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13. SUPPLEMENTARY NOTES

14. ABSTRACT The primary outcome of our research has been the elucidation of gene network between *scribbler*, *Merlin* and *Cyclin E*. We have shown that *Merlin* functions upstream of both *scribbler* and *Cyclin E.* We have shown that both *Merlin* and *scribbler are* dominant second site modifiers of Cyclin E phenotypes. Curiously enough, Merlin dominantly suppresses hypomorphic Cyclin E phenotypes, while, null and hypomorphic scribbler mutations dominantly enhance Cyclin E phenotypes. The two scribbler isoforms have slightly different functions: the smaller SbbA expression promotes ectopic proliferation/Cyclin E expression, while the larger SbbB represses proliferation/Cyclin E expression. Using these data we have constructed an intriguing pathway for *scribbler/Merlin* regulation of proliferation. In our model we propose that Merlin regulates the intracellular level or activity of scribbler isoforms. In undifferentiated/actively proliferating cells, the expression of the smaller, proliferagenic SbbA isoform predominate regulating gene expression. However in cells that have differentiated, Merlin may down-regulate the expression of SbbA and promote the expression of SbbB. Merlin may be regulating alternative splicing, the stability/instability of the mRNA or protein of scribbler isoforms, or altering the activity of sbb isoforms. However, Northern analysis clearly shows no alteration to sbb transcripts in a Merlin mutant background.

15. SUBJECT TERMS
Scribbler, merlin, cyclin E

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Introduction

Scribbler is a genetic modifier of *Merlin,* the *Drosophila* homologue of the *Neurofibromatosis type II* tumor suppressor gene. *Scribbler* is required for a number of different processes including larval foraging behavior, axon guidance, and regulation of *dpp* signaling. *Scribbler* encodes two novel nuclear proteins that appear to function as transcriptional repressors. We show that the two *scribbler* isoforms have distinct roles regarding cell proliferation in the *Drosophila* wing. Overexpression of SbbA results in a larger wing and higher levels of Cyclin E protein, while ectopic expression of SbbB results in a smaller wing with defects in venation and a reduction in Cyclin E expression. However, co-expression of both isoforms ameliorates the effects of expression of either isoform alone, suggesting that a balance of *scribbler* isoforms is required to ensure proper development. We also show that *Merlin* and Cyclin E act as dominant modifiers of *scribbler* overexpression phenotypes, suggesting *Merlin, scribbler,* and *Cyclin E* are part of a pathway that regulates proliferation in the *Drosophila* wing.

Scribbler (sbb) is involved in a number of varied biological processes in Drosophila including foraging behavior (Yang et al, 2000), axon guidance, (Senti et al, 2000; Rao et al., 2000), *dpp* regulation via transcriptional regulation of thick veins (Funakoshi et al., 2001), and growth regulation (LaJeunesse et al., 2000). The *sbb* gene is highly expressed in the embryonic and larval nervous systems and in larval imaginal discs and encodes two novel nuclear proteins, a small 934 amino acid protein isoform called SbbA, and a larger 2023 amino acid isoform called SbbB (Yang et al., 2000, Senti et al, 2000.) Structurally, both isoforms contain a well conserved region of unknown function which resides at the C-terminal end of SbbA and in the middle of SbbB while the larger scribbler isoform contains a zinc-finger domain and another conserved region at its C-terminus (Senti et al, 2000).
Both *sbb* isoforms appear to function as transcriptional repressors. The axon guidance phenotype observed in the developing retina has been shown to be the result of derepression of the *runt* gene in R2 and R5 photoreceptors; this is due to the loss of *sbb* function resulting in the misprojection of all photoreceptor axons into the optical lamina (Kaminker et al., 2002). Furthermore, *sbb* was identified as a hedgehog-responsive transcriptional repressor of the dpp receptor *thick veins* in the Drosophila wing imaginal disc (Funakoshi et al., 2001). An initial characterization of *sbb* revealed that either isoform could rescue the axon guidance phenotype in the eye that was observed in *sbb* mutants, although the larger SbbB isoform rescued more completely than the smaller SbbA (Senti et al., 2000). As part of Specific Aim 1 of our Statement of Work, we have concentrated on characterizing the signal transduction pathway defined by *Merlin, scribbler* and *Cyclin E*. We believe this to be a major pathway involved in Neurofibromatosis type II tumor growth. We have shown that the two *scribbler* isoforms (*sbbA and sbbB*) are not functionally equivalent as previously suggested (Yang et al, 2000; Senti et al, 2000). SbbA appears to promote cell proliferation transcription of *Cyclin E*, while ectopic SbbB appears to repress proliferation. However, we do not know whether SbbB is repressing *Cyclin E* transcription or SbbA activating *Cyclin E* transcription. From genetic epistasis, we know that *Merlin* functions upstream of *scribbler* and *Cyclin E*. We are currently in the process of determining whether *Merlin* regulates the stability of *scribbler* protein

isoforms or the alternative splicing of scribbler to generate altered ratios of scribbler isoforms. From our model we predict that cells undergoing rapid proliferation possess larger amounts of the small *scribble* SbbA isoform than cells that have undergone differentiation and that Merlin regulates the levels of *scribbler* isoforms. To test this hypothesis we have constructed RNA probes specific to each *scribbler* isoform have used them to determine whether the expression of each isoform is altered in a *Merlin* mutant background. In a Northern Blot we have shown that loss of Merlin function does not alter the pattern or expression of scribbler transcripts (data not presented). Therefore the mechanism by which Merlin regulates scribbler is still unclear and may be through protein-protein interactions.

We have identified a human homologue to *scribbler* (*HSbb*) which shares a similar genomic organization and appears to be alternatively spliced like the *Drosophila* gene. Northern analysis of several different cancer cell lines demonstrates the presence of two major isoforms in a pattern reminiscent to what is observed in *Drosophila.* We have generated FLAG and HA epitope-tagged versions of the HSbb isoforms in a human CMV expression vector (Invitrogen) which are for experiments that are designed to determine whether human cells have the same requirements for the *HSbb* as flies do for *scribbler* regarding the regulation of proliferation. We are also planning to generate *HSbb* transgenic *Drosophila* to test whether the fly and human proteins are functional homologues. In addition to further characterize the cellular function of *scribbler* we have generated three antibodies to *scribbler* proteins: one specific to the larger SbbB isoform, one antibody to the highly conserved Region A of the protein, and an antibody to the human scribbler conserved region.

As part Specific Aim I, we have attempted to characterize Merlin's subcellular vesicular bodies using enhanced yellow fluorescent protein constructs that have been targeted to various intracellular membrane bound compartment: the endoplasmic reticulum, the Golgi and the mitochondrion. Although none of this effort resulted in the elucidation of the exact nature of Merlin bodies, this work was published in **Biotechiques** in May, 2004 (see Appendix).

Body

Over expression of *sbb* isoforms results in altered patterns of growth in the wing

Scribbler was identified as a dominant modifier of *Merlin* wing phenotypes (LaJeunesse et al., 2000). Merlin is the *Drosophila* homologue of the Neurofibromatosis type II tumor suppressor gene and encodes a member of the ezrin, radixin, moesin (ERM) family of actin-binding membrane associated adaptor proteins that regulate a variety of adhesive and signal transduction processes (Bretscher et al, 2002). The *Drosophila* and human Merlin proteins are functionally and structurally conserved and function to regulate cellular proliferation in the *Drosophila* wing (LaJeunesse et al., 1998). To show that that *sbb* plays in the regulation of cellular proliferation in the *Drosophila* wing we have characterized the overexpression phenotypes of SbbA and SbbB. We ectopically expressed SbbA and/or SbbB in the wing imaginal disc using the Gal4/UAS expression system (Brand and Perrimon, 1993). In these experiments we used either the *engrailed-Gal4* driver which expresses in the posterior compartment of the wing imaginal disc (Fig. 1A) or the *apterous-Gal4* driver which expresses in the dorsal compartment of the wing imaginal disc. Overexpression of the smaller SbbA isoform resulted in a significant enlargement of the wing area (+9.7%; Fig. 1B, table 1). Since the spacing of the wing hairs in these wings remained unchanged when compared to the hairs on wild type wing, we interpret the observed increase in size as being due to an increase in the number of cells and not an increase in cell size. Moreover, ectopic expression of SbbA under the *apterous* Gal4 driver resulted in an outheld wing phenotype similar to that observed with loss of Merlin activity (data not shown, LaJeunesse et al., 1998). Interestingly, we observe a non-autonomous effect with over expression of sbbA (Table 1), which is similar to what was observed with ectopic expression of a Merlin Dominant negative (LaJeunesse et al, 1998).

In contrast to SbbA, ectopic expression of the larger SbbB isoform resulted in the expression of a small wing phenotype (-8.4%; Fig. 1C, table 1). Again the spacing of the wing hairs remained unchanged from wild type wings and no ectopic cell death was observed using an acridine orange assay, so we interpreted that the smaller wing phenotype was due to a decrease in the number of cells and not smaller cells or missing cells. We also observed venation defects in the posterior compartment mostly in the forms of a loss of the posterior cross vein (Fig. 1C arrow) and in ectopic vein material along longitudinal vein four and five (Fig. 1C large arrow heads) in all flies ectopically expressing SbbB. The SbbB over-expression phenotypes are more severe in homozygous flies (figure 1D). These wings are dramatically smaller when compared to wild type (-58%; Table 1) and are missing anterior as well as posterior cross vein structures. We never recovered homozygous flied expression SbbA. Curiously, we also observe apparent instances of growth compensation. While growth induction/retardation appears to be mostly cell-autonomous we do observe some non autonomous effect of ectopic sbb expression. Ectopic expression of the SbbB isoform in the posterior compartment results in a posterior wing, the anterior portion of the same wing is significantly larger (+7.1%; Table 1) when compared to wild type. This increase is observed in all cases where growth in the posterior is retarded by expression of SbbB.

Interestingly, co-expression of SbbA and SbbB resulted in a reversion of observed gain of function phenotypes such as ectopic wing material and loss of posterior cross vein (Fig. 1E, arrow heads and arrows) as well as a significant reduction in surface area when compared to ectopically expression SbbA wings alone (+7.7% compared to 12.7%; table 1).

Merlin is a dominant modifier of sbb gain-of-function and CycE

Merlin acts as a dominant modifier of *sbb* gain-of-function phenotypes. Heterozygosity for a recessive null *Merlin* mutant allele (mer^4) enhances the overgrowth phenotypes expressed by ectopic expression of SbbA (Fig. 1F, compare to Fig. 1B, table 2) and suppress the small wing phenotype expressed by ectopic SbbB (Figure 1G, compare to Fig. 1C, Table 2). However, reduction of Merlin activity does not alter the penetrance or extent of venation defects observed with ectopic SbbB expression. *Merlin* is also dominant modifier of *Cyclin* E^{JP}, a viable hypomorphic allele of *Cyclin E* that expresses a small eye and small wing phenotype (Secombe et al., 1998; Fig. 2C, D). Heterozygosity for recessive mutant alleles of *Merlin* suppresses the recessive $CycE^{JP}$ small eye phenotype and small wing phenotype. The wing size increases significantly from -14.9% compared to wild type controls in $CycE^{JP}$ homozygote to deviations of -8% and -0.2% respectively in flies heterozygous for mer^4 and mer^3 and homozygous for $CycE^{JP}$ (Table 2).

***sbb* is a dominant modifier of hypomorphic *CycE* phenotypes**

To test whether sbb modifies CycE phenotypes we recombined a marked P-element allele of *CycE* ($CycE^{k050217}$) onto a null *sbb* allele (sbb^4; Rao et al, 2000) and a hypomorphic sbb allele (sbb^{256}). Sbb256 is a hypomorphic allele that expresses a strong enhancement of Merlin phenotypes (LaJeunesse et al, 2000). These recombinant double mutant chromosomes where then crossed to the hypomorphic $CycE^{JP1}$ allele and there wings were examined both for a change in area and a change in morphologic markers. Transheterozygous $CycE^{JP1}$/ $CycE^{k050217}$flies express a small wing phenotype (Table 3) with defects in the wing morphology such as notching of the wing (22% of the wings), defects in the posterior crossvein (2.2% of wings) and in the longitudinal veins (2.2% Figure 3; Table 4). Heterozygousity of the null allele of sbb results in a slight but significant decrease in area (Table 3), but an enormous increase in the frequency of notching, posterior crossvein defects and defects (Table 4). Curiously, heterozygosity for the hypomorphic Merlin interacting *sbb* allele, sbb^{256} has a different modification of these CycE phenotypes. While sbb^{256} heterozygosity also slightly enhances the $CycE^{JP1}$/ $CycE^{k050217}$ small wing phenotype (table 3), it appears to suppress the notched wing phenotype and result in an increase in the amount of posterior cross vein defects (Table 4).

***CycE* is a dominant modifier of ectopically induced SbbA overgrowth**

An amorphic allele of *CycE* ($CycE^{AR95}$)genetically suppress the overectopic SbbA over growth phenotype. A null *CycE* allele ($CycE^{AR95}$) dominantly suppresses *SbbA* over expression phenotypes (Fig. 4). Heterozygosity of *Cyclin E* resulted in a significant reduction in the wing area of flies over-expressing SbbA with a reduction of -5.5% from engrailed Gal4 SBBA wings lone (Figure 4C, table 1). However, reduction of *Cyclin E* gene dose had no significant effect on SbbB size phenotypes (Table 1) suggesting that SbbB over-expression phenotypes are below a threshold effect of *Cyclin E* dose. To follow up these genetic experiments, we examined at Cyclin E protein levels within wing imaginal discs over-expressing either SbbA or SbbB (Figure 4D). Ectopic expression of SbbA resulted in levels of Cyclin E protein roughly twice the amount observed in wild type wing imaginal disc (Figure 4D, compare lane 3 with lane 2). In contrast over-expression of expression of SbbB resulted a reduction of Cyclin E levels (Figure 4D, lane 4) to observed in $CycE^{JP}$ mutants (Figure 4D, lane 1).

sbb isoforms have significantly different roles in the regulation of proliferation of the *Drosophila* wing. Overexpression of the smaller SbbA isoform resulted in a larger wing and

raised levels of Cyclin E protein, while over expression of SbbB resulted in a small wing phenotype and reduced levels of Cyclin E protein. While over-expression of either isoform resulted in an altered wing size and patterns of venation, co-expression of both *scribbler* isoforms ameliorates the phenotypes produced from over-expression of each *scribbler* isoform alone. This result suggests that a balance between the isoforms may be maintained during development for proper proliferation and differentiation. We have also shown that mutations in *Merlin* and *Cyclin E* genetically interact with *sbb* over-expression. Although the molecular nature of these interactions is unclear, these genetic interactions suggest a potential novel pathway for the regulation cell proliferation. *Merlin* regulates a number of signal transduction cascades including Rac signaling, PI-3 signaling, and N-WASP (Shaw et al., 2001; Xiao, et al., 2001, Kissil et al., 2002, Rong et al., 2004, Manchanda et al., 2005). However, the exact mechanism of its growth regulation is not fully understood. Given the data presented in this report, one may hypothesize that *Merlin* might regulate proliferation by regulating the relative levels, stability, or activity of *sbb* isoforms transcripts or *sbb* proteins during development. In cells that are actively proliferating, SbbA may be expressed at higher levels relative to SbbB thus elevating the expression of Cyclin E and other genes required for proliferation, while in cells that are differentiating the opposite might be occurring. Differential expression of *sbb* isoform transcripts has been observed as more of the SbbA transcript is found during the larval period, while during pupal development higher levels of the SbbB transcript are expressed (Yang et al., 2000). In any case, *sbb*, *Merlin* and *Cyclin E* appear to define a novel pathway for the regulation of growth and differentiation in the *Drosophila* wing.

Table 1: Wing measurements in flies over expressing SbbA and/or SbbB

Genotype	n	Average Area in pixels (Anterior top/ Posterior bottom)	% deviation from wild type with significance	% deviation from SbbA overexpression with significance	% deviation from SbbB overexpression with significance
***w^{1118}* (wild type)**	**33**	Ant: 190912±4402	-	+2.5%$^{\#}$	-6.6%
		Post: 254279±10840	-	-8.9%	+9.1%
enGal4/+	61	Ant:186231±16958	-2.45% $^{\#}$	-	NA
UAS-SbbA/+		Post: 278872±15156	+9.7%	-	NA
enGal4/+	**32**	Ant:204433±14228	+7.1	NA	-
UAS-SbbB/+		Post: 233008±15585	-8.4%	NA	-
enGal4/+	18	Ant: 178885±21584	-6.3%*	NA	-12.5%
UAS-SbbB/UAS-SbbB		Post: 106810±14025	-58%	NA	-54.1%
enGal4/+	**43**	Ant: 202270±14610	+6%*	-5.6%	-1.0%$^{\#}$
UAS-SbbA/+					
UAS-SbbB/+		Post: 266549±15079	+4.8%	-4.8%	+14.4%
Mer^{4}/+	21	Ant:214684±17511	+12.5%	+15.3%	NA
enGal4/+					
UAS-SbbA/+		Post: 335489±8409	+31.9%	+20.3%	NA
Mer^{4}/+	**23**	Ant:218131±11364	+14.2%	NA	+6.7%
enGal4/+					
UAS-SbbB/+		Post: 255686±11003	+0.6%$^{\#}$	NA	+9.7%
enGal4/+	20	Ant: 247075±11454	+29.4%	+32.3%	NA
UAS-SbbA					
UAS Mer^{DN}		Post: 333979±10182	+31.3%	+19.7%	NA
enGal4/+	**26**	Ant:237043±12218	+24.2%	NA	+16%
UAS-SbbB					
UAS Mer^{DN}		Post: 264353±15335	+3.9%	NA	+13.4%
CycE^{AR95}/+	24	Ant:181171±15223	-5.1%*	-2.7%$^{\#}$	NA
enGal4/+		Post: 264019±10415	+3.8%*	-5.5%	NA
UAS-SbbA/+					
CycE^{AR95}/+	**26**	Ant:205240±12648	+7.5%	NA	+0.4%$^{\#}$
enGal4/+		Post: 241702±10780	-4.9%	NA	+3.7%$^{\#}$
UAS-SbbB/+					
en GAL4/+	20	Ant: 196312±10515	+2.8%$^{\#}$		
		Post:258724 ±12531	+1.7%$^{\#}$		
UAS-Sbb B/+	**20**	Ant: 192089±12004	+0.6%$^{\#}$		
		Post: 257498±13732	+1.3%$^{\#}$		
UAS-Sbb A/+	22	Ant:190480±10435	-0.22%$^{\#}$		
		Post: 265333±13552	+4.3%$^{\#}$		
UAS-Sbb B/UAS-Sbb B	**23**	Ant:191010±8895	+0.05%$^{\#}$		
		Post: 258342±11957	+1.6%$^{\#}$		

All significance levels are at P<0.001 unless otherwise noted. * denotes a significant level of P<0.05, #denotes non-significant difference between the comparison.

Table 2: Modification of $CycE^{JP}$ phenotypes by *Merlin* mutations

Genotype	n	Area	% deviation from wild type with significance
w^{1118}	22	485578±23799	-
$CycE^{JP1}/CycE^{JP1}$	41	413128±20296	-14.9%
$Mer^{4}/+$; $CycE^{JP1}/CycE^{JP1}$	24	446024±15458	-8%
$Mer3/+$; $CycE^{JP1}/CycE^{JP1}$	27	484550±18470	-0.2%

All significance levels are at P<0.001 unless otherwise noted. * denotes a significant level of P<0.05, #denotes non-significant difference between the comparison.

Table 3: Wing area data for scribbler modification of hypomorphic *CycE* mutant phenotypes.

Genotype	n	Area in pixels	% difference in wing area from wild type with significance	% difference wing area from $CycE^{JP}/CycE^{K050217}$ with significance
w^{1118} *(wild type)*	20	445191±21171	N.A.	+49.33% (P>0.001)
$\frac{CycE^{K050217}}{CycE^{JP}}$	45	330765±15632	-25.7% (P>0.001)	N.A.
$\frac{sbb^{4}\ CycE^{K050217}}{+\quad CycE^{JP}}$	39	322880±9383	-27.5% (P>0.001)	-2.38% (P>0.05)
$\frac{sbb^{256}\ CycE^{K050217}}{+\quad CycE^{JP}}$	36	316675±25371	-28.9% (P>0.001)	-4.25% (P>0.05)

Table 4: Wing area data for *scribbler* modification of hypomorphic *CycE* mutant phenotypes.

Genotype	n	Notching in the wing	Defects in posterior crossvein	Defects in anterior crossvein	Defects in fourth and fifth longitudinal veins
w^{1118} *(wild type)*	20	0%	0%	0%	0%
$\frac{CycE^{K050217}}{CycE^{JP}}$	45	22.2%	2.2%	0%	8.8%
$\frac{sbb^{4}\ CycE^{K050217}}{+\quad CycE^{JP}}$	39	56.8%	21.6%	2.7%	21.6%
$\frac{sbb^{256}\ CycE^{K050217}}{+\quad CycE^{JP}}$	36	2.9%	88.2%	0%	38.4%

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Genetic and Molecular characterization of *Drosophila brakeless*

Figure 1 Over-expression of *sbb* isoforms modifies wing size. All wings shown are from female flies. A) Wild type wing. The posterior compartment is shaded. B) Wings expressing SbbA and C) SbbB in the posterior compartment of the wing. Note general increase in area of the posterior compartment in B) and the decrease in the area of the posterior compartment C). The thin arrow points out disrupted posterior crossvein, the bold arrow heads points to ectopic vein deltas along margin at longitudinal veins four and five, the only two veins of the posterior compartment. D) Wing from a fly homozygous for *engrailed Gal4 UAS Sbb,* note very diminished size of posterior compartment when compared to wild type and completely missing posterior crossvein. E) Wing expressing both SbbA and SbbB in posterior compartment. Note the more wild type venation pattern and size (compare to the arrow and arrowheads in C). F) Wing heterozygous for recessive null Merlin allele (mer^{4}) and expressing SbbA in posterior compartment of wing, notice the large increase in size of the wing. G) Wing heterozygous for recessive null Merlin allele (mer^{4}) and expressing SbbB in posterior compartment of wing, notice the increase in wing size but no alteration to the incomplete posterior cross vein (arrow).

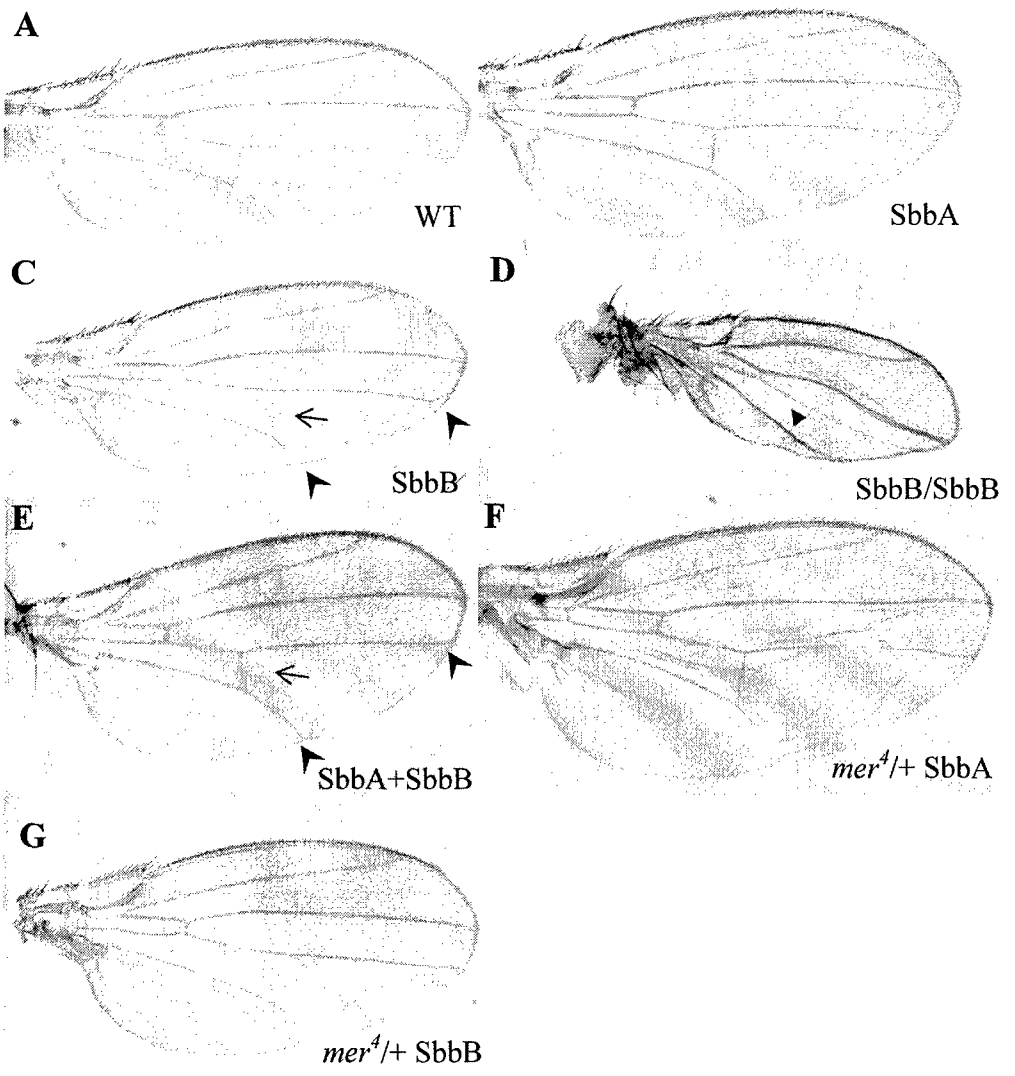


Figure 2: *Merlin* is a dominant suppressor of *Cyclin E* phenotypes. Wild type eye (A) and wing (B). Eye and wing from homozygous $CycE^{JP}$ female (C and D respectively). E) Eye from a female fly heterozygous for a recessive, null mer^4 allele and homozygous for $CycE^{JP}$. Note the decrease in general roughness and increase in size of eye when compared to $CycE^{JP}$ eye alone. F) Wing from mer^4 allele and homozygous for $CycE^{JP}$ Note size significantly larger than the wing in D.

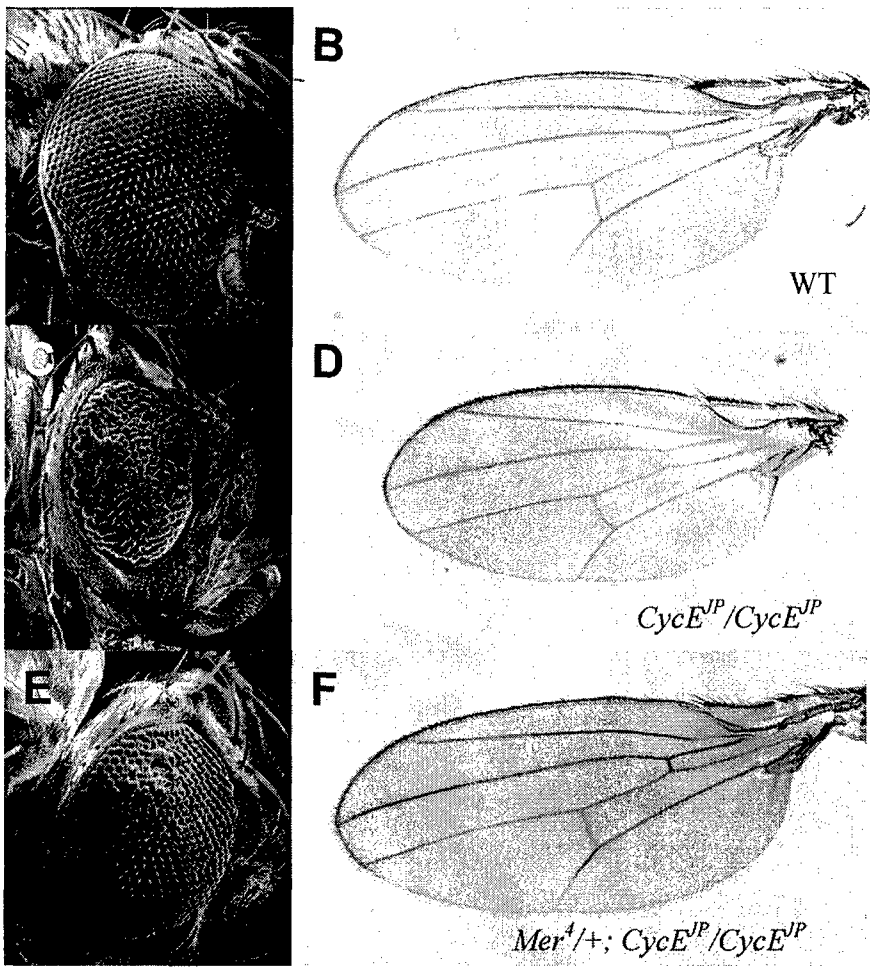


Figure 3: *sbb* is a dominant suppressor of *Cyclin E* phenotypes. A) Wild type wing. B) wing from a $CycE^{JP}/CycE^{K05217}$ fly. Note the smaller size or the wing. C) Wing from a fly heterozygous for sbb4 (a null sbb allele) and transheterozygous for $CycE^{JP}/CycE^{K05217}$. Note the large notch at the tip of the wing (arrow) and the incomplete fifth longitudinal vein (arrow head). D) Wing from a fly heterozygous for sbb256 (*Merlin* interacting *sbb* allele) and transheterozygous for $CycE^{JP}/CycE^{K05217}$. Note the ectopic vein material along the posterior cross vein (arrow head).

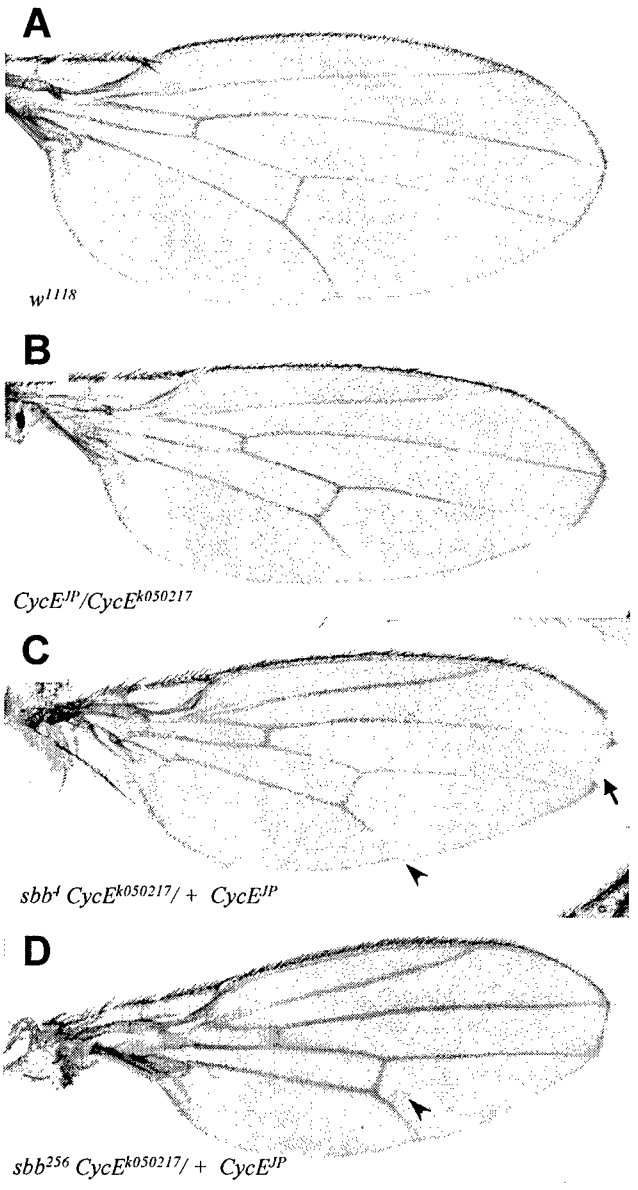
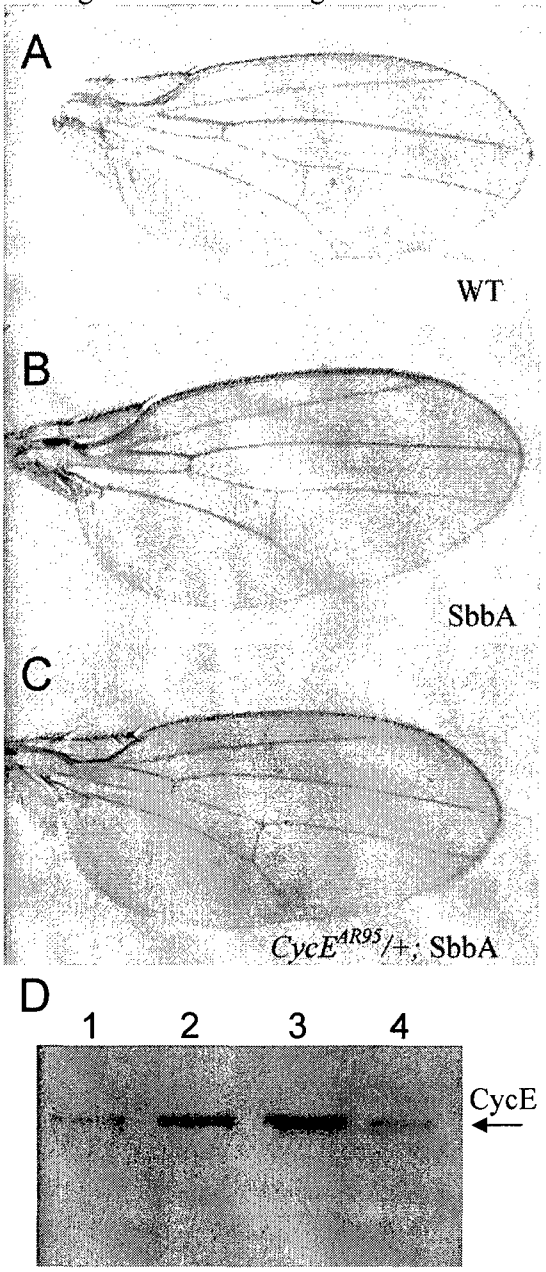


Figure 4: Cyclin E is a dominant suppressor of SbbA overgrowth phenotypes. A) Wild type wing. B) Wing expressing SbbA in posterior compartment. C) Wing from a flies heterozygous for a recessive null $CycE^{AR95}$ that is also expressing SbbA in the posterior compartment of the wing. Notice the significant decrease in size. D) Western blot of thirty wing imaginal discs. Lane 1: $CycE^{JP}$, Lane 2: wild type, lane 3; *SbbA*, Lane 4, *SbbB.* Note the increase in the amount of Cyclin E in *engrailed SbbA* (lane 3) versus wild type (lane 2*). B;ot was probed with mouse monoclonal α Cyclin E (8B10; gift of H. Richardson).* Note the decrease in the amount of Cyclin E to levels comparable to the hypomorphic *CycE* wing imaginal discs (compare lane 4 to lane *2 (wild type)* and lane 1 *($CycE^{JP}$).* Thirty discs were used for each condition and equal loading was verified using anti-actin antibody (Data not shown).



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Genetic and Molecular characterization of *Drosophila brakeless*

Summary of our results:

- Establishment that alternatively splice *sbb* isoforms have unique roles in differentiation and proliferation
- Demonstration that *Merlin* is a dominant suppressor of *Cyclin E* phenotypes.
- Demonstration that *scribbler* is a dominant enhancer of *Cyclin E* phenotypes.
- Identification of a signal transduction pathway by which *Merlin* and *scribbler* take part to regulate *Cyclin E* expression and cellular proliferation.
- Aberrant *Merlin* protein localization observed in *scribbler* mutant epithelium is not a component of the Golgi, endoplasmic reticulum or mitochondrial network.
- Determine Merlin sensitive period during development: an early proliferation requirement and a pupal differentiation requirement.

Key accomplishments July 2001- June 2005:

Meetings/poster abstracts

- **Poster presentations at the 44th annual National *Drosophila* convention in Chicago, Ill: Poster #259A**

Lake, J., Na, C., Buckner, S., LaJeunesse, D.
Organization of intracellular membrane bound compartments. University of North Carolina Greensboro, 231 Eberhart Bldg., Greensboro, North Carolina 27455

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 retiuculum, 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.

- **Poster presentations at the 44th annual National *Drosophila* convention in Chicago, Ill: Poster #458C**

Jamshidi, F., Lake, J., LaJeunesse, D. Genetic analysis of the Scribbler, a dominant second site modifier of the Merlin/NF2 tumor suppressor gene. University of North Carolina Greensboro, 231 Eberhart Bldg., Greensboro, North Carolina 27455

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.

- **Poster presentations at the 45th annual National *Drosophila* convention in Washington D.C: Poster #465C**

***Merlin* and *scribbler* isoforms regulate *Cyclin E* expression and cellular proliferation**
Dennis LaJeunesse, Stephanie Buckner, Rebecca Atwell, Amanda Brown, and Amanda Pirt. University of North Carolina Greensboro, 231 Eberhart Bldg., Greensboro, North Carolina 27455

Abstract: Scribbler was identified as a dominant second site modifier of the *Drosophila Neurofibromatosis type II* tumor suppressor gene homologue, *Merlin*. Mutations in *Drosophila Merlin* result in defects in the regulation of proliferation as well as defects in differentiation. We have continued the work on the circuit between *scribbler*, *Merlin* and *Cyclin E*. Using epistasis, we show that *Merlin* functions upstream of *scribbler*. We also demonstrate that the two scribbler isoforms (SbbA and SbbB) are not equivalent in function. Ectopic SbbA expression appears to promote cell proliferation, while ectopic SbbB appears to repress proliferation. Moreover, both *scribbler* and *Merlin* genetically interact with both loss and gain of function mutations of *Cyclin E* suggesting a common mechanism in the regulation of proliferation. In addition to this, we demonstrate that Sbb regulates Cyclin E transcription. With these data we have constructed an intriguing hypothetical pathway for sbb/Merlin regulation of proliferation: *Merlin* may regulate the alternative splicing *of scribbler*, which in turn affects Cyclin E transcription. We have also identified a human homologue to scribbler (HSbb) which shares a similar genomic organization to the *Drosophila scribbler* gene. We will present preliminary data on whether HSbb is alternatively spliced like its Drosophila homologue and plays a similar role in the regulation of proliferation.

Manuscripts

- **BioTechniques entitled: "Three new *Drosophila* markers of Intracellular Membranes and Fixation Artifact." Dennis Richard LaJeunesse, Stephanie M. Buckner, Jeffrey Lake, Charles Na, Amanda Pirt and Kathryn Fromson May issue Volume 36, No. 5**
Abstract: The need for cellular markers that permit a quick and accurate evaluation of a protein's subcellular localization has increased with the surge of new data generated by the *Drosophila* genome project. In this report we present three ubiquitously-expressed *Drosophila* transgenes that expressed a green fluorescent protein variant (EYFP) that has been targeted to different intracellular membrane targets: the Golgi, mitochondria and the endoplasmic reticulum. These markers serve as an internal standard for characterizing a proteins subcellular localization or as a means of tracking the dynamics of intracellular organelles during normal or abnormal cellular or developmental processes. We have also examined fixation artifacts using these constructs to illustrate the effects that fixation and permeablization have on intracellular membrane organization.

- **Manuscript submitted to *genesis*: "*Merlin* and *scribbler* regulate *Cyclin E* expression and cellular proliferation."**
Abstract: *Scribbler* is a genetic modifier of *Merlin,* the *Drosophila* homologue of the *Neurofibromatosis type II* tumor suppressor gene. *Scribbler* is required for a number of different processes including larval foraging behavior, axon guidance, and regulation of

dpp signaling. *Scribbler* encodes two novel nuclear proteins that appear to function as transcriptional repressors. We show that the two *scribbler* isoforms have distinct roles regarding cell proliferation in the *Drosophila* wing. Overexpression of SbbA results in a larger wing and higher levels of Cyclin E protein, while ectopic expression of SbbB results in a smaller wing with defects in venation and a reduction in Cyclin E expression. However, co-expression of both isoforms ameliorates the effects of expression of either isoform alone, suggesting that a balance of *scribbler* isoforms is required to ensure proper development. We also show that *Merlin* and Cyclin E act as dominant modifiers of *scribbler* overexpression phenotypes, suggesting *Merlin*, *scribbler,* and *Cyclin E* are part of a pathway that regulates proliferation in the *Drosophila* wing.

- **Manuscript in preparation: ""Sequence Analysis between human and Drosophila scribbler genes reveals conserved genomic organization and isoform expression." Amanda Brown and Dennis LaJeunesse, manuscript in prep. Will be submitted to *Gene, fall 2005***

Reportable outcomes

- Three poster presentations at a National meetings
- Three manuscripts one published, one in review, one in preparation
- Two master's thesis: Farnaz Jamshidi, "The potential role of Scribbler gene in the regulation of cell proliferation" 2003 and Amanda Brown "Characterization of human scribbler," pending fall 2005.
- Generation of two polyclonal antibodies to *scribbler*., recognizes the human and Drosophila proteins.
- Generation of five RNA probes specific to *SbbA*, *SbbB and HSbb*.
- The generation of FLAG tagged HSbb transgenes for examination in human tissue culture cells.

Conclusions:

- Establishment that *sbb* protein isoforms have unique roles in differentiation and proliferation.
- Demonstration that *Merlin* is a dominant suppressor of *Cyclin E* phenotypes.
- Demonstration that *scribbler* is a dominant enhancer of *Cyclin E* phenotypes.
- Identification of a signal transduction pathway by which *Merlin* and *scribbler* take part to regulate *Cyclin E* expression and cellular proliferation.
- Aberrant *Merlin* protein localization observed in *scribbler* mutant epithelium is not a component of the Golgi, endoplasmic reticulum or mitochondrial network.
- Merlin doesn't regulate scribbler expression through the regulation of alternative splicing of scribbler.
- There are critical periods during developmental that require Merlin for different processes which include proliferation control during larval development and differentiation during pupal.

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Three new *Drosophila* markers of intracellular membranes

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BioTechniques 36:784-790 (May 2004)

The need for cellular markers that permit a quick and accurate evaluation of a protein's subcellular localization has increased with the surge of new data generated by the Drosophila *genome project. In this report, we present three ubiquitously expressed* Drosophila *transgenes that expressed a green fluorescent protein variant (enhanced yellow fluorescent protein) that has been targeted to different intracellular membrane targets: the Golgi apparatus, mitochondria, and endoplasmic reticulum. These markers serve as an internal standard for characterizing a protein's subcellular localization or as a means of tracking the dynamics of intracellular organelles during normal or abnormal cellular or developmental processes. We have also examined fixation artifacts using these constructs to illustrate the effects that fixation and permeabilization have on intracellular membrane organization.*

INTRODUCTION

With the completion of the *Drosophila* genome project, there has been a trend in *Drosophila* research toward assessing gene function at the cellular level. As the field of functional genomics races toward a comprehensive understanding of gene function, a need arises for intracellular standards that permit a quick and accurate evaluation of a novel protein's localization within the cell. Fusion proteins to green fluorescent protein (GFP) and its variants have been used to detect a target protein's subcellular localization in both living and fixed cells (1–3). Here we describe three *Drosophila* transgenes that ubiquitously express a GFP variant, enhanced yellow fluorescent protein (EYFP), which has been targeted to different intracellular membrane-bound compartments: the Golgi apparatus, the endoplasmic reticulum, and the mitochondria. While extensive work has been performed on fixation and fixation artifacts in electron microscopy, there is little or no literature dealing with such topics in fluorescent light microscopy, especially regarding fixation of whole tissues in a multicellular model organism. Because EYFP can be examined in living and fixed preparations, we took the opportunity to examine the effects of fixation and permeabilization on the expression and localization of the targeted EYFP moiety.

MATERIALS AND METHODS

Microscopy

All images (both live and fixed) were captured using a fluorescein isothiocyanate (FITC) filter (Chroma High Q filter; Chroma Technology, Rockingham, VT, USA) and a 60× UPlanFl oil-emersion objective [numerical aperture (NA), 1.25] with oil on an Olympus BX51 compound fluorescent microscope (Olympus America, Melville, NY, USA) equipped with a Cool Snap *fx* charge-coupled device (CCD) digital camera (Photometrics, Tucson, AZ, USA). The camera and microscope were controlled using Image Pro® Software (Media Cybernetics, Silver Spring, MD, USA). Captured images were imported into Microsoft® PowerPoint® for presentation.

Sample Preparation

Tissue from wandering third instar larvae was dissected in Shields and Sang M3 Insect tissue culture media (Sigma, St. Louis, MO, USA). Living tissue was mounted on a glass microscope slide in a wet mount of 20 µL cell culture media under a 22-mm coverslip prepared with clay feet to prevent damage to the tissue. Fixed tissue was mounted using the ProLong® Antifade Kit (Molecular Probes, Eugene, OR, USA) and examined within 24 h of preparation because formaldehyde-based fixes tend to degrade. We observed little difference in tissue quality within 24 h of fixation.

Cloning and P-Element Germline Transformation

The membrane-targeted moieties were PCR-amplified from mammalian expression vectors pEYFP-Mito, pEYFP-ER, and pEYFP-Golgi (for the mitochondria, endoplasmic reticulum, and Golgi apparatus, respectively; BD Biosciences Clontech, Palo Alto, CA, USA) and cloned into a *Drosophila spaghetti squash* (*sqh*) CASPER 4 P-element transformation vector (gift of D. Kiehart, Duke University, Department of Biology, Durham, NC, USA) (Figure 1). EYFP is a brighter, red-shifted variant of the original jellyfish GFP. The *sqh* promoter expresses ubiquitously, permitting examination in a wide variety of tissue types without induction. P-element-mediated germline transformation was performed as previously described (3).

Initial Characterization of EYFP Constructs in Insect Cells

Because the EYFP constructs were designed for expression in mammalian tissue culture cells, we first determined whether the same organelles were labeled in *Drosophila* cells. Although the localization of each construct appeared to be similar in both human HeLa tissue culture cells and *Drosophila* Schneider line 2 tissue culture cells, we confirmed the localization in *Drosophila* tissue culture cells by double labeling the transfected cells with a fluorescent dye that specifically labeled each organelle, using MitoTracker® Orange CMTMRos for the mitochondria, $NBDC_6$-ceramide for the Golgi apparatus, and ER-Tracker™ Blue-White DPX for the endoplasmic reticulum (all from Molecular Probes). In each case, we found that the signals overlapped perfectly, suggesting that our constructs labeled the intended structures in *Drosophila* cells (data not shown). Three independent insertions of each construct were characterized, and each displayed subcellular patterns of localization to specific compartments that we interpreted

as analogous to the structures we identified in the tissue culture.

Buffer Preparation

Buffer compositions are as follows: phosphate-buffered saline (PBS); phosphate lysine periodate (PLP), 0.037 M sodium phosphate, 0.075 M lysine, 0.01 M sodium periodate, pH 7.2; and PIPES/EGTA/magnesium (PEM), 0.1 M PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.9. An 8% paraformaldehyde stock solution was prepared by dissolving 80 g of paraformaldehyde in 80 mL of warmed water and 10 µL of 10 M NaOH and heated to 55°C for 2–3 h, distilled water was added to 100 mL, and the solution was vacuum-filtered to remove the flocculent nonsoluble fraction. To make fixatives, an appropriate amount of this paraformaldehyde stock solution was diluted in the desired concentrated buffer solution. In 1× PEM and 1× PBS buffers, a concentration of either 2% or 4% of paraformaldehyde was used; PLP fixative was made with 2% paraformaldehyde. Samples were fixed for 20 min at either 4°C or 21°C. To test for the effects of permeabilization, a 30-min wash of 1× PBS, 1% bovine serum albumin (BSA), and 0.1% Triton® X-100 (Sigma), called PBT, was performed on fixed samples.

RESULTS

We examined the expression of each construct in several different tissues, including muscles, neuronal cells, and epithelial cells (imaginal discs, guts, and salivary glands). In general, *Drosophila* cells are small; thus, in this report, we used the giant epithelial cells of the third instar larval salivary gland to demonstrate our results. These cells are large (approximately 100 µm in diameter) and permit easy observation of the subcellular localization of each of the organellar/intracellular membrane-targeted EYFP constructs. Considering that all immunological protocols involve a fixation step and that EYFP can be visualized in both living and fixed tissues, we examined the effects of fixation on the organization and distribution of intracellular membrane structure. We tested two concentrations of paraformaldehyde-based fixative (2% or 4%) in three buffer solutions commonly used in *Drosophila* research: PBS, PEM, and PLP, which has been reported to work well for membrane-associated epitopes (4,5). Not surprisingly, in all cases, we observed some degree of alteration of the subcellular EYFP patterning after fixation, regardless of the buffer used. We also examined the effects of fixation at two different temperatures [room temperature and on ice (4°C)] but saw no difference in results between these conditions (data not shown).

EYFP-Golgi showed a punctate distribution through the cytoplasm of a wide range of cells types, including salivary gland (Figure 2A), neuron, muscles, and intestinal cells (data not shown). While fixation does not alter the overall distribution of the EYFP-Golgi punctuate-labeled bodies, we observed subtle differences in the morphology of these structures after fixation, especially in the PLP buffer (compare Figure 2, A and D). In this buffer, we consistently observed finer distribution of Golgi throughout the cell. We do not know the basis for this observation. We also observed alteration in the EYFP-Golgi body organization as a function of paraformaldehyde concentration. Living EYFP-Golgi are composed of irregularly shaped, lobular structures (Figure 3A, inset). Fixation in 2% paraformaldehyde in PBS buffer resulted in a bloated, swollen appearance to these structures, perhaps as a consequence of incomplete fixation (Figure 3B, inset). Fixation in 4% paraformaldehyde fixative in a PBS buffer resulting in a rounded, smaller, more regularly shaped appearance (Figure 3C, inset).

EYFP-Mito was also distributed in punctate structures throughout the cytoplasm in salivary gland cells (Figure 2I). Furthermore, we noticed that EYFP-Mito also had a strong localization at cell cortex (Figure 2I, arrowhead) and an apical localization in the salivary gland epithelium basal to the adherens junction region, as determined by the localization of Merlin (data not shown; D.R. LaJeunesse, unpublished observations). The fixation of EYFP-Mito in all buffer conditions used resulted in loss of the cortical mitochondrial localization, while overall, the punctuate distribution of the mitochondria seemed relatively unperturbed (compare Figure 2I with J, K, and L).

In living salivary gland epithelial cells, EYFP-ER labeled a reticular network found throughout the cytoplasm (Figure 2E). This reticular pattern was observed in different tissue types in varying degrees, ranging from completely filling the cytoplasm to a fine diaphanous network (data not shown). In this case, the pattern resembled that

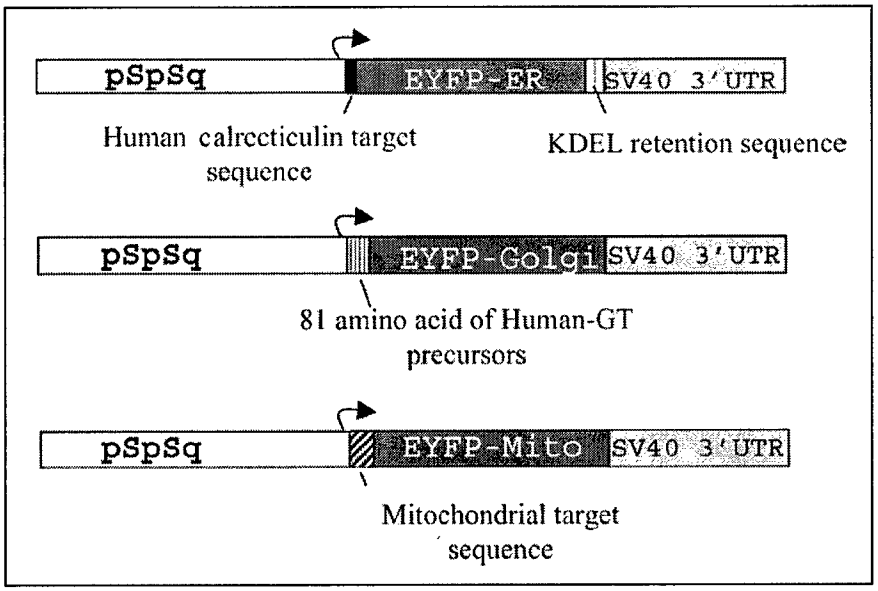

Figure 1. *Drosophila spaghetti squash* enhanced yellow fluorescent protein (EYFP) membrane-targeting constructs. Mammalian expression vectors pEYFP-ER, pEYFP-Golgi, and pEYFP-Mito were used to target sequence for the endoplasmic reticulum, Golgi apparatus, and the mitochondria, respectively. The arrows mark the start of transcription. SV40, simian virus 40; UTR, untranslated region.

of a GFP fusion with an endoplasmic reticulum-specific protein disulfide isomerase (6). Interestingly, while the Golgi and mitochondria maintained some of the structural integrity that was observed in living tissue after fixation with a paraformaldehyde-based fixative, fixation of EYFP-ER resulted in complete loss of all observable structure. The result is an amorphous mass that occupies the cytoplasm and that is completely devoid of any discernable reticulum organization (compare Figure 2E with F, G, and H).

Since many immunological localization techniques contain a permeabilization step that usually involves a wash with a buffered solution containing a detergent, we wished to examine the effects on the organization and appearance of our labeled structures by treating the fixed tissue with a detergent wash. As our detergent, we used standard concentration 0.1% of a common reagent, Triton X-100. After fixation, we exposed the samples (either EYFP-Golgi or EYFP-Mito salivary glands) to a 30-min incubation/wash with PBT. This step is similar in both composition and duration to the blocking and incubations steps found in many common immunohistological protocols used in *Drosophila* research. As in the previous experiments, we fixed under several different buffered conditions and used either a 2% or 4% concentration of paraformaldehyde. These results are shown in Figure 3. As stated before, fixation with paraformaldehyde resulted in alterations of Golgi morphology (compare Figure 3A and inset with B and C). Furthermore, poorer preservation translated into a complete loss of label after incubation with buffer containing 0.1% Trition X-100 (compare Figure 3, B and D), while stronger fixation preserved the Golgi morphology better (compare Figure 3, C and E).

DISCUSSION

Subcellular localization is a powerful aid in determining gene function. Here we report the characterization of three new EYFP intracellular membrane markers for use in the *Drosophila* model system (*Sqh::EYFP-Golgi*, *Sqh::EYFP-ER*, and *Sqh::EYFP-Mito*) that label the appropriate intracellular organelles in living and fixed tissues and that permit the assessment of subcellular structures in living and fixed tissues and cells. These constructs will be of great use to the *Drosophila* research community, and stocks containing these constructs have been deposited at the Bloomington *Drosophila* Stock Center at Indiana University (Bloomington, IN, USA). These tools offer a unique opportunity for the researcher studying intracellular organization and organelle structure. Although there are antibody markers and other GFP fusion proteins to endogenous endoplasmic reticular, mitochondrial, and Golgi-targeted proteins for use in *Drosophila* research (6–9), the new intracellular membrane markers reported in this report have distinct advantages over these

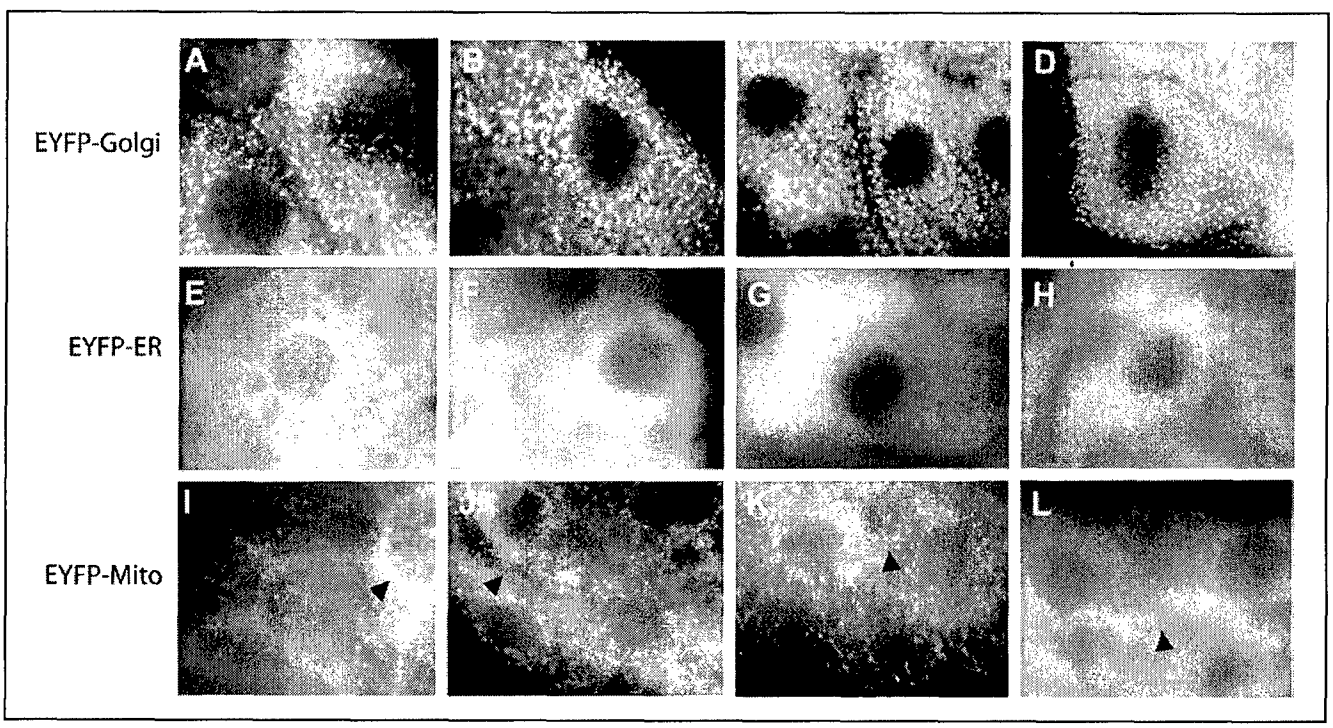

Figure 2. Living versus fixed: *Drosophila* salivary gland epithelial cells expressing membrane-targeted enhanced yellow fluorescent protein (EYFP) fixed under different buffer conditions. Living salivary gland epithelial cells expressing ubiquitously expressed *Drosophila* transgenes (A–D) EYFP-Golgi, (E–H) EYFP-ER, and (I–L) EYFP-Mito. (B, F, and J) Cells fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS). (C, G, and K) Cells fixed with 2% paraformaldehyde in PIPES/EGTA/magnesium (PEM) buffer. (D, H, and L) Cells fixed with 2% paraformaldehyde in phosphate lysine periodate (PLP) buffer. Fixation under a variety of buffer conditions results in little alteration to the overall distribution of EYFP-Golgi localization (compare A and B, C and D). However, we observed consistent reduction in the size of EYFP-Golgi when fixed in the PLP buffer. In living larval salivary gland epithelial cells, EYFP-ER localizes to a large cytoplasmic network (E), but fixation destroyed all discernable structure of the label (F, G, and H). EYFP-Mito localization remains generally unaltered after fixation, however, the cortical localization of EYFP-Mito is lost with fixation (I) to cells borders as defined by arrowheads in (J, K, and L).

tools. Since these constructs are merely EYFP moieties targeted to a specific organelle, they will therefore be less likely to affect the structure and organization of these organelles than ectopic overexpression of an endogenous protein. Moreover, the targeted EYFP constructs described in this paper are ubiquitously expressed and are not constructed in the bipartite UAS/GAL system (10); therefore, observation of the EYFP does not require the addition of further genetic elements, thus expediting mutant analysis and phenotypic characterization.

Using these constructs, we also addressed the topic of fixation artifact. An overwhelming majority of information regarding protein subcellular localization has been determined by common immunohistochemical techniques in which a tissue is fixed (usually with a formaldehyde-based fixative), incubated with a primary antibody specific to the target protein, and then labeled with secondary antibodies for detection (11). However, astonishingly little work has been published regarding the extent and role that fixation artifact plays in this process, although differences between living and fixed tissue have been alluded to in the literature and anecdotally. Traditionally, fixation artifact has been of greater concern in electron microscopy, and through the development of some advanced procedures such as freeze substitution, a few of these obstacles have been overcome (12). In most immunohistochemical procedures, there is a conflict between the fixation and preservation of the epitope. Generally, longer and stronger fixations preserve more of the overall cellular and intracellular structure, but the cost is the loss of the sensitive epitopes that are recognized by the primary antibodies. As shown with immunoelectron microscopy, the fixation of membrane-bound organelles with chemical cross-linking agents such as paraformaldehyde presents an unusual problem because biological membranes are not entirely composed of proteins, moreover, it has been suggested that fixation may even alter the apparent membrane structure through aberrant chemical cross-linking (12).

Here we show that fixation alters the morphology of intracellular organelle structure. However, the extent of artifact was dependent on a fixative as well as organelle. Some structures fixed well under a variety of conditions (Golgi and mitochondria), while the endoplasmic reticulum was extremely labile to any fixation protocol. In all procedures, we observed differences between fixed and living specimens. Some features such as the intracellular organization of EYFP-Golgi and the intracellular distribution of EYFP-Mito changed after fixation. However, the most striking results were the effects that a short wash with a buffered detergent solution had on our EYFP markers. Since most immunohistological techniques that are used to investigate intracellular antigens involve a permeabilization step to allow the antibody access to the target protein inside the cell, we examined the effects this procedure has on the localization of our membrane tags. In every case that a permeabilization/wash step was used after fixation with a 2% paraformaldehyde in any buffer formulation, all EYFP expression was lost. This artifact was solely dependent on paraformaldehyde concentration as the use of a 4% solution in any buffer resulted in the preservation of EYFP. The point of these experiments was not to define the conditions of fixation of a given organelle but to provide experimental caveats for future investigation and demonstrate the artifacts that such procedures generate. In general, we found that a 4% paraformaldehyde fixative in a PBS buffer preserved most of the structure and integrity of EYFP-Golgi and EYFP-Mito localization even after a PBT wash, while EYFP-ER fixed poorly under all conditions. However, these fixation conditions might not be appropriate for all epitopes found in an intracellular membrane compartment, and therefore it is appropriate to test a variety of different fixation conditions.

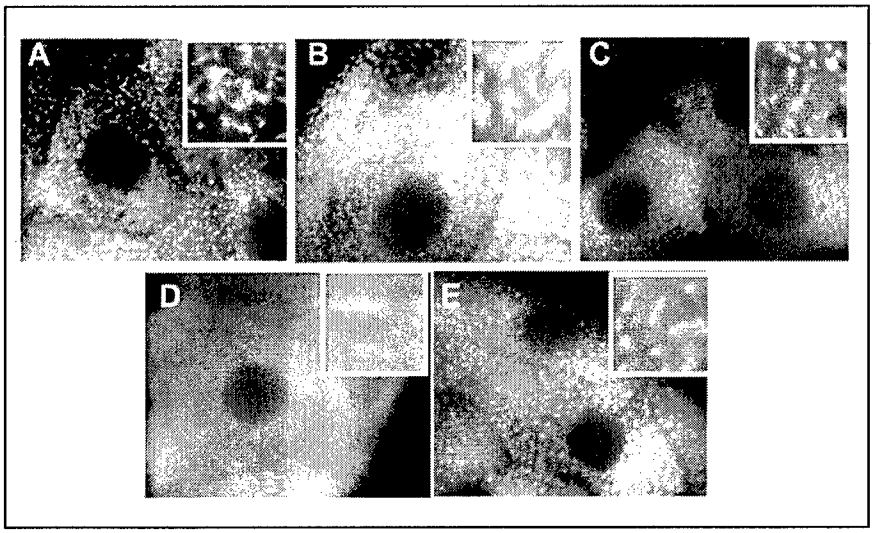

Figure 3. Effect of permeabilization on enhanced yellow fluorescent protein (EYFP)-Golgi in *Drosophila* larval salivary epithelial cells. Living larval salivary gland epithelial cells with (A) EYFP-Golgi; inset, the EYFP-Golgi structures are not round but are composed of multiple lobed structures. (B) Larval salivary gland epithelial cells fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) buffer; inset, amorphous, swollen appearance of Golgi bodies. (C) Larval salivary gland epithelial cells fixed with 4% paraformaldehyde in PBS buffer; inset, small, more discrete appearance of EYFP-labeled structures. (D) Cells fixed with 2% paraformaldehyde and then treated with 30 min incubation with 1× PBS, 1% bovine serum albumin (BSA), and 0.1% Triton X-100 (PBT); inset, no Golgi labeling. (E) Cells fixed with 4% paraformaldehyde in PBS buffer and then treated with 30 min incubation with PBT; inset, preservation of some Golgi staining.

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Microarray results improve significantly as hybridization approaches equilibrium

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Dual-channel long oligonucleotide microarrays are in widespread use. Although much attention has been given to proper experimental design and analysis regarding long oligonucleotide microarrays, relatively little information is available concerning the optimization of protocols. We carried out a series of microarray experiments designed to investigate the effects of different levels of target concentration and hybridization times using a long oligonucleotide library. Based on principles developed from nucleic acid renaturation kinetics studies, we show that increasing the time of hybridization from 18 h to 42 h and 66 h, especially when lower than optimal concentrations of target were used, significantly improved the quality of the microarray results. Longer hybridization times significantly increased the number of spots detected, signal-to-noise ratios, and the number of differentially expressed genes and correlations among replicate arrays. We conclude that at 18 h of incubation, target-to-probe hybridization has not reached equilibrium and that a relatively high proportion of nonspecific hybridization occurs. This result is striking, given that most, if not all, published microarray protocols stipulate 8–24 h for hybridization. Using shorter than optimal hybridization times (i.e., not allowing hybridization to reach equilibrium) has the consequence of underestimating the fold change of differentially expressed genes and of missing less represented sequences.

INTRODUCTION

Major parameters that affect nucleic acid hybridization equilibrium include labeled target concentration and the incubation time allowed for hybridization, both of which are critical because maximum specificity and sensitivity are reached with equilibrium. Typical protocols propose hybridization times of 8–24 h (1) and 16–20 h (2); however, there is little or no experimental data in the literature to justify a given incubation time.

Recent reports have examined different parameters that affect hybridization equilibrium for microarrays. Using a long oligonucleotide platform (60-mer), it was shown that with high complexity targets (i.e., a highly diverse population of RNA), specific target-to-probe hybridization equilibrium took much longer to attain than did nonspecific hybridization equilibrium (3). Increasing the incubation time to approach maximum hybridization substantially improved fluorescent intensities for specific targets. However, other critical criteria that help determine the quality of microarray results, such as differential expression, signal-to-noise ratios, and correlations among replicates, were not directly assessed. In other studies, using short oligonucleotide platforms (20–30 bases), it was shown that a considerable amount of cross-hybridization occurred under various conditions (4) and that longer hybridization times improved accuracy (5). Our experimental results are in complete agreement with and expand on the findings of these earlier studies (4,5). Using a long oligonucleotide platform, we applied multiple criteria to assess the quality of the microarrays that were carried out under laboratory conditions. We show that as hybridization equilibrium is approached by allowing longer incubation periods and/or approached more rapidly by increasing the concentration of labeled target, microarray quality is significantly improved.

MATERIALS AND METHODS

Microarray Hybridization

Total RNA isolated from mouse (strain Balb/c) heart was purchased