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During the 2nd year, we have continued the work on the circuit defined by genetic interactions, we have observed among *scribbler*, *Merlin* and *Cyclin E*. During this year, we have shown that: 1) the two *scribbler* isoforms (*SbbA* and *SbbB*) are not completely equivalent in their requirements for the regulation of proliferation; 2) *scribbler* regulates Cyclin E expression at the transcriptional level and 3) *Merlin* functions upstream of *scribbler*. In addition to this, we have identified a human homologue to *scribbler* (*Hsbb*) which remarkabley shares a similar genomic organization to Drosophila *scribble* and we have also performed a genetic screen to identify genes involved in proper Merlin localization within epithelial cells.

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Genetic and Molecular characterization of *Drosophila brakeless(scribbler)*

Introduction

This is the annual report for the work year, July 1, 2002 to June 31, 2003 for Award DAMD17-01-1-0718 entitled: "Genetic and molecular characterization of *Drosophila brakeless* (*scribbler*): a novel modifier of Merlin Phenotypes." During this year as part of Specific Aim 1 of our Statement of Work, we have continued the work on the circuit defined by genetic interactions we have observed among *scribbler*, *Merlin* and *Cyclin E*. In this process, we have shown the two *scribbler* isoforms (*SbbA and SbbB*) are not completely equivalent in their requirements for the regulation of proliferation. Ectopic SbbA expression appears to promote cell proliferation, while ectopic SbbB appears to repress proliferation. In addition to this, we demonstrate that ectopic expression of SbbA transcriptionally activates Cyclin E expression while ectopic expression of SbbB does not. This result provides us with a potential explanation for the observed phenotypes which we are currently investigating further. As part of Specific Aim 2 we also show that *Merlin* functions upstream of *scribbler*. Ectopic expression of SbbB rescues the Merlin overgrowth phenotype. Given these two sets of data, these results suggest that Merlin may regulate the relative expression of *sbb* isoforms. This work regarding the interactions among *Merlin*, *sbb* and *Cyclin E* is being written up for publication and we are very close to getting all of the data we need to get a solid publication which we are planning to send to Development within the next few months. We have also identified a human homologue to *scribbler* (*Hsbb*) which remarkably shares a similar genomic organization to Drosophila *scribbler*. Curiously, *HSbb* was also identified as the human NY-REN-36 ANTIGEN in patients with renal cancer (Scanlan, et al., 1999).

During this year as part of a separate objective in Specific Aim 1, we have also examined the nature of the intracellular Merlin bodies that are found in *sbb* mutant epithelial cells. To do this we constructed three different Green Fluorescent Protein intracellular membrane markers and are examining their co-localization with Merlin within various tissues. So far this work has resulted in a technical publication that has been recently submitted to BioTechniques. To complement this effort, we conducted a small genetic screen using the *Drosophila* deficiency kit and uncovered several genomic regions that contain a gene or genes involved with proper localization of Merlin.

Body

Over proliferation and under-proliferation with expression of different SbbA and B isoforms

Result: Last year we noted that ectopic expression of the large isoform of *sbb* in the wing produced a phenotype that suggested a defect in proliferation. We used the Gal4/UAS expression system, which allows us to express *sbb* UAS transgenes at different levels and patterns in a target tissue (Brand and Perrimon, 1993). On closer inspection, we noticed that expression of UAS::SbbB transgene resulted in a smaller wing when expressed using both the *apterous::Gal4 driver*, which expresses throughout the dorsal surface of the developing wing (Figure 1D), and the *engrailed::Gal4* driver, which expresses in the posterior half of the wing (data not shown). In addition we noticed some defects in venations, particularly the loss of the posterior cross vein. The venation defects appeared to be the more sensitive of the two phenotypes often expressing with other wing specific Gal4 drivers that did not express an overt growth defect phenotype. The small wing phenotype produced by ectopic SbbB expression is temperature sensitive and at 29°C the wing is very small but anatomically correct with much of its venation patterning still present (data not shown). This suggested that the defect was in growth and not something like cell death, although this is currently being tested. Expression of UAS::SbbA with the *engrailed::Gal4* and *apterous::Gal4* drivers resulted in the opposite phenotype (Figure 1C). The wings were considerably larger and were held out from the body in a fashion similar to flies expressing a dominant negative Merlin (LaJeunesse et al., 1998). Flies ectopically expressing SbbA have no defects in wing venation.

Implication: The *scribbler* isoforms has been shown to be redundant regarding the axon guidance phenotype in the eye (Senti, et al., 2000; Rao et al., 2000), the behavior phenotypes and viability (Yang et al., 2000). However, we see an obvious difference in the behavior of these two isoforms regarding proliferation in the wing. Since there are both overlapping functions and unique functions, it is possible that the alternative expression of each *sbb* isoform is dependent on factors such as differentiation or point in the cell cycle.

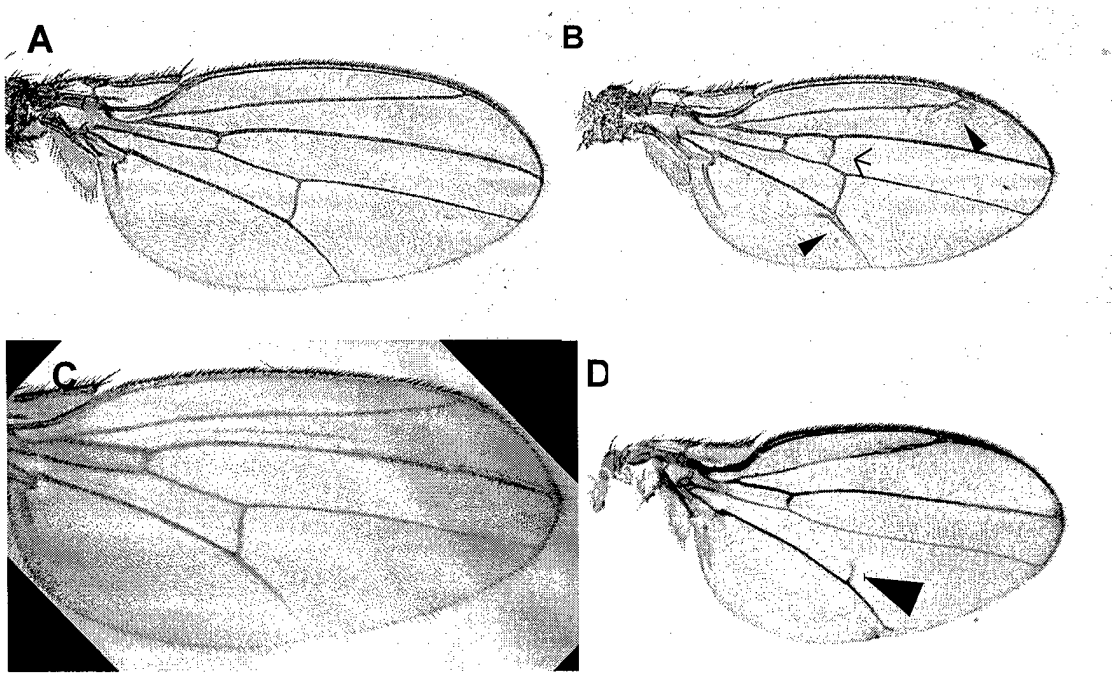
Next steps:

- Confirm the wing results with clonal analysis using an Frt/Gal4 system. In these experiments we will generate single cell expression of SbbA or SbbB and compare the rates of proliferation.

- Brd-U labeling of tissue. Determine whether more cells expressing either isoform are undergoing division than in controls.

- Co-expression of SbbA and SbbB, test whether one isoform can cancel out the growth defects of the other isoform. This experiment would also test the idea that there is a balance between the two isoforms in the cell.

Figure 1. Scribbler gain and loss-of-function wing phenotypes



A) Wild type adult female wing. *B) Wing from a rare* sbb^{270}/sbb^{324} escaper. Note the smaller size and numerous defects in venation: ectopic anterior cross veins (arrow) and ectopic wing vein material around the second and fifth vein termini (thin arrow heads). C) *A wing from a fly (apGal4 UAS::SbbA)* ectopically expressing the smaller SbbA isoform in the wing. The wing is substantially larger than its wild type counter part. The wings are often held out like those seen in *Merlin* dominant negative expression (LaJeunesse et al., 1998). D) A wing from a fly *(apGal4 UAS::SbbB)* ectopically expressing the larger SbbB isoform in the wing. The wing is significantly smaller than its wild type counter part and there are numerous defects in wing venation, particularly the posterior cross vein (large arrow head.)

Genetic Epitasis experiments

Result: As part of Specific Aim 2, we wished to determine the order or Merlin and scribbler functions genetically. To do this we asked whether expression of SbbB could suppress Merlin loss of function phenotypes. Since ectopic expression of SbbB results in a small wing phenotype and loss of Merlin function (either through expression of a dominant negative form or hypomorphic mutations) results in a larger wing, we could test to see which phenotype is expressed when both conditions are imposed on the system. Since SbbB is a gain of function, these experiments must be interpreted differently from the standard double mutant analysis. For instance, expression of a Merlin "large wing" phenotype would suggest that Merlin is downstream of SbbB activity. If the SbbB "small wing phenotype persists then SbbB is downstream of Merlin. The idea was to add back to a defective system SbbB and test the outcome. In the experiment shown in Figure 2, we used an *apterous::Gal4* driver to express dominant negative Merlin alone and then with SbbB. The result is a suppression of the overgrowth phenotype (compare Fig. 2B with 2C) which suggests that Merlin is upstream of SbbB activity. Simply put, by adding SbbB back to Merlin deficient flies we rescue the overgrowth phenotype.

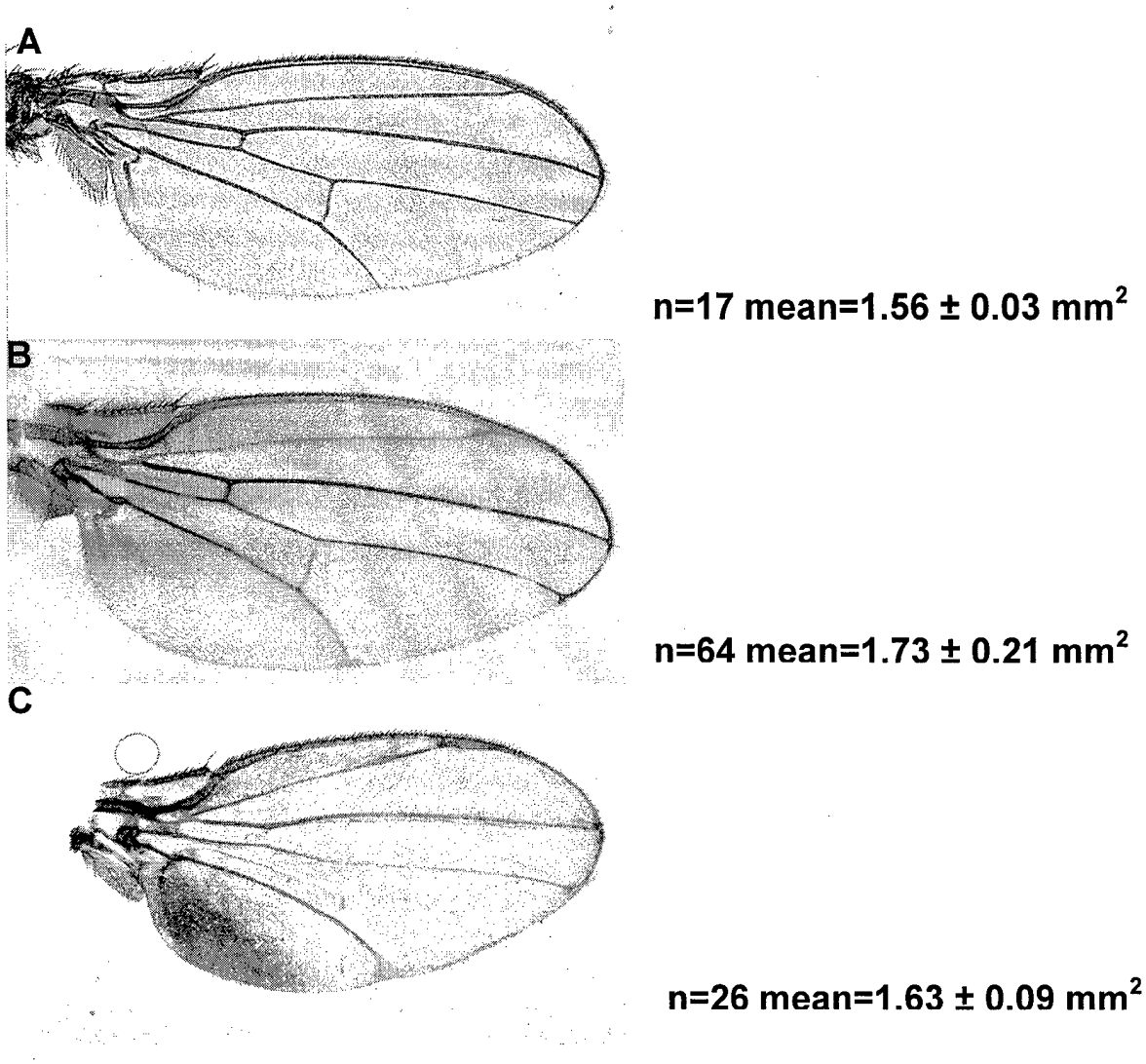
Implication: These results suggest that Merlin might be regulating the activity of SbbB. Taken with the previous results we hypothesize that Merlin may be regulating the alternative splicing or stability of the two *sbb* isoforms. For instance, too much SbbA isoforms may build up in Merlin mutant cells thus resulting in an overgrowth phenotype.

Next steps:

- Perform more epistasis experiments (standard double mutant analysis) using Merlin loss of function mutations and null and hypomorphic *sbb* alleles to confirm these results.

- Examine the levels of SbbA and SbbB in a Merlin mutant background. Is Merlin regulating the expression levels of these isoforms?

Figure 2: Over expression of *sbbB* suppresses MerΔBB over proliferation



SbbA drives Cyclin E::lacZ expression

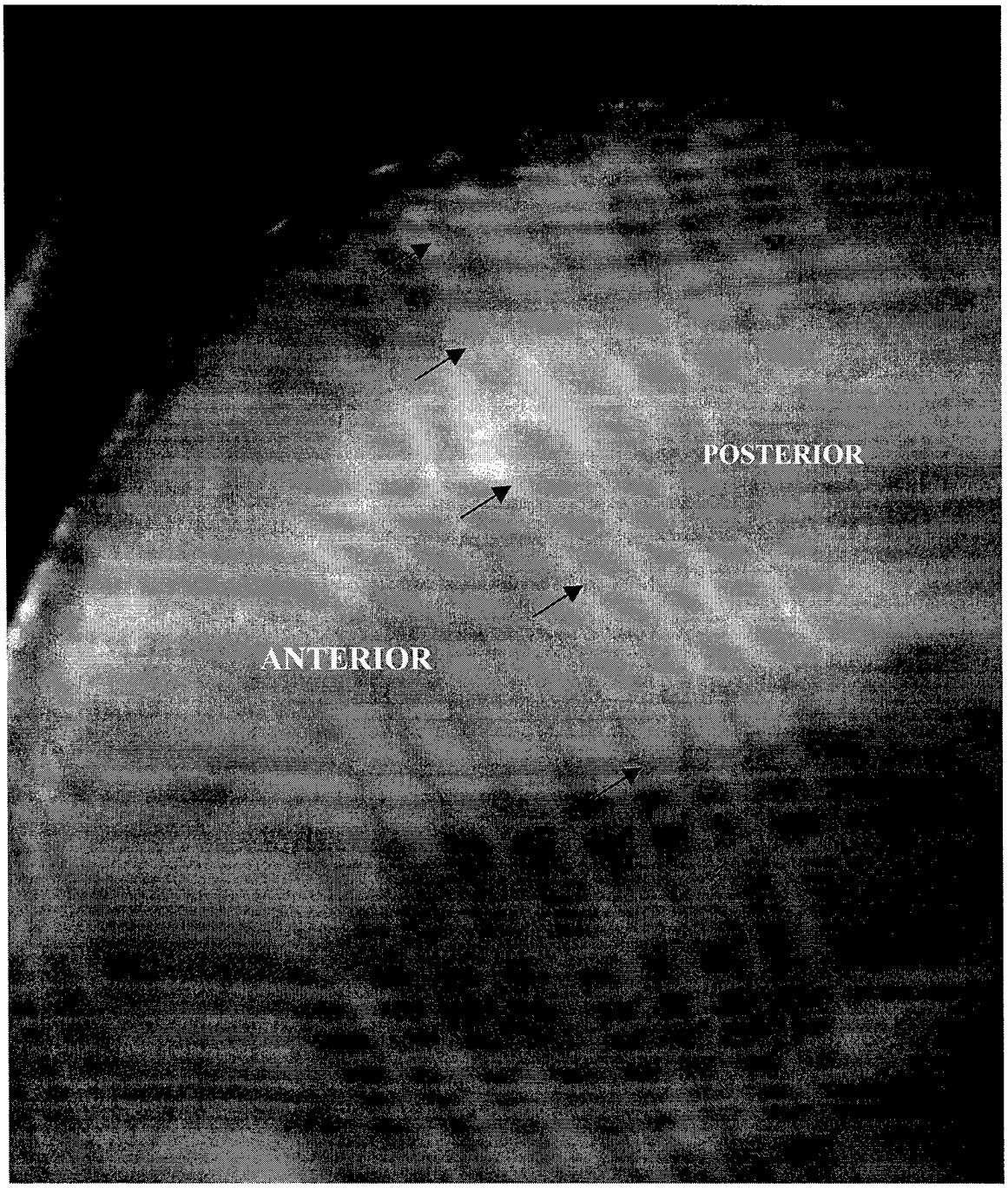
Result: As described in last year's report, we have observed genetic interactions with *Merlin* and *Scribbler* with gain and loss-of-function mutations of *Cyclin E.* These genetic interactions suggest an underlying pathway. Recently *sbb* has been shown to function as a transcriptional repressor of *thick vein* transcription in the wing and *runt* expression in the second and fifth photoreceptor in the developing ommatidia (Funakoshi et al., 2001; Kaminker et al., 2002). In the simplest scenario we wished to test whether *sbb* also regulates expression of Cyclin E. To test this we examined the expression of a Cyclin E::LacZ reporter that contained 16.4kb of the upstream activation sequence of Cyclin E. This reporter gene expresses lacZ in an pattern reminiscent of the endogenous expression pattern of the Cyclin E gene (Secombe et al., 1998). We examined the effects on Cyclin E::LacZ expression of both over-expression of SbbA and SbbB. However, only over expression of SbbA resulted in ectopic expression of the reporter (Fig. 3). Expression of SbbA in an *engrailed* pattern results in the posterior half the disc expressing more lacZ than the anterior. The same can be observed in all discs and tissues where the engrailed gene is expressed.

Implication: We might have found another target gene for *sbb* besides *runt* and *thick veins* and because SbbA had an effect on reporter gene expression we might know target sights within the promoter. Furthermore, these results confirm the phenotypic results we observed from over expression of *sbb* isoforms.

Next step:

- Examine loss-of-function *sbb* mutant effects on Cyclin E expression.
- To confirm reporter gene result, we will examine Cyclin E transcript by *in situ* hybridization and Cyclin E protein levels using antibodies.
- To confirm the genetic interactions, Test the effects that sbb has (using both over expression and loss of function mutations) on a weak hypomorphic CyclinE mutation known as a $CycE^{JP1}$ (Secombe et al., 2000).

Figure 3: Ectopic expression of SbbA results in ectopic expression of 16.4 Cyclin E::LacZ reporter gene



Human Scribbler Homologue

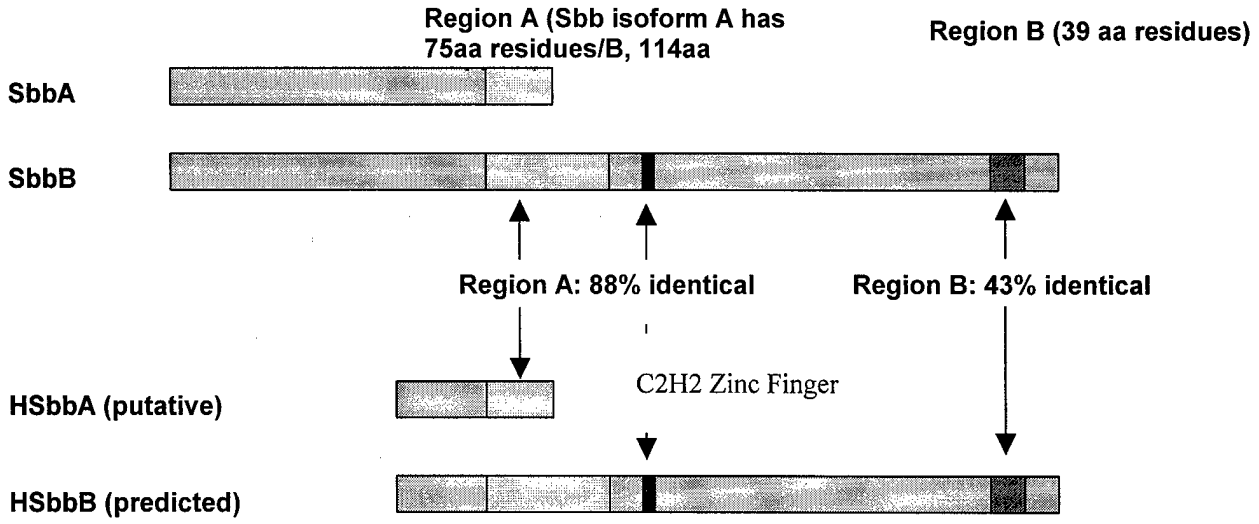
Result: We have found a human homologue to the *Drosophila scribbler* gene while searching a human cDNA database (HUGE; Kazusa DNA Research Institute, http://www.kazusa.or.jp/huge/; Kikuno et al, 2002). The clone name was KIAA1281. The *HSbb* gene is 29% identical from to *Drosophila sbb.* More importantly *HSbb* contains the two well conserved regions, A and B, and the zinc finger domain. The only difference is that the human gene is that HSbb protein is 453 amino acids residues shorter in the N-terminus than the fly protein. Within the well conserved regions the conservation is very great. Over the 117 amino acids of Region A between the sbb homologues, there is 88% identity with 100% similarity and over the 39 amino acids of Region B there is 43% identity (Fig. 4). Interestingly, the intron/exon composition is well also conserved including the exons involved in alternative spliced variants in *Drosophila*. A preliminary Northern analysis shows two transcripts in human tissue culture cells that may correspond to the two message variants seen in flies (Senti et al., 2000; Yang et al., 2000). We have cloned putative versions of these human isoforms into human and *Drosophila* expression vectors and are currently testing their ability to regulate proliferation in human tissue culture cells and complement mutations in the fly gene respectively.

Implication: We have discovered a very similar human sbb homologue that may be spliced in a fashion similar to what has been observed in the fly. Given that Merlin and Cyclin E are also well conserved it is possible that the *sbb* portion of the circuit we are uncovering in flies is also conserved as well.

Next step:

- Determine whether homologous splicing occurs in *HSbb*.
- Determine whether the human and fly genes operate in a functionally homologous fashion by transformation of human gene into *Drosophila* system and testing for genetic rescue.
- Determine whether HSbb regulates cell proliferation and *Cyclin E* expression in human tissue culture cells.

Figure 4: Alignment of *Drosophila* and Human *Scribbler* Protiens



Screen for genomic regions that alter Merlin's Subcellular localization

Results: To determine the nature of the strange Merlin bodies observed in sbb mutant epithelial cells we generated three membrane targeted GFP transgenes that label the endoplasmic reticulum, the Golgi apparatus, and mitochondria. The idea was to test whether Merlin co-localized with any of these structures in normal or *sbb* mutant cells. We have examined all three but do not have any conclusive evidence one way or the other. We have currently acquired a new confocal microscope facility, which will aid in these investigations. Furthermore, we have sent a publication of these three constructs for review to *BioTechniques*.

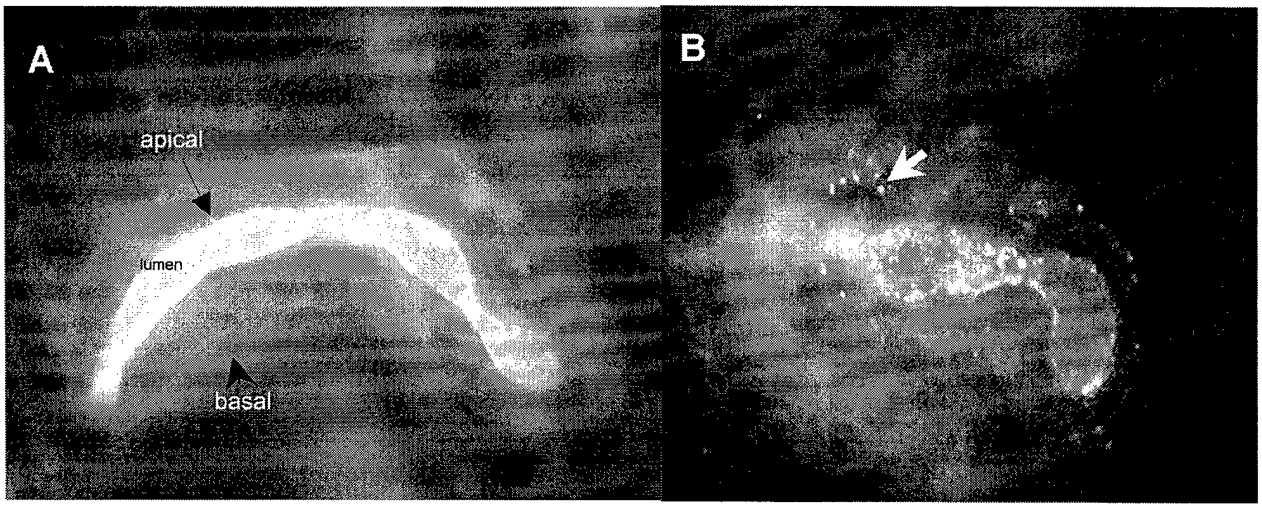
To complement this effort, we conducted a small genetic screen using the *Drosophila* deficiency kit . The *Drosophila* deficiency kit is a collection of ~200 deficiencies that represent ~75-85% of the *Drosophila* genome (LaJeunesse et al, 2001). To examine Merlin in these deficiencies we used a Merlin::GFP transgene that has proper Merlin localization in the cell and functions like wild type Merlin (LaJeunesse et al., 1998). Merlin typically localizes to the apical membrane and to small cytoplasmic vesicular structures in the apical portion of the cell (McCartney and Fehon, 1996). We noticed that in living embryos Merlin::GFP could be easily visualized in the larval salivary gland. The salivary gland is a classic epithelial tube with an apical and basal ends (Fig. 5A). To perform the screen, we crossed the Merlin::GFP transgene into deficiency one stock at a time, collected embryos and examined Merlin::GFP in the salivary glands of embryos. Since there was no way of determining homozygous deficiency embryos from heterozygous or homozygous wild type, we had to look at batches of embryos and if in a batch 25% of the embryos displayed an altered Merlin phenotype we scored the deficiency as containing a gene which is required for Merlin's proper subcellular localization. We have screened 123 of the ~200 deficiencies in the kit and have discovered three deficiencies in which Merlin localization is altered in a reproducible fashion. One example is shown in figure 5B. In *Df(3R)D605* Merlin::GFP localizes to various punctate structures throughout the cell.

Implications We don't know what gene or genes may be found within these deficiencies, but since Merlin function is linked to its proper localization in the cell, by identifying genes required for Merlin localization we will have identified genes that

Next step:

- Finish screening with the rest of the deficiency kit.
- Define the localization of the genes within the deficiencies.

Figure 5: Localization of Merlin::GFP in Epithelial cells of an Embryonic salivary gland



A) A living embryonic salivary gland epithelium from a wild type embryo labeled with Merlin::GFP. This is an epithelial tube of cells with an apical surface (thin arrow) that borders the lumen of this tube, the basal surface (thicker arrow head) forms the outer surface of the epithelial tube. Merlin::GFP localizes primarily to the apical surface, creating a bright outline of this membrane domain. **B)** A living embryonic salivary gland epithelium from a homozygous deficiency, *Df(3R)D605/Df(3R)D605*. Although Merlin::GFP still can be found at the apical domain its localization there is far more punctuate than that observed in wild type embryos. Furthermore small punctuate Merlin condensations (white arrowhead) never observed in wild type embryos can be seen in the basal cytoplasm of the epithelium.

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Key Research Accomplishments

- **Discovery that alternatively spliced *sbb* isoforms play different roles regarding proliferation**

- **Demonstration of the transcriptional activation of Cyclin E by Ectopic SbbA expression.**

- **The ordering of *scribbler* as a downstream component of Merlin function using genetic epistasis.**

- **The identification of a human *scribbler* homologue (*HSbb*) with 29% identify overall and higher homology in several well conserved regions within the protein. Furthermore in the *Human scribbler* gene several important intron/exon boundaries are conserved, suggesting a possible conserved mode of splice regulation between isoforms.**

- **Identification of six genomic deficiencies that when homozygous result in the alteration of Merlin's Subcellular localization.**

- **Generation of Sbb antibodies and RNA probes.**

Reportable Outcomes

- **Two presentations at the 44th annual National *Drosophila* convention in Chicago, Illinois:**

Poster abstract # 259A: Organization of intracellular membrane bound compartments Jeff Lake, Charles Na, Stephanie Buckner, Dennis LaJeunesse , Department of Biology, University of North Carolina Greensboro, 27402
Abstract: Although a great deal is known regarding the organization of the plasma membrane, little is known about the mechanisms that organize intracellular membranes. We have constructed GFP fusion proteins that label the endoplasmic reticulum, the Golgi, apparatus, the plasma membrane, and the mitochondrion to examine the organization of intracellular membrane. In this poster we present live cell images of the endoplasmic reticulum, Golgi apparatus, the mitochondrion and Merlin::GFP in various cell types and present the fixation conditions that preserve these membrane bound compartments. We will also present data from drug experiments in which we disrupt either the actin or microtubule based cytoskeleton using **latrunculin A** and **Nocodazol** and show the effect that these disruptions have on the structure of these membrane bound organelles. (Supported by DOA NF program new investigator award: DAMD17-01-1-0718)

Poster Abstract # 458B: Genetic analysis of the Scribbler Faith Jamshidi, Jeff Lake, Dennis LaJeunesse
Department of Biology, University of North Carolina Greensboro, 27402
Abstract: Scribbler was identified in a screen as a dominant second site modifier of Merlin phenotypes. Merlin is the Drosophila homologue of the human Neurofibromatosis type 2 gene. Like its human counterpart, mutations in the Drosophila Merlin result in defects in the regulation of proliferation as well as defects in differentiation. Scribbler encodes two isoforms, both of which are nuclear proteins of unknown function. Both loss of function mutation that remove both isoforms and gain of function for the large scribbler isoform (SbbB) but not the small isoform (SbbA) results in wing phenotype that suggest defects in the regulation of proliferation. Furthermore ectopic expression of SbbB in the wing results in a small wing phenotype. Although Merlin and Scribbler genetically interact, the relationship between a membrane-associated/cytoplasmic protein and a nuclear protein remains unclear. Recently, we have shown that both Scribbler and Merlin genetically interact with both loss and gain of function mutations of Cyclin E, which suggest a common cell cycle regulatory link between them in their regulation of proliferation. In this poster we present the proliferation phenotypes of Scribbler lost and gain of function mutants as well as characterize the genetic interaction between Merlin, Scribbler and Cyclin E. (Supported by DOA NF program new investigator award: DAMD17-01-1-0718)

- **Paper submitted to BioTechniques entitled: "Three new *Drosophila* markers of Intracellular Membranes and Fixation Artifact."**
- **Generation of three new fusion 6His-Sbb proteins and antiserum to them.**
- **Generation of RNA probes specific to SbbA and SbbB alternatively-spliced isoforms.**
- **Identification of human sbb homologue and the generation of three CMV::HSbb transgenes for examination in human tissue culture cells.**

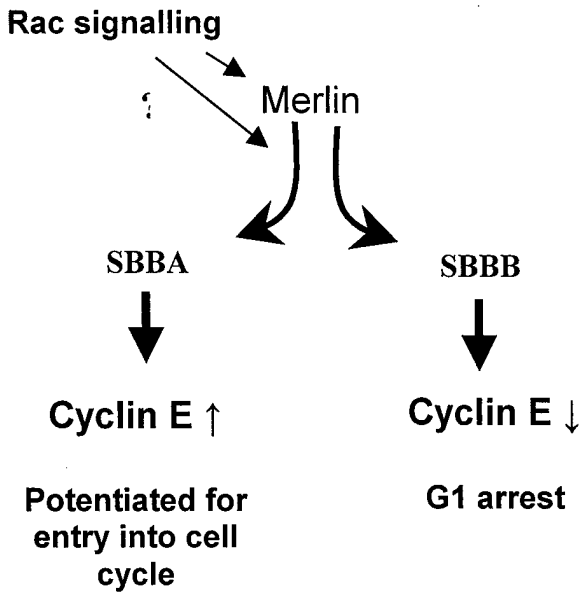
Conclusions

- **Using a *Cyclin E* reporter gene, we have found that Sbb regulates *Cyclin E* expression at the transcriptional level. We are verifying this result using RNA in situ hybridization and antibodies, but if it is true we might have found a mechanism by which sbb regulates proliferation.**

- **We have shown that Merlin is upstream of sbb and we have found that the two isoforms function differently regarding proliferation. SbbA promotes proliferation, while SbbB represses it. Again this finding suggests an interesting model of *Merlin/scribbler* regulation of proliferation in which Merlin regulates the amount of each sbb isoform in the cell. In our model a Merlin regulated signal alters either the splicing of *sbb* or the relative stability of the *sbb* protein isoforms (Fig. 6). We are planning to test this model by examining the levels of SbbA and SbbB in Merlin mutant backgrounds. Recently, it has been shown that Merlin regulates Rac-based signaling (Shaw et al, 2001; Kissel et al, 2003). Along these lines we will also test whether *sbb* isoforms expression is altered when Rac signaling is perturbed.**

- **We have found a human homologue to sbb, HSbb, that has a similar genomic organization and may be regulated in a similar fashion. We are planning to examine the *HSbb* role in Cyclin E expression in human tissue culture cells and test whether the human and fly genes are functionally homologous by P-element mediated transformation of the *HSbb* into the *Drosophila* system.**

Figure 6: Model of *Merlin* regulation of *sbb*



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Appendices