced blistering and ectopic vein material in a significant fraction of the flies (Figure 6F). In contrast, the original *scribbler P*-element alleles (Rao *et al.* 2000; Senti *et al.* 2000; Yang *et al.* 2000), which are likely hypomorphic, completely complement *blistered* phenotypes. However, the deficiency that uncovers *sbb*, *Df(2R)PC4*, showed weaker second-site noncomplementation with *blistered* mutations (data not shown), suggesting that the *sbb* alleles identified in our screen are distinct from null or strongly hypomorphic *sbb* alleles.

DISCUSSION

Merlin is the Drosophila homologue of the human *NF2* gene and is required for the regulation of proliferation and differentiation of epithelial tissues. However, the mechanisms of Merlin function are unknown. To identify genes involved in Merlin's cellular functions we performed genetic screens for mutations that modify *Merlin* wing phenotypes caused by expression of a dominant negative form of *Merlin*, *en*Δ*BB*, and a hypomorphic allele of *Merlin*, *Mer*^{3}. In a screen of the deficiency kit, we identified 20 chromosomal regions that contain dose-sensitive modifiers of *en*Δ*BB* phenotypes and 23 chromosomal regions that modify the *Mer*^{3} phenotype. Eight regions were identified in both screens, suggesting that they contain *Merlin* modifiers of particular interest. However, we were unable to identify the individual genes within the cytological regions that modify *Merlin* phenotypes. To complement the deficiency screen, we performed a dominant second-site modifier screen designed to identify dose-sensitive modifiers of *Merlin* wing phenotypes generated by *en*Δ*BB*. In a screen of 100,000 progeny from mutagenized flies, we identified 29 recessive mutations that modify *Merlin* phenotypes. Twenty-three of the mutations fall into five complementation groups. Three of the complementation groups are new alleles of previously identified genes, *sbb*, *bs*, and *net*. Two groups, *Group IIb* and *Group IIIa*, are novel genes. Genetic tests suggest that mutations in all five complementation groups interact with *Merlin* to regulate proliferation or differentiation.

None of the modifying mutations were in the regions that were identified by the *Mer*^{3} deficiency kit screen, including one region (*In(2R)bw*^{VDe2L}) with a very strong genetic interaction. Moreover, no new alleles of *expanded*, another Drosophila 4.1 family member and a previously characterized *Merlin* modifier (McCartney *et al.* 2000), were identified, despite the fact that an existing amorphic *expanded* allele interacts with both *Mer*^{3} (McCartney *et al.* 2000) and *en*Δ*BB* (data not shown). There are several explanations to account for these results. It is possible that the *In(2R)bw*^{VDe2L} deficiency (or the chromosome that carries it) contains additional mutations that singly do not exhibit an interaction above the phenotypic threshold used in this screen. Similarly, putative mutations in *expanded* may have fallen below the level of detection. Furthermore, mutations in other potential dose-sensitive modifiers of *Merlin* phenotypes may have generated dominant sterile or lethal interaction phenotypes with *en*Δ*BB* and would not have been recoverable from this screen. Interest-

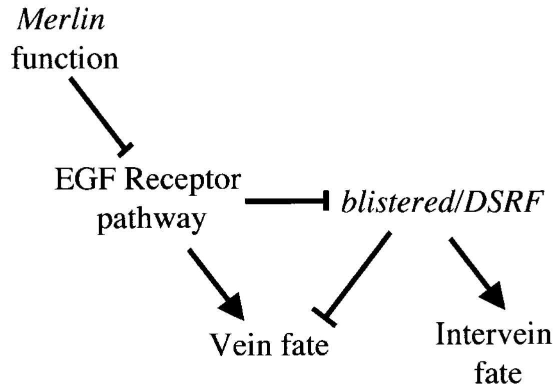

Figure 8.—Merlin may function antagonistically to EGFR pathway function. *blistered* expression is required for the formation of intervein regions within the wing and is negatively regulated by EGF signaling in presumptive vein tissue. Alteration in Merlin activity may hyperactivate EGF signaling in intervein regions, thus disrupting the differentiation of intervein regions and promoting the formation of ectopic veins.

ingly, we also found that while point mutations in the modifier groups displayed interactions with the Mer^3 allele, in some cases deficiencies that uncovered these genes did not show similar interaction with Mer^3 (Table 1). In these cases the observed genetic interactions may be allele specific (resulting from neomorphic, hypermorphic, or antimorphic mutations), although it seems likely that many, if not most, of the modifier mutations isolated are simple hypomorphic mutations. Regardless, this report illustrates that there are qualitative differences between a deficiency kit screen and a random mutagenesis screen for second-site modifiers. Although deficiency kit screens have been successful in identifying functionally related genes (Halsell and Kiehart 1998), the ability to screen a range of mutations in a common genetic background can allow for more complete and less ambiguous identification of interacting loci.

Merlin clearly has a role in the regulation of proliferation and differentiation; however, the proteins and pathways that are involved with Merlin function remain unknown. Therefore, the intent of our genetic screens was to identify genes that functionally and/or physically interact with Merlin and thus define the molecular context in which Merlin functions. Of the five complementation groups identified in this screen, *sbb* and *bs* were characterized molecularly and at this point hold the most potential in understanding Merlin function. In addition, we showed that mutations in *blistered* and *sbb* disrupt the subcellular localization of Merlin and that both mutations exhibit strong second-site noncomplementation, suggesting an underlying functional relationship between *sbb* and *bs* gene products and Merlin.

In our screen we identified seven new alleles of *sbb*; allelism was based on noncomplementation with a null *sbb* allele and the presence of nonsense mutations in two *sbb* alleles identified. Null and strong hypomorphic mutations in *scribbler* result in aberrant axon guidance and behavioral phenotypes (Rao *et al.* 2000; Senti *et al.* 2000; Yang *et al.* 2000). However, none of the *sbb* alleles identified in our screen display either of these phenotypes (data not shown). In addition, none of the previously identified *P*-element insertional mutations in *sbb* modify *Merlin* phenotypes, although the null sbb^4 allele and *Df(2R)PC4* do enhance Merlin phenotypes (data not shown and Table 1). These data suggest that *sbb* has two distinct functions, one in axon guidance of photoreceptor cells and the other in regulation of proliferation in epithelial cells, and that these functions are independent. Consistent with this model, previous studies showed that *sbb* encodes two novel proteins of unknown function, SBB-A and SBB-B (Figure 7; Senti *et al.* 2000; Yang *et al.* 2000). Although it was shown that the two SBB isoforms are functionally redundant in axon guidance (Senti *et al.* 2000), the presence of a zinc finger domain and a novel Region B in the larger SBB-B isoform suggests that it may have functions distinct from SBB-A. Sequence analysis indicates that two of the alleles we isolated as *Merlin* modifiers correlate with nonsense mutations that affect the SBB-B product but leave the BSKA product intact (the lesions in the other alleles have not yet been determined).

The identification of SBB-B mutations that specifically modify *Merlin* phenotypes but do not affect photoreceptor axon guidance supports a model where SBB-B has distinct functions in the proliferation and differentiation of wing tissue. Both SBB isoforms are reported to be nuclear proteins and the presence of a zinc finger in SBB-B suggests that it may be involved in transcriptional regulation. How SBB proteins interact with Merlin, a membrane-associated cytoplasmic protein, is unclear. The observation that Merlin subcellular localization is disrupted in *sbb* mutant cells makes this question particularly intriguing and suggests that *sbb* may play a role in a cellular pathway that regulates Merlin function. The identity of this pathway is currently unknown.

While *sbb* encodes novel proteins with unknown function, the *bs* gene product, also known as the Drosophila serum response factor (BS/DSRF), is a well-characterized transcription factor (Affolter *et al.* 1994; Guillemin *et al.* 1996). *bs* is required for formation of terminal tracheal branches and differentiation of the adult wing (Fristrom *et al.* 1994; Guillemin *et al.* 1996; Montagne *et al.* 1996; Roch *et al.* 1998). BS/DSRF activity, like that of its mammalian homologue, is regulated by the epidermal growth factor receptor (EGFR) signaling pathway (Roch *et al.* 1998). During development of the wing imaginal disc, cells can adopt one of two fates; most cells form wing blade (intervein tissue), while a subset form the characteristic longitudinal veins. BS/DSRF is believed to promote the intervein cell fate—loss-of-function *bs* mutations result in wings in which all cells develop as vein tissue. Activity of the EGFR pathway is believed to promote the vein cell fate by downregulating

BS/DSRF function in the vein primordia and promoting the expression of vein-specific genes. Thus interactions between the EGFR pathway and BS/DSRF play a crucial role in wing development.

The identification of *bs* as a dominant modifier of *Merlin* phenotypes suggests that Merlin, like Blistered, is involved in EGFR signaling. Specifically, the observation that *bs* mutations enhance *Merlin* dominant-negative and loss-of-function phenotypes suggests that Merlin may function antagonistically to EGFR pathway function (Figure 8). Although this hypothesis should be considered as tentative, several lines of evidence support this notion. First, developing wing cells that have lost both *Merlin* and *expanded*, which appear to function redundantly, produce abundant ectopic vein material adjacent to endogenous veins (McCartney *et al.* 2000). Second, *net,* which was also identified as a *Merlin* modifier, has been shown to modify phenotypes of components of EGFR signaling in the wing (Sturtevant and Bier 1995; Biehs *et al.* 1998). Third, a role for Merlin in negatively regulating EGFR function is consistent with the observation that Merlin mutations result in overproliferation phenotypes (LaJeunesse *et al.* 1998). Finally, a hypermorphic *EGFR* mutation called *Ellipse* enhances phenotypes expressed by dominant-negative and hypomorphic *Merlin* alleles (data not shown). However, despite these intriguing indications that Merlin may function to regulate EGFR pathway activity, it should be noted that *Merlin* does not interact genetically with several other known pathway members (*Star*, *asteroid*, and *rhomboid),* nor does it interact with hypomorphic *EGFR* mutations (data not shown). In addition, because other signaling pathways, including *dpp*, *wingless*, and *Notch*, are involved in vein specification (Roch *et al.* 1998), it is possible that Merlin functions to regulate one or more of these either instead of or in addition to the EGFR pathway. In support of this notion, *Merlin* and *expanded* have both been shown to genetically interact with *dpp* (McCartney *et al.* 2000). Further experiments are required to determine the significance of these genetic interactions. Nonetheless, the identification of *Merlin* modifiers suggests testable hypotheses regarding Merlin cellular functions and opens new avenues for further investigation of the molecular basis of the NF2 disorder.

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