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*Brakeless*: A Novel Modifier of Merlin Phenotypes

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<b>13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)</b> In the first year of our grant we have concentrated on determining the nature of <i>scribbler</i> . As presented in our Statement of Work the first specific aim deals with the "Molecular and genetic analysis of <i>bks</i> ( <i>sbb</i> ) function." We proposed four experimental approaches to this problem and have started on three. In a screen of ~5000 mutant second chromosomes, we have identified five new alleles of <i>scribbler</i> . The new alleles of <i>scribbler</i> are all lethal in trans with the null <i>scribbler</i> mutation, <i>sbb</i> <sup>4</sup> ; however, there is a range of genetic interaction phenotypes between these alleles and mutations in <i>Merlin</i> . We are currently determining the molecular lesions in these <i>scribbler</i> mutations and correlating them with their interaction with <i>Merlin</i> . We have also generated eight FLAG tagged mutant versions of <i>scribbler</i> . The transgenic animals are currently being generating. One interesting and intriguing result is that over expression of the larger but not the smaller wild-type isoform of <i>sbb</i> in the <i>Drosophila</i> wing results in overgrowth phenotypes similar to that observed with mutations in <i>Merlin</i> . As part of Specific Aim II, we have also shown a genetic interaction of mutations in <i>Cyclin E</i> with <i>scribbler</i> and <i>Merlin</i> mutations.				
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## Introduction:

In the first year of our grant we have concentrated on determining the role of *scribbler* (the *brakeless* gene had been officially recognized as *scribbler* by the *Drosophila* community) in the regulation of proliferation and differentiation in the epithelium of the *Drosophila* wing imaginal disc. Our goal is to determine the relationship between Merlin, the product of the *Neurofibromatosis type II* gene and the scribbler protein, a novel, nuclear protein of unknown function. The *scribbler* gene was identified as a genetic modifier of *Drosophila Merlin* phenotypes (LaJeunesse et al, 2001) and the relationship of its product with Merlin, a cytoplasmic/membrane-associated protein remains unclear. To address this issue we proposed a molecular and genetic analysis of the scribbler gene. As presented in the Statement of Work our first specific aim deals with the "Molecular and genetic analysis of *brakeless(scribbler)* function." We proposed four experimental approaches.

The first year has been productively spent with the generation of the tools needed for this research and has begun to result in some interesting discoveries, which will be detailed in later sections. Given the similarities, both functionally and structural, between *Drosophila* and human Merlin this work will be important for understanding the molecular basis of the human NF2 disorder. Furthermore, the discovery of a human *scribbler* homologue has made the proposed research far more relevant to the study of NF2.

## Body of the Report:

Our first goal as outlined in Specific Aims 1 was to determine the genetic requirement of the two scribbler isoforms:

**"(Specific Aim I) Question 1:** What are the genetic requirements for the two *scribbler* isoforms? ...**(Specific Aim I) Question 2:** Do mutations in a specific *scribbler* isoform sensitize the background to *Merlin* loss of function?"

Preliminary results suggested that there might be different functions for each isoform regarding the interaction with *Merlin*. Mutations the larger *scribbler* isoform had stronger genetic interactions with *Merlin* mutants than null *scribbler* alleles that removed both *scribbler* isoforms (LaJeunesse et al, 2001). Several other groups have recently reported on the roles of the two *scribbler* isoforms. The expression of either *scribbler* isoform appears to satisfy loss-of-function equally regarding viability, the axon guidance phenotype, and the behavioral phenotype (Senti et al, 2000; M. Suster personal communication). Furthermore, it was Funakoshi et al reported that the larger isoform appears to be predominant form in imaginal tissue (Funakoshi et al, 2001). This last result may partly explain our initial allele specific *scribbler* interactions with *Merlin*. Although both isoforms can substitute for one another, only the larger one is present in imaginal discs and therefore mutations in it should have a specific genetic interact with *Merlin*. However, none of these data supports an argument that the larger isoform has specific differential roles in imaginal development, specifically wing development. Moreover, none of these data support an argument explaining why a specific set of mutations in

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*scribbler* result in genetic interactions with *Merlin*, which are stronger than the interactions with null *scribbler* alleles.

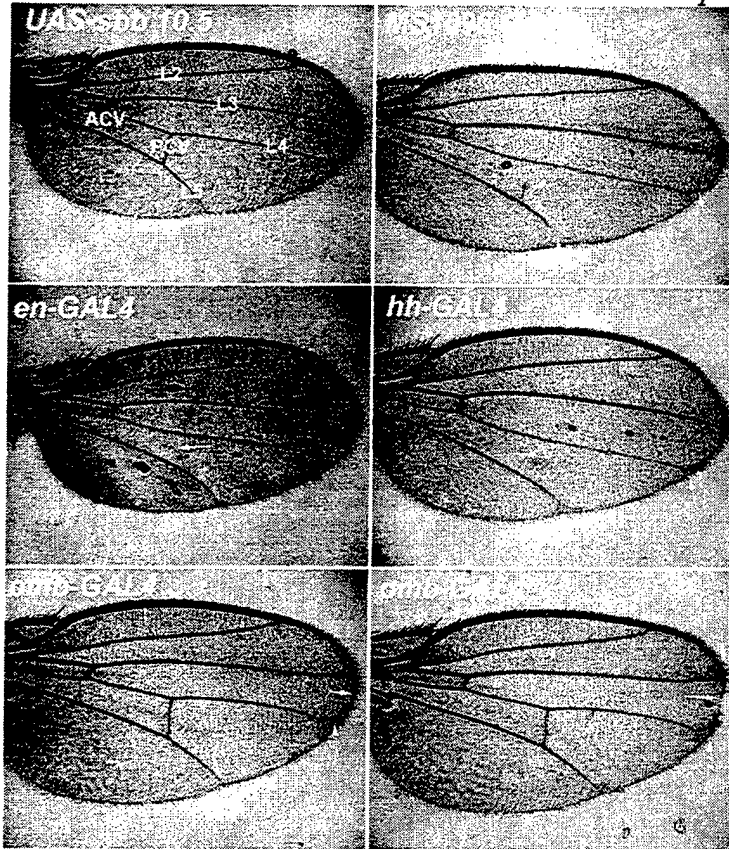
To address these issues, we set out to identify more alleles of *scribbler* on the basis of non-complementation to a null allele and independently test their genetic interaction with *Merlin*. In a genetic screen of ~3000 mutant chromosomes we have identified 6 new alleles of *scribbler*. The screen went perfectly. Our goal for this aspect of the project is to identify at least 15 more. All 5 new *scribbler* alleles are lethal *in trans* with the null *scribbler*<sup>4</sup> allele and in preliminary studies these alleles show a range of genetic interaction phenotypes with the hypomorphic *Merlin*<sup>3</sup> allele and the antimorphic *Merlin*<sup>ABB</sup> allele (Table 1). We are in the process of finishing the phenotypic characterization of the genetic interactions with *Merlin* and are beginning to sequence each allele. The idea will be to see if we can correlate specific mutations in *scribbler* to the strength of genetic interactions with Merlin thereby identifying crucial functional regions in the gene.

**Table 1: Results from the mutagenesis, 5 new alleles of *scribbler*.**

Allele	Interaction with <i>Mer</i> <sup>3</sup>	Interaction with <i>Mer</i> <sup>ABB</sup>
scribblerM78	ND	**
scribblerF40	*	*
scribblerT19	ND	***
scribblerN73	ND	*
scribblerFF26	**	***

N.D. – not determined, “—” no interaction, \* weak, \*\* medium, \*\*\* strong interaction

This past year we have also characterized a gain-of-function *scribbler* phenotype. Loss-of-function for *scribbler* results in a *Drosophila* wing phenotype characterized by smaller wing with ectopic wing vein structures such as extra crossveins, smaller wing size, and ectopic vein material along longitudinal veins (LaJeunesse et al, 2001). Interestingly, ectopic expression of the **larger** *scribbler* isoform, **but not the smaller** *scribbler* isoform, results in an alteration of wing morphology (Figure 1). Wings expressing the larger *scribbler* isoform using a number of different Gal4 drivers are significantly larger than wild-type control wings and display alterations to patterning with missing cross veins, most notably the posterior cross vein, notching of the wing margin and the formation of terminal vein deltas at the margin. These phenotypes are very similar to ectopic expression of a dominant negative Merlin protein (LaJeunesse et al, 1998) and suggest that *scribbler* may be positively regulate proliferation. Currently, we are determining the nature of the gain-of-function *scribbler* large wing phenotypes and whether there is proliferation defect in *scribbler* loss-of-function.



**Figure 1: Over expression of UAS::SbbB isoform under several different Gal4 results in disruption of patterning and increase in overall wing size. The top right image shows the phenotype of a wing that carries but does not express the larger SbbB isoform. The top left image is a wing expression the larger isoform throughout the developing wing under the *MS1096-GAL4* driver. Note that the wing is larger and there are disruptions to the posterior cross vein and along the wing margin. Similar phenotypes are seen with different Gal4 drivers that express in different part of the developing tissue: *en-GAL4* and *hh-GAL4* both express in the posterior half of the wing, *omb-GAL4* expresses in the central region between L2 and L5. In each case an obvious alteration in overall wing size can be observed, as can disruptions to venation at the cross veins and along the wing margin (arrows).**

A major problem with interpreting the *scribbler*/*Merlin* genetic interaction arises from the fact that scribbler encodes a novel protein with unknown function and the Merlin protein is a membrane associated cytoplasmic protein. To address this problem, we are in the process of performing a structure/function analysis of the scribbler proteins as proposed in question 3 of specific aim I:

**“(Specific Aim I) Question 3: What are the functions of conserved domains found within BKS? Approach: Generate transgenic animals and *in vitro* expression constructs to genetically and biochemical determine the role of these domains.”**

The idea of this analysis is to define the requirements of specific region in the scribbler proteins and correlate this to Merlin function. As mentioned earlier, *scribbler* has two isoform as the result of alternative splicing. The smaller isoform A is made up of 929 amino acids while the larger isoform B is made up of 2023 amino acids (Rao, et al, 2000; Yang et al, 2000; Senti, et al, 2000). Both proteins contain a putative N-terminal PEST sequence which is involved with protein turnover and degradation and a novel but well conserved Region A of unknown function (Senti et al, 2000; Funakoshi et al, 2001). The larger *scribbler* isoform has two additional motifs: a C2H2 zinc finger domain, and another novel but well conserved region of unknown function, Region B. To determine the importance and the role that each of these functional motifs have in *scribbler* function, we have constructed the six mutant *scribbler* transgenes and

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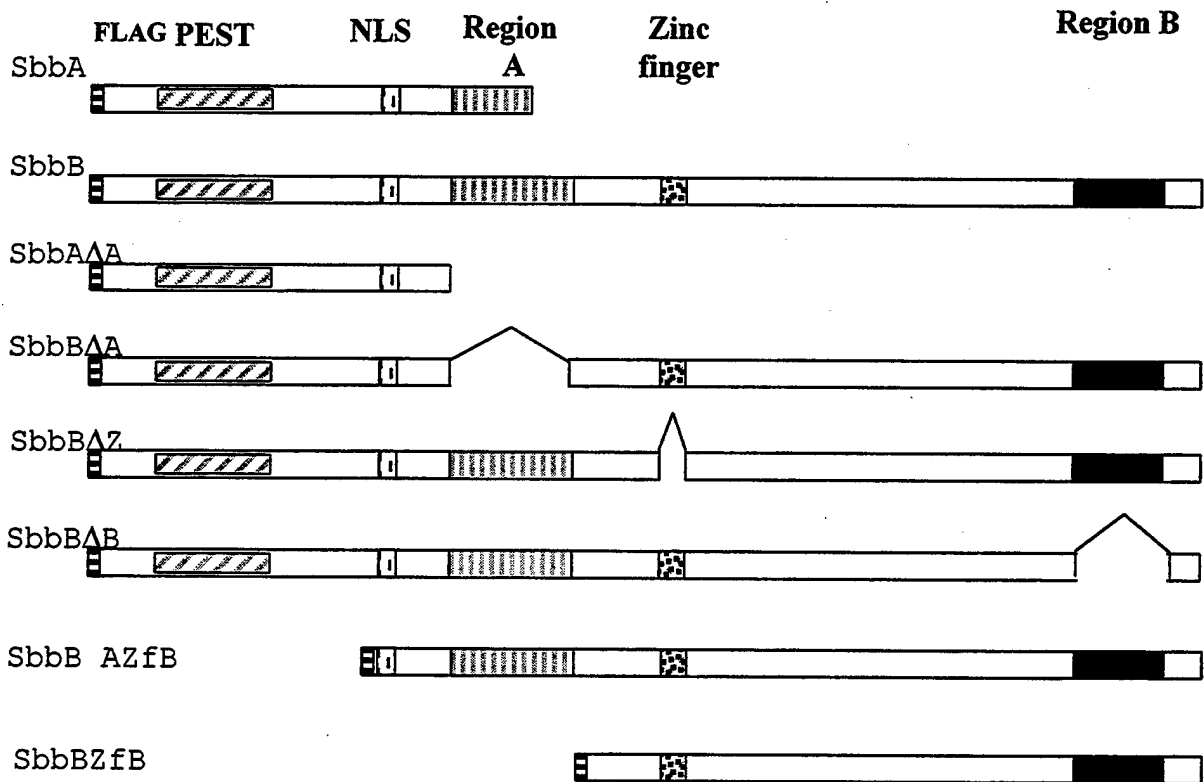
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two wild type control transgenes (Figure 2). All of these constructs have a FLAG epitope tag on their N-terminus to permit detection of our mutant protein independent of the endogenous Scribbler. We are in the process of generating the transgenic animals. This is our goal for the summer of 2002.

To date we have several constructions in the G0 generation and are injecting ~600 embryos daily per construct. We have experienced several setbacks regarding viability of injected embryos. Initially, our survivability was <3%, making injection unfeasible and the expectations transgenic animals low. However, upon purchase of a new lot of halocarbon oil (used to cover embryos to prevent desiccation during the injection process) these have been resolved. Our plan is to generate 3-5 transgenic lines for each of the constructs. These transgenic lines will be used to address the following four questions. **1) What are the genetic requirement for scribbler?** We will answer this by genetic rescue experiments of *scribbler* loss-of-function. In these experiments we will also be able to determine whether any of our mutations express a dominant phenotype, which will also help us determine the cellular role of *scribbler*. **2) What are the requirements for localization of Scribbler protein within the cell?** Using the an antibody to the FLAG epitope tag we will examine the localization of the new *scribbler* mutant proteins in both in *Drosophila* Schneider Line 2 tissue culture cells and endogenous tissues such as imaginal discs and brains. **3) What are requirements of scribbler for the genetic interaction with Merlin?** We will do this by examining the influence of expression of the mutant forms of *scribbler* on *Merlin*

**Figure 2: Schematic of FLAG tagged versions of scribbler**



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phenotypes. **4) What is the cellular and genetic context of scribbler expression?** This last question falls more into specific aim II, but the idea will be to examine how our *scribbler* mutations interact with other genes that have a genetic interaction with Merlin such as *blistered* and *Cyclin E*. The goal of these experiments will be to determine the role that these motifs have in *scribbler* function and in particular, in the *scribbler*/*Merlin* interactions.

In the past year we have also addressed a portion of specific aim 2:

**“Specific Aim 2: To elucidate the molecular basis of *Bks (scribbler)* genetic interactions with Merlin... Question 2: What other genes are involved in the *scribbler*/*Merlin* pathway?”**

We have been able to show a genetic interaction between *Merlin*, *scribbler*, and *Cyclin E*, both loss-of-function *Cyclin E* mutations and gain-of-function *Cyclin E*, via ectopic expression using the Gal4/UAS system. *Merlin* phenotypes are modified with both loss and gain of *Cyclin E* function. Both *scribbler* and *Merlin* interactions with loss-of-function mutations in *Cyclin E* produce wings that are missing cross-veins, while interactions with gain-of-function for *Cyclin E* results in the ectopic vein material, especially along vein 5 (data not shown). We are planning to test several other mutations in cell cycle genes such as *string* and *Cyclin A* for interactions with *scribbler* and *Merlin* to determine whether this is a check-point specific interaction or a general cell cycle phenotype. We are in the process of characterizing these results at the cellular level. One interesting experiment will be to test whether *scribbler* or *Merlin* mutants have defects in a G1-S arrest. Preliminary work has shown that *scribbler* mutants do have a cell proliferation defect in the brain (M. Suster, personal communication.) These results suggest that *Merlin* and/or *scribbler* may be regulating the cell cycle, possibly by regulating the entry into S-phase. We are current examining whether this is at the transcriptional level, by examining *Cyclin E* expression using a reporter construct in *scribbler* and *Merlin* mutant backgrounds.



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**Key research Accomplishments:**

- Identification of five new mutant alleles of *scribbler*.
- Determination that over-expression of the larger scribbler isoform (SbbB) results in phenotypes similar to loss of *Merlin* function, suggesting that an putative oncogenic role for *scribbler*.
- Generation of eight deletions mutations of *scribbler* for creation of eight new transgenic *Drosophila* strains.
- Demonstration of genetic interactions between *Cyclin E*, *Merlin* and *scribbler*.

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**Reportable Outcomes**

- Five new strains of *Drosophila* carrying mutations in the scribbler gene. These strains will be kept in the lab and available to all researchers, once they have been fully characterized and published.
  
- Creation of eight FLAG versions of scribbler (two wild-type controls versions and six deletion mutants named: FLAG<sup>SbbA</sup>, FLAG<sup>SbbB</sup>, FLAG<sup>SbbA</sup>ΔA, FLAG<sup>SbbB</sup>ΔA, FLAG<sup>SbbB</sup>ΔZf, FLAG<sup>SbbB</sup>ΔB, FLAG<sup>SbbAZfB</sup> C-term truncation and FLAG<sup>SbbZfB</sup> C-term truncation.

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**Conclusions:**

We have a great deal of preliminary evidence. We are continuing the screen for more alleles of *scribbler*. These experiments are important, because they allow us a means of generating mutant forms of *scribbler* independent of a genetic interaction with *Merlin*. This allows us to determine how *scribbler* loss of function directly correlates with loss of *Merlin* function. This cannot be done in the human system. Furthermore, this kind of data will be important for evaluating the role different polymorphisms in the human *scribbler* locus may have in NF2 progression and severity.

The *scribbler* over-expression result is very exciting and we are interested in understanding how this fits in with *scribbler*'s genetic interactions with *Merlin*. What is unusual and for now unexplainable is the fact that the *scribbler*/*Merlin* interaction was defined as a mutual loss-of-function interaction (LaJeunesse et al, 2001). Further work must be done to characterize the *Merlin*/*scribbler* interaction in this regard. One possibility is that *scribbler* regulates cell proliferation. Over expression of *scribbler*'s large isoform results in a large wing phenotype that is similar to phenotypes expressed by mutations in *Merlin*. Whether *scribbler* is downstream of a signal cascade regulated by *Merlin* or upstream regulating *Merlin* activity is unclear. However, both genes express similar interaction phenotypes with mutations in *Cyclin E*, suggesting that regulation of the G1-S transition may be crucial. Our plan for the next year is to characterize the phenotypes expressed by our new *scribbler* mutants, focusing in on cell cycle defects and the interactions with *Merlin*.

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**Appendices:**

None