RNAi knockdown of the *flightless-I* transcript In *Drosophila melanogaster*

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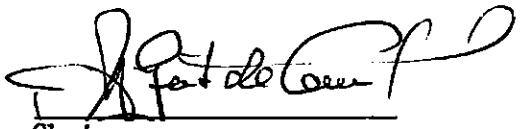
We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Biomedical Sciences (Cell and Molecular Biology).

Thesis Committee



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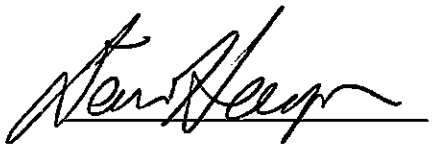

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I. Abstract

This study is part of a basic research endeavor to investigate the function of the *flightless-I* gene and its encoding protein in its cellular context. The model organism used for this purpose is the vinegar fly *Drosophila melanogaster*.

The *Drosophila flightless-I* gene encodes a protein that shares significant sequence similarity with members of the gelsolin family of actin severing and capping proteins. Genomic deletions of the *flightless-I* locus result in lethality at pupal stages, indicating that *flightless-I* is essential in flies. Viable mutant alleles lead to structural deformities in actin-related structures such as indirect flight musculature and bristles. Flightless-I is thus hypothesized to be an actin-regulatory protein that functions in cellular processes pivotal in fly development. In order to investigate the cause of lethality, a combination of GAL4/UAS and RNAi technology is applied to produce *flightless-I* loss-of-function phenotypes in select tissue. In this thesis, an RNAi construct was tested for knockdown efficiency of the *flightless-I* message. It was demonstrated that this RNAi construct did not effectively downregulate *flightless-I* expression. Residual protein levels remained that rescued the wild type phenotype. This indicated limitations of RNAi experiments, and it was concluded that novel RNAi constructs should be designed to test for enhanced knockdown efficiency.

II. Introduction

II.1. The Gelsolin Superfamily

The actin cytoskeleton plays an important role in multiple aspects of cell behavior, such as cytokinesis, muscle contraction, vesicular transport, and formation of filopodia and lamelipodia during axonal pathfinding (Schmidt & Hall 1998, Stamnes 2002, Dent & Gertler 2003). The actin cytoskeleton also participates in transmembrane signaling where Rho-GTPases are thought to be key regulators in signaling pathways that link extracellular signals to the assembly and organization of the actin cytoskeleton (see Schmidt & Hall 1998 for review).

In these processes, the structure of actin filaments is modulated by actin binding proteins. One class of actin binding proteins is comprised of the gelsolin superfamily of actin severing and capping proteins. Members of this family share extensive sequence homology and have been identified in *Dictyostelium*, *Caenorhabditis elegans*, *Drosophila*, and mice (see Silacci et al. 2004 for review). These proteins consist of either three or six repeats of homologous domains, in addition to unique domains at the amino or carboxy termini.

Gelsolin, the founding member of this superfamily, is a protein of 80-84kDa that can sever actin filaments and cap the (+)-end of actin filaments (figure 1) (Kwiatkowski et al. 1989). These processes are activated by Ca^{2+} and inhibited by phosphatidylinositol 4,5-bisphosphate (PIP_2), suggesting that gelsolin activity is initiated by extracellular signals. Gelsolin contains six repeats of homologous gelsolin-like domains (see figure 2), and binding of Ca^{2+} induces multiple conformational changes, which eventually render

the protein capable to undertake its actin binding, regulatory activities. In vertebrates as well as *Drosophila*, a secreted form and a cytoplasmic form of gelsolin exist (Kwiatkowski et al. 1988, Stella et al. 1994). Both are derived from the same coding region by differential splicing. Gelsolin is expressed in a wide variety of cell types, and its activity leading to changes in actin filament organization is involved in various processes such as cell motility, phagocytosis, and even apoptosis, where gelsolin has been shown to be a target of caspase-3 (Kwiatkowski 1999, Silacci et al. 2004).

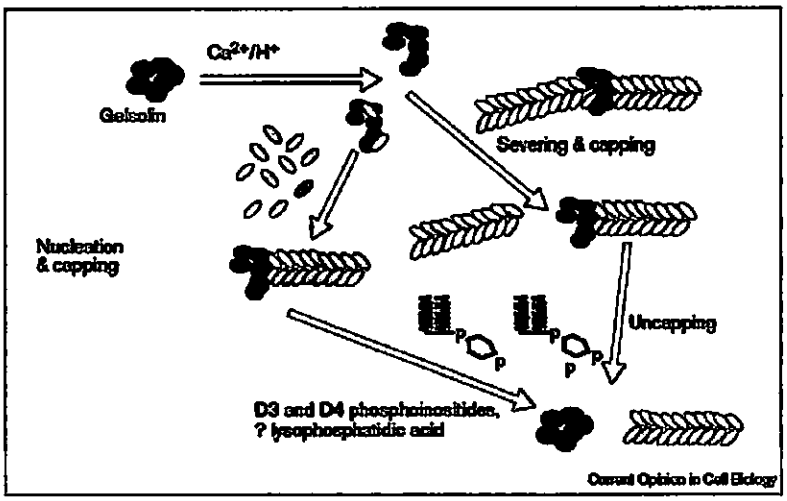


Figure 1. Actin-regulatory activity of gelsolin. In its inactive state, gelsolin is depicted as a compact six domain structure. In response to micromolar Ca^{2+} or pH < 6.5, gelsolin opens up to become active in either severing or binding to actin monomers, resulting in a capped actin filament. In response to clustered phosphoinositides, gelsolin uncaps from the filament, and resumes a closed configuration in low Ca^{2+} and normal pH (image from Kwiatkowski 1999).

II.2. The *Drosophila flightless-I* Gene and its Encoded Protein

The *flightless-I* gene of *Drosophila* maps to subdivision 19F of the X-chromosome (de Couet et al. 1995). The subdivision 19F is 67kb in length and contains 12 transcription units as shown in figure 2 (Maleszka et al. 1998).

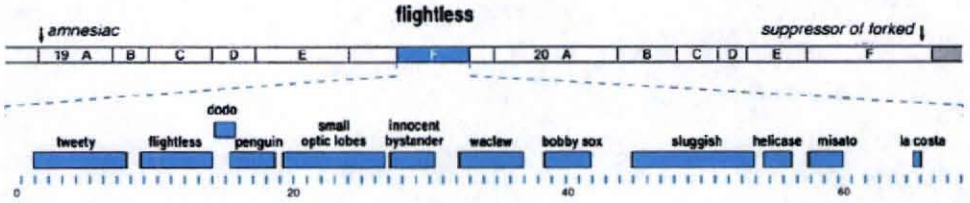


Figure 2. A model of the 19F subdivision of the *Drosophila* X-chromosome in which the *flightless-I* gene is located (from Maleszka et al. 1998).

The *flightless-I* gene encodes a 145kDa protein that shares significant sequence similarity with the members of the gelsolin superfamily (figure 3) and is conserved across animal phyla, sharing 58% identity with human flightless-I and 48% identity with *Caenorhabditis elegans* (*C. elegans*) flightless-I, respectively (Campbell et al. 1993). Like other members of the gelsolin superfamily, the flightless-I protein contains six homologous gelsolin-like domains (GLDs) at its C-terminus, whereas its unique feature is the presence of a leucine-rich 16 tandem repeat domain (LRR) at its N-terminus (Campbell et al. 1993) (figure 3 and 4).

A leucine-rich repeat domain is a horseshoe-shaped structure which is present in about 2000 proteins identified in viruses, bacteria, and eukaryotes (Enkhbayar 2003). Proteins carrying this domain are implicated in various protein-protein interactions, such as signal transduction, cell adhesion, apoptosis, transcription, and immune responses. Several binding partners, found to be components of nuclear receptor signaling pathways as well as the β-catenin signaling pathway, of the mammalian flightless-I LRR have been determined via yeast-two-hybrid screens (Liu & Yin 1998, Fong & de Couet 1999, Lee et al. 2004, Liu et al. 2005, Lee & Stallcup 2006). The implication of a possible direct or indirect involvement of the flightless-I protein in nuclear receptor signaling and β-catenin signaling, as well as other cellular processes, will be discussed in detail in the last section of this thesis.

As will be outlined in the following section, the *flightless-I* gene appears to play an actin-regulatory role, and, since deletion of this gene result in a late embryonic lethal phenotype, seems to be essential in *Drosophila* development. Yet, in spite of its suggested significance in development, its cell-specific roles remain elusive, which makes this gene an important candidate to investigate in order to broaden our understanding of conserved developmental mechanisms.

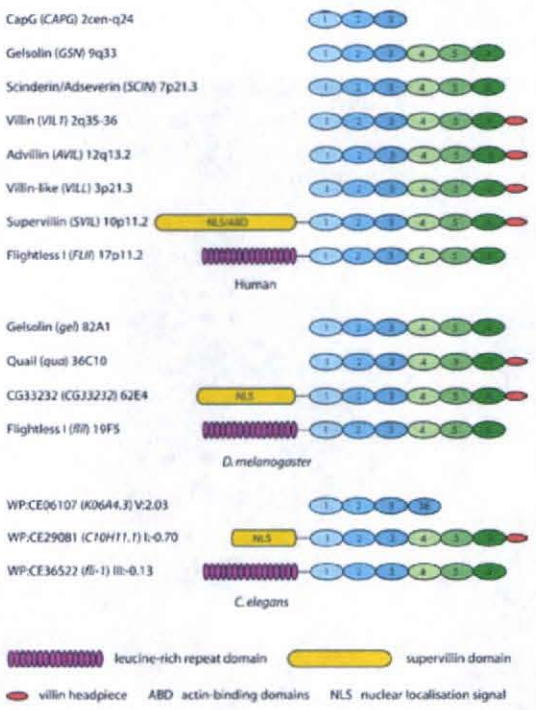


Figure 3. Structure of *Drosophila* flightless-I compared to other members of the gelsolin superfamily (from Archer et al. 2004).

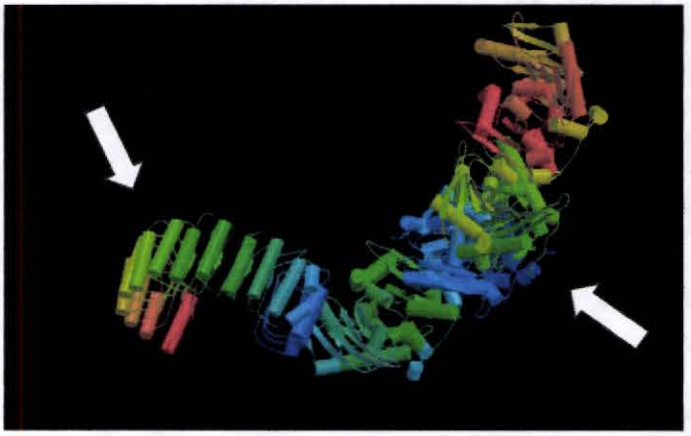


Figure 4. A hypothetical 3D model of the flightless-I protein structure. 16 tandem leucine rich repeats at the N-terminus are shown on the left (left block arrow). The six gelsolin-like domains at the C-terminus are shown at the right (right block arrow) (image courtesy of H. G. de Couet).

II.3. Flightless-I as an Actin Regulator

The *Drosophila* flightless-I protein is a ubiquitous component of developing and differentiated tissues and appears to play a pivotal role in actin organization during embryogenesis and myogenesis.

In indirect flight muscles, flightless-I was shown to target plus-ends of actin filaments in sarcomeres (figure 5) and to reduce sarcomere length from an average of 3μm in wild type to an average of 2.7μm. Overexpression of the gelsolin-like domains without the leucine-rich repeat domain in indirect flight muscles caused severe structural deformities (figure 6) (Meada & de Couet, unpublished data).

This difference in activity of the complete flightless-I protein and the gelsolin-like domains in indirect flight musculature implied that the leucine-rich repeat domain might perform a regulatory function. Without the leucine-rich repeat domain, the gelsolin-like domains were possibly unrestricted in their activity, thus leading to a more severe distortion of indirect flight muscle structure.

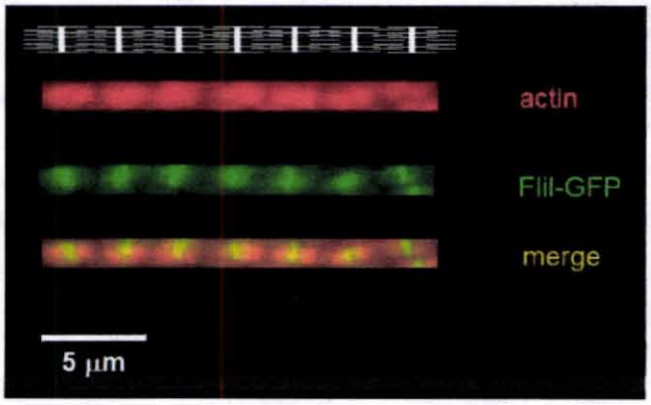


Figure 5. Flightless-I-GFP colocalizes with Z-bands in the indirect flight muscle (image courtesy of H. G. de Couet).

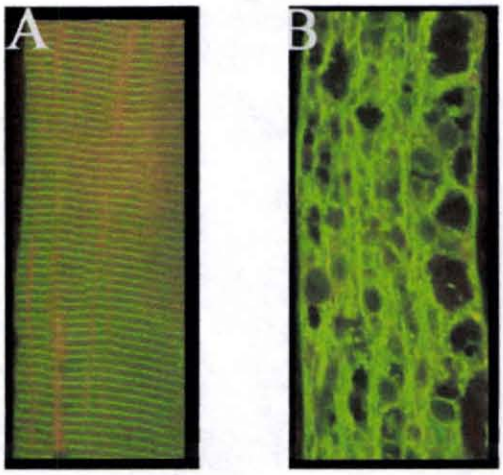


Figure 6. GLD-GFP overexpression leads to indirect flight muscle degeneration. (A) wild type. (B) GLD-GFP overexpression (image courtesy of H. G. de Couet).

Furthermore, overexpression of flightless-I in bristles led to a distinct, singed-like bristle phenotype (figure 7). This suggested that excessive amounts of severing activity due to flightless-I overexpression truncated the actin filament network that mediate bristle formation, thus leading to the bristle phenotype.

Figure 7. Overexpression of Fli-I-GFP in bristles causes singed-like bristle phenotype (image courtesy of H. G. de Couet).

Several *flightless-I* mutant alleles that imply a role of flightless-I in myogenic actin organization are known. For example, *flightless-I*^{3} and *flightless-O*^{2} alleles are caused by point mutations in the gelsolin-like domain 1. They are characterized by flightlessness due to disrupted Z-bands, leading to distorted sarcomere structure of the indirect flight muscles (figure 8) (Perrimon et al. 1989, Miklos & de Couet 1990, de Couet et al. 1995).

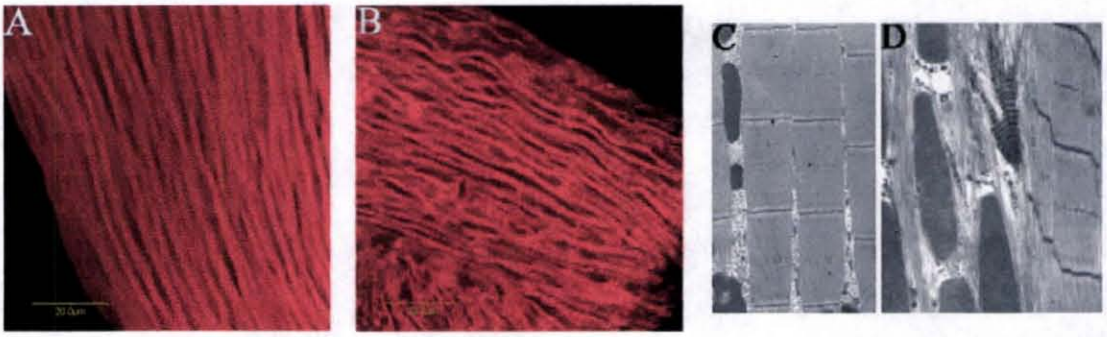


Figure 8. (A) Confocal image of wild type *Drosophila* indirect flight muscle. (B) Confocal image of *flightless-O*^{2} *Drosophila* indirect flight muscle; disruption of sarcomere structure can be seen. (C) TEM image of wild type *Drosophila* indirect flight muscle. (D) TEM image of *flightless-I*^{3} *Drosophila* indirect flight muscle; disruption of Z-bands can be seen (A stained with phalloidin, C and D courtesy of H. G. de Couet).

Null-alleles of the *flightless-I* gene, e.g. the *fli-I*^{W2} and *fli-I*^{D44} alleles, have been demonstrated to cause lethality at pupal stages, illustrating the significance of the *flightless-I* gene during development (Perrimon et al. 1989, Miklos & de Couet 1990, de Couet et al. 1995). The significance of *flightless-I* during development was furthermore demonstrated by Straub et al. (1996). In *flightless-I* null-mutant *Drosophila* eggs lacking maternal flightless-I protein, development was severely disrupted during early embryogenesis. In these embryos, nuclei lost their positioning during cellularization of

the syncytial blastoderm, which consequently led to abnormal gastrulation and lethality in early development (figure 9).

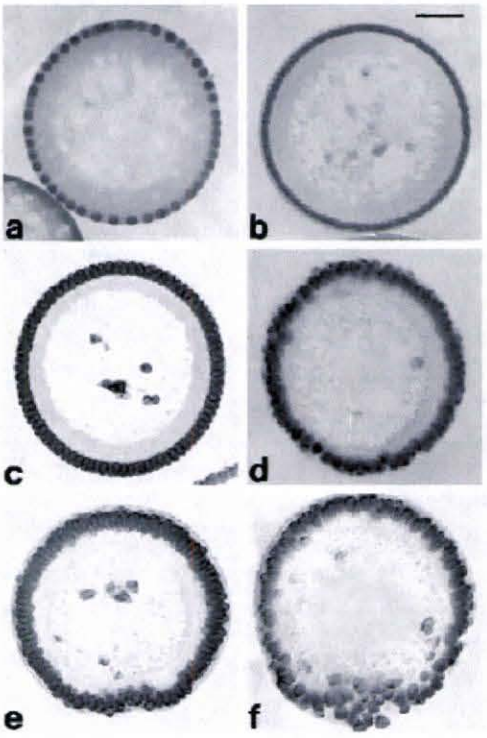


Figure 9. Defects in nuclear positioning in *flightless-I* null mutants. (a,c,e) Wild type; (b,d,f) mutant. (d) The layer of nuclei in mutant eggs begins to look disorganized soon after the nuclei have begun to elongate and cellularization has begun. (e) When gastrulation begins, nuclei in ventral cells move away from the apical cell surface. (f) In mutant embryos, nuclei move out of the incompletely cellularized peripheral layer of cytoplasm into the inside of the egg. Bar: 50 μm (image from Straub et al. 1996).

Modifications of submembraneous actin filaments are known to be involved in formation of the furrow canals during cellularization (see Mazumdar & Mazumdar 2002 for review). Actin forms complexes with myosinII and accumulates at the furrow canals which then extend into the interior of the egg. Straub et al. (1996) suggested that a loss of flightless-I led to a disruption of the accumulated actin filament network which, in turn, disrupted delivery of vesicles needed to extent the plasma membrane into the interior of the egg.

II.4. Research Hypothesis and Objective

The flightless-I protein is hypothesized to bind to the barbed ends of actin filaments via its gelsolin-like domains to mediate actin filament organization in cellular processes during *Drosophila* development, whereas the leucine-rich repeat domain is hypothesized to regulate gelsolin-like domain activity. The presence of the leucine-rich repeat domains in the flightless-I protein also suggests a possible role in protein-protein interaction (Claudianos & Campbell 1995, Liu & Yin 1998, Enkhbayar et al. 2004). Thus, it is also postulated that the flightless-I protein might bind to other factors or even dimerize via its LRR to regulate actin organization.

In this context, the precise tissue-specific function of the flightless-I protein is difficult to determine due to lethality of *flightless-I* null-alleles ($fli\text{-}I^{W2}$ and $fli\text{-}I^{D44}$) at pupal stages. Hence, the overall objective of this research endeavor is to suppress expression of the *flightless-I* gene in select tissues only, leading to cell-specific phenotypes which could provide further insight into flightless-I actin-regulatory activity and the causes of lethality. A method commonly used to give rise to cell-specific mutations is the flip-recombinase (FLP-FRT) system. However, application of this technique to generate mosaic phenotypes is problematic due to the proximity of the *flightless-I* locus to the centromere. There are no known FRT sites between the *flightless-I* locus in 19F and the centromere.

Therefore, an alternative methodology, a combination of RNA interference (RNAi) and GAL4-UAS technology, is applied in the attempt to generate tissue-specific mutants. These techniques are described in detail in sections III.1. and III.2. Application of this methodology employs an RNAi plasmid construct inserted into the fly's genome which

generates double stranded RNA (dsRNA) in select fly tissues, such as indirect flight muscles, bristles and central nervous system (e.g. mushroom bodies). The presence of dsRNA leads to downregulation of the *flightless-I* messenger RNA (mRNA) in that select tissue. Furthermore, this methodology also allows for ubiquitous downregulation of the *flightless-I* mRNA in all tissues as well as downregulation in imaginal discs during early development.

The specific objective of this thesis was to test for activity of an RNAi construct targeting *flightless-I* mRNA. For that purpose, *flightless-I* expression was first attempted to be downregulated in indirect flight muscles, in bristles, as well as ubiquitously in all tissues. *flightless-I* phenotypes in indirect flight muscles and bristles were already known and could serve as controls. Downregulation of *flightless-I* expression in indirect flight muscles was expected to lead to a flightless phenotype similar to the *flightless-I*^{3} or *flightless*-O^{2} alleles (see figure 8). Of specific interest were abnormalities in sarcomere length and arrangement of the Z-bands. Since overexpression of *flightless-I* in bristles led to a singed-like bristle phenotype (see figure 7), loss of *flightless-I* in bristles was expected to lead to a bristle phenotype also. Ubiquitous downregulation of *flightless-I* was expected to mimic the null alleles, leading to lethality at pupal stages. Knockdown efficiency of the RNAi construct was furthermore set out to be determined by Western blot analysis and a control cross with a fly line that overexpresses *flightless-I* in bristles, which was expected to lead to suppression of the singed-like bristle phenotype. In the case of high knockdown efficiency of the RNAi construct, flies carrying that construct were planned to be crossed with other tissue-specific GAL4 driver lines (e.g. specific for central nervous system) to further investigate the causes of lethality. In the case of low or

no knockdown efficiency, additional RNAi constructs of various lengths targeting different regions of the *flightless-I* mRNA were planned to be constructed.

III. Research Design and Methods

III.1. RNA Interference and Plasmid Construction

The use of double-stranded RNA as a powerful tool for inducing downregulation of targeted gene expression was first described in *C. elegans* (Fire et al. 1998). Generally speaking, RNAi technology is based on the presence of double stranded RNA (dsRNA) which triggers post-transcriptional silencing of a corresponding gene by causing degradation of its complementary messenger RNA (mRNA) via a complex pathway outlined in figure 10 (see Dyxhoorn et al. 2003 for review).

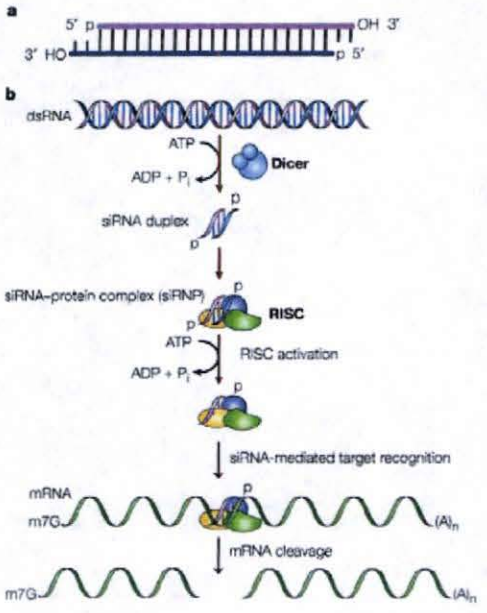


Figure 10. siRNA Pathway. dsRNA is cleaved into siRNAs by Dicer, a member of the RNase III family. These siRNAs are then incorporated into the RNA-inducing silencing complex (RISC), followed by unwinding of the siRNA duplex. The single stranded siRNA then guides RISC to the mRNA that has a complementary sequence, followed by endonucleolytic cleavage of the target message (image from Dyxhoorn et al. 2003).

In this research project, generation of dsRNA was induced using the pWiz vector. This vector consists of a *white* marker gene as well as a *white* intron which is flanked by

cloning sites for complementary forward and reverse DNA fragments of the gene to be downregulated (Lee & Carthew 2003).

Primers for the amplification of *flightless-I* fragments were designed by the *E*-RNAi internet database of the German Cancer Research Center, Heidelberg, Germany (web address: http://www.dkfz.de/signaling2/e-rnai/).

The construct encoding *flightless-I* inverted repeats was generated by amplifying the *flightless-I* coding sequence (GenBank Accession Number LD21753) from positions 1853-2172bp by polymerase chain reaction (PCR), using primers that introduced unique restriction sites at the product ends. The forward DNA fragment was cloned into the *Eco*RI and *Bgl*II sites of the pWiz plasmid. The primer sequences used for the forward fragment were:

- 5'-primer: 5'-CGAGCGAATTCTGTCCCTCTTCGAGACG-3', and
- 3'-primer: 5'-ACTGGATTTCAGATCTGTTCTTGCGCTC-3'.

The reverse DNA fragment was cloned into the *Nhe*I and *Xba*I sites. The primer sequences used for the reverse fragment were:

- 5'-primer: 5'-GATTTCACGCTAGCTCTTGCGCTCCG-3', and
- 3'-primer: 5'-CGAGCAGTTTCTAGACCTCTTCGAGAC-3'.

General cloning techniques of polymerase chain reaction, DNA digestion, SAP-treatment, DNA ligation, and transformation according to Sambrook et al. (1989) were applied. Gel extraction was performed using the Qiagen MinElute™ Gel Extraction Kit (50) (Catalog #28604), and Qiagen QIAquick™ Gel Extraction Kit (50) (Catalog #28704). Plasmid extraction was performed using the Qiagen QIAprep® Spin Miniprep Kit (50) (Catalog #27104).

III.2. GAL4/UAS Technology

GAL4 is a well-known yeast transcriptional activator that binds to a response element, the upstream activation sequence (UAS), to activate transcription of any target gene that is fused to that UAS. Crossing a fly line that expresses GAL4 in specific tissue with a fly line that carries a target gene fused to UAS, leads to offspring that will express the target gene in the tissue in which GAL4 is expressed (Brand & Perrimon 1993) (figure 11).

In pWiz, upstream of the two insertion sites for the DNA fragments a UAS is located. When GAL4 binds to the UAS, it triggers expression of the forward and reverse DNA fragments. Since these fragments are complementary, dsRNA with a hairpin formed by the *white* intron is produced. After splicing of the *white* intron, a loopless dsRNA for RNAi on the *flightless-I* message is generated (figure 12).

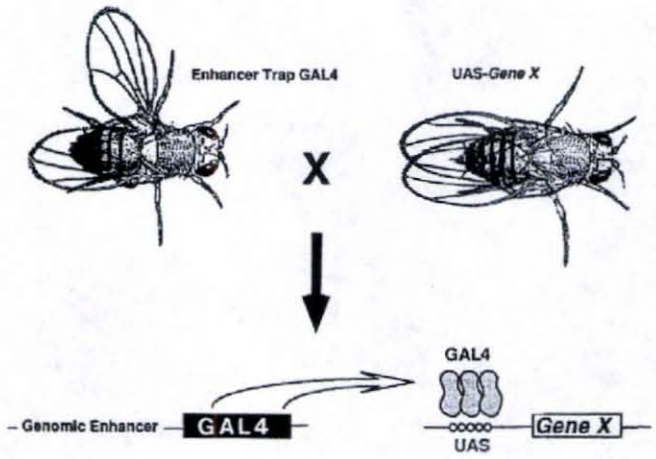


Figure 11. General principle of GAL4/UAS technology. If both fly lines are homozygous for GAL4 and UAS, respectively, all offspring will contain GAL4 as well as its response element fused to the target gene, leading to expression of that gene (image from Brand & Perrimon 1993).

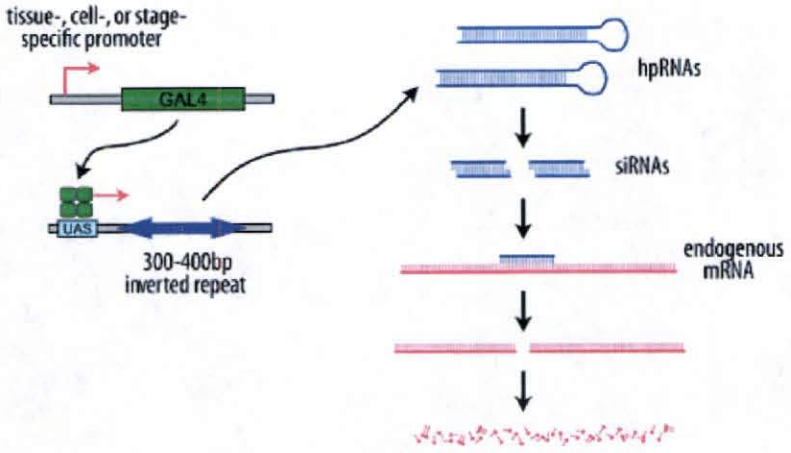


Figure 12. Combination of GAL4/UAS technology and RNA interference leads to tissue specific downregulation of the target mRNA (image from Vienna Drosophila RNAi Center, http://www.vdrc.at/library.htm)

III.3. Fly Stocks and Crossings

P-element mediated transformation of the RNAi construct with Δ2-3 transposase into y;w⁻ *Drosophila* germ line by was performed commercially. The service used was Genetic Services, Inc., 16 Craig Rd. Sudbury, MA 01776. The injected larvae were raised to adulthood (G_0). These flies were then crossed with w^{1118}, and transformed flies were collected to generate five independent insertion lines from the RNAi construct. Four of these five lines were mapped according to the mapping scheme in appendix VII.1. For these mapping crosses, the balancer stock used was provided by the laboratory of Steven Robinow at the Zoology Department of the University of Hawaii at Manoa.

Fly lines were kept on basic food medium (protocol on file and available upon request) at 22°C. Crosses were performed at 28°C to enhance GAL4 production. The GAL4 lines were obtained from the Bloomington Stock Center: indirect flight muscle-GAL4 (Stock #8609), bristle-GAL4 (Stock #1122), tubulin-GAL4 (Stock #5138), heat

shock (hs)-GAL4 (Stock #1799), nanos-GAL4 (Stock #7303), and mushroom body-GAL4 (Stock #854).

Heat shock crosses of the RNAi lines and hs-GAL4 to downregulate maternal *flightless-I* were conducted as follows: The RNAi lines were crossed with the hs-GAL4 driver. Female virgin offspring of that cross were exposed to 37°C for 1-2 hours twice a day for 5 days. These females were crossed with wild type (Canton S) males.

The flightless-I overexpression line used in the control cross was designed by H. G. de Couet and is included in the stocks of the de Couet laboratory. This line contains the bristle GAL4 driver as well as the UAS-Fli-I response element on the 3rd chromosome. One stock is balanced with TM6 (named B11-Fli/TM6), another is balance with TM3 (named B11-Fli/TM3). The TM3 balanced line was used in the control cross.

If homozygosity of the *gal4* gene is lethal, GAL4 fly lines are usually balanced with a balancer chromosome. A balancer chromosome has three advantages. First, it carries multiple inversions, which prevent crossing over. Secondly, it carries recessive lethal mutations, which hinder homozygosity of the balancer. Thirdly, it carries dominant, phenotypically visible marker genes. Due to these characteristics, balancer chromosomes are not only used for GAL4 fly lines but also for any recombinant line that needs to be kept heterozygous due to a lethal mutation, or that carries an otherwise invisible recessive mutation on one of its alleles. Balancer chromosomes are widely used and are available for the sex chromosomes and autosomes 2 and 3.

Flightlessness was tested as follows: 30 flies of each offspring of the crosses between the RNAi lines and the indirect flight muscle specific GAL4 driver were collected. The upper inside of a 1000mL beaker was lubricated with Teflon to prevent

flies from crawling out of the beaker. The 30 flies were collected in a food vial and the vial was held 30cm above the beaker while the flies were poured into the beaker. Flies that were not flightless could fly away and flightless flies dropped into the beaker. This process was repeated 5 times for each sample.

III.4. Detection of UAS and RNAi Fragments via Sequencing and PCR

In order to verify the presence and correct orientation of the DNA fragments in pWiz via sequencing the following sequencing primers targeting regions of pWiz flanking both fragments were used:

- 5'-primer: 5'-ACTGAAATCTGCCAAGAAGT-3', and
- 3'-primer: 5'-TGTCACACCACAGAAGTAAG-3'.

To verify the presence and correct orientation via PCR, two sets of primers were used that target regions flanking each fragment. These were for the forward fragment and reverse fragments, respectively:

- 5'-primer: 5'-ACTGAAATCTGCCAAGAAGT-3' (forward fragment), and
- 3'-primer: 5'-AGCCGACTGCGAATAGAAAC-3' (forward fragment).
- 5'-primer: 5'-GTCGGCTGATCTGTGTGAAA-3' (reverse fragment), and
- 3'-primer: 5'-TGTCACACCACAGAAGTAAG-3' (reverse fragment).

These two sets of primers were also used to verify the presence of the RNAi construct in the genomes of the mapped independent transformation lines. The protocol applied to extract fly DNA was provided online by E. Jay Rehm of the Berkeley Drosophila Genome Project (http://www.fruitfly.org/about/methods/inverse.pcr.html). In order to

detect the presence of the GAL4 response element UAS in the RNAi construct, primers flanking the five repeats of UAS (CGGAGTACTGTCCTCC) were used for sequencing:

- 5'-primer: 5'-CAAGCTTGCATGCCTGCAGG-3', and
- 3'-primer: 5'-GCGCTCGCTAGAGTCTCCGC-3'.

III.5. Western Blotting

Offspring of the crosses of tubulin-GAL4 drivers with the RNAi lines were decapitated and the heads were homogenized for sample preparation. Sample preparation was performed with SDS sample buffer: 7mL 4x TrisCl/SDS in pH 6.8, 3mL glycerol, 1g SDS, 0.93g DTT, 1.2mg bromphenol blue.

Protein samples were run on SDS-polyacrylamide gels (8% resolving and 5% stacking) and transferred onto Millipore Immobilon™-P PVDF transfer membrane (Catalog #IPVH00010). Running buffer (1x): 25mM Tris, 192mM glycine, 0.1% SDS. Transfer buffer: 48mM Tris base, 39mM glycine, 200mL MetOH, 0.0375% SDS. The membrane was pre-treated with 100% MetOH and dH_20 for 1-2 minutes each.

Western blots were performed following standard protocol (Ausubel et al. 1994). Blocking buffer: 1x PBS, 0.3% Tween-20, 5% non fat dry milk. Primary antibodies were α-actin #JLA-20 (1:3000) from the Developmental Studies Hybridoma Bank at the University of Iowa, and monoclonal α-flightless-I 114C #16 (1:3000) manufactured by H. G. de Couet. Secondary antibody was anti-mouse HRP-conjugated IgG (1:10,000 for flightless-I and 1:5000 for actin) from Jackson ImmunoResearch Laboratories, Inc. (code # 715-036-150). Detection was carried out utilizing SuperSignal® West Pico Chemiluminescent Substrate from Pierce Biotechnology (catalog #34077).

IV. Results

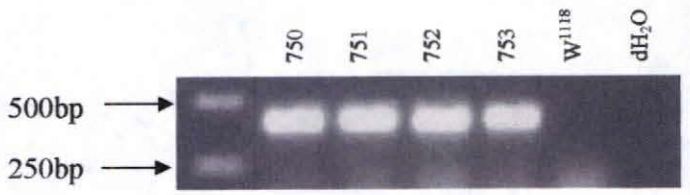
IV.1. RNAi Insertion Lines

The cloning of the RNAi construct was successfully completed and five independent insertion lines were generated commercially from that construct via P-element mediated transformation.

The RNAi construct targeted the C-terminal gelsolin-like domain 1 from cDNA positions 1853-2172bp. Three of the obtained RNAi Lines (labeled 750, 751, and 752) were determined to be homozygous viable and were mapped to the 3rd chromosomes. A fourth RNAi Line (labeled 753) was also determined to be homozygous viable and was mapped to the 2nd chromosome. The 5th RNAi line has yet to be mapped and was not used in these experiments. The crossing scheme for mapping and generation of homozygotes is listed in appendix VII.1.

Sequencing of the construct, polymerase chain reaction (PCR), as well as digestions with the respective digestion enzymes confirmed the presence and correct orientation of the two inserted *flightless-I* forward and reverse fragments in the pWiz construct. DNA was extracted from insertion lines 750, 751, 752, and 753 and the presence of the inserted forward and reverse fragments in the pWiz RNAi construct in the genomes of the four fly lines was furthermore verified via PCR (figure 13).

A



B

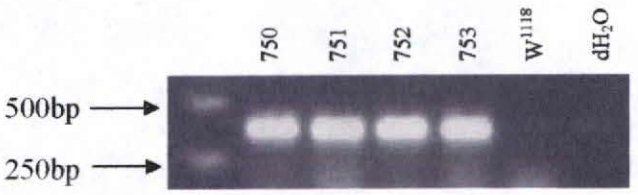


Figure 13. DNA fragments generated by polymerase chain reaction (PCR) to verify the presence of forward and reverse fragments of the RNAi construct in insertion fly lines 750, 751, 752, and 753. w^{1118} and dH_2O were negative controls. The primers used targeted sequences flanking each fragment (see section II.4.). The expected size of each PCR product was 350-400bp. A: forward fragments. B: reverse fragments.

IV.2. Initial GAL4 Crossing Experiments

In order to test for activity of the RNAi construct, the four independent RNAi insertion lines were crossed with a ubiquitous GAL4 driver as well as GAL4 drivers specific for indirect flight musculature and bristles. These GAL4 drivers were chosen for the test crosses because potential phenotypes due to a loss of *flightless-I* expression could be predicted based on already known mutant phenotypes in these tissues. These predictions were as follows:

- Cross of RNAi lines with an indirect flight muscle-GAL4 driver
    - Here, the expectation was to observe a flightless phenotype, possibly due to disruption of sarcomere structure.

- Cross of RNAi lines with a bristle-GAL4 driver
    - It was expected that downregulation of *flightless-I* gene expression in bristles would lead to a phenotype of abnormal bristle morphology.

- Cross of RNAi lines with a ubiquitous tubulin-GAL4 driver
    - This cross was expected to mimic null-alleles; that is, it was expected to observe a lethal phenotype.

Offspring of the crosses of the RNAi lines and the indirect flight muscle GAL4 driver did not reveal the expected flightlessness. Both the RNAi lines and the indirect flight muscle GAL4 driver were homozygous viable. Therefore, all offspring were expected to carry one RNAi allele and one GAL4 allele, and thus all offspring were expected to be flightless. The flightlessness test as described in section III.3. did not demonstrate flightlessness.

The bristles GAL4 driver was heterozygous and balanced with TM3. Thus 50% of the offspring were expected to carry one copy of the bristle specific GAL4 construct and one copy of the RNAi construct, whereas the other 50% of the offspring did not carry a GAL4 copy. The 50% offspring carrying both the RNAi and GAL4 copies did not show any difference in bristles structure compared to wild type.

The tubulin GAL4 driver was also heterozygous and balanced with TM3. Thus, 50% of the offspring carried one copy of the RNAi and GAL4 constructs and were expected to show changes in viability, whereas the other 50% not carrying tubulin GAL4 served as control. The results are summarized in figures 14-17. As can be seen, expected lethality of the 50% of offspring carrying both the RNAi construct and the tubulin GAL4 construct did not occur. However, hatching of the offspring from the 750 cross and the 752 cross carrying both the RNAi construct and the tubulin GAL4 construct was observed to be delayed by 2 days. That is, no offspring carrying both constructs emerged during the first two days of collection.

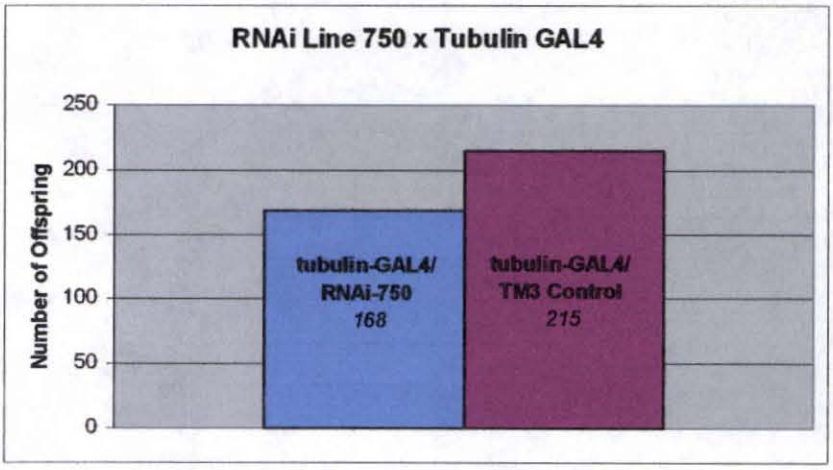


Figure 14. Summary of cross of RNAi line 750 with tubulin GAL4. Offspring were collected over a period of 14 days. Hatching of offspring carrying both a copy of the RNAi construct and the tubulin GAL4 construct was observed to be delayed for 2 days.

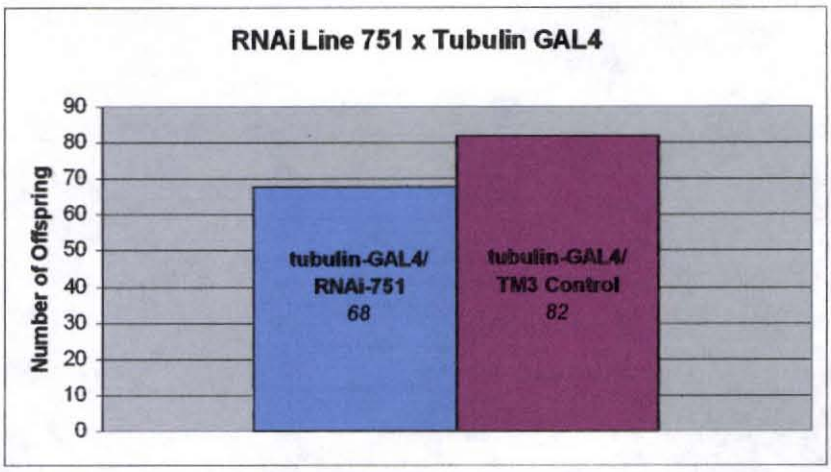


Figure 15. Summary of cross of RNAi line 751 with tubulin GAL4. Offspring were collected over a period of 10 days. Even though a difference in the number of offspring occurred, hatching of offspring carrying both a copy of the RNAi construct and the tubulin GAL4 construct was not delayed.

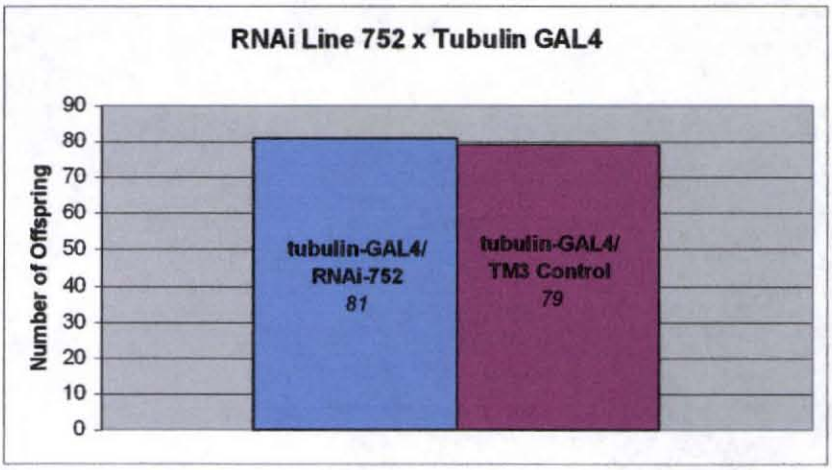


Figure 16. Summary of cross of RNAi line 751 with tubulin GAL4. Offspring were collected over a period of 8 days. Even though the number of offspring did not differ, no offspring carrying both the RNAi construct and tubulin GAL4 construct were observed to emerge during the first two days of collection.

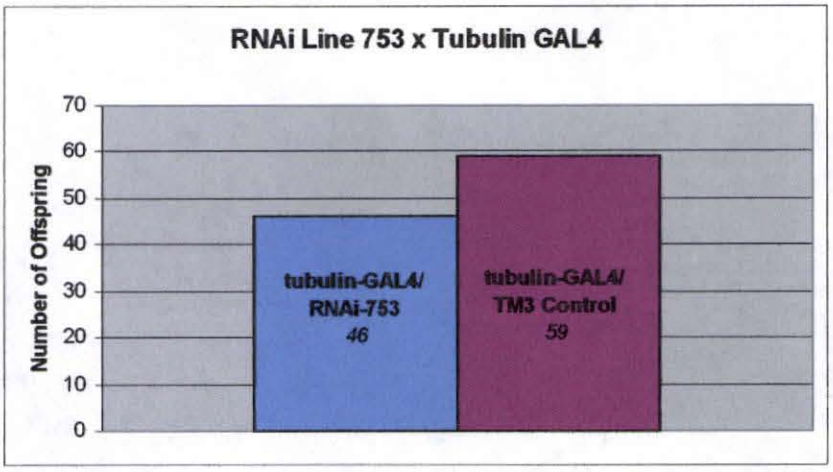


Figure 17. Summary of crosses of RNAi line 753 with tubulin GAL4. Offspring of this cross were collected for 7 days. Even though the number of offspring between those having both the RNAi and the tubulin GAL4 constructs and those not having the tubulin GAL4 construct differed, no delay in hatching occurred.

To summarize, the initial control crosses did not reveal any phenotype or changes in viability. That is, no flightlessness, no bristle phenotype, and no lethality were observed. Considering that the presence of the RNAi construct inside the flies' genomes was verified (see figure 13), this lack of any phenotype suggested the following:

1) The RNAi construct was not active; for instance, not responsive to GAL4 due to a loss of the GAL response element (UAS), or
2) The RNAi construct was not effective in its downregulation of the target; for instance, access of RISC to the *flightless-I* mRNA might be blocked by unknown factors

IV.3. Verification of the Sequence Integrity of the RNAi Construct and Germline Insertion

In order to further test for its activity, the sequence of the RNAi construct was determined for detection of the UAS. Also, additional crosses with heat-shock GAL4 and nanos-GAL4 drivers were performed. Furthermore, a control cross with a *flightless-I* overexpression line as well as Western Blots were conducted. The results of these experiments are described in the following sections.

IV.3.1. Detection of UAS

The sequencing primers listed in section III.4. were used to sequence the RNAi construct for the presence of the GAL4 response element. The presence of the UAS was thereby confirmed.

IV.3.2. Additional Crosses with heat shock-GAL4 and nanos-GAL4

The RNAi lines were crossed with a heat shock (hs)-GAL4 driver to downregulate maternal flightless-I (see section III.3. for details). It was expected that lethality occured at early embryonic stages during cellularization of the syncytial blastoderm. However, offspring of this cross developed and hatched normally and lethality was not observed.

As an alternative experiment to downregulate maternal flightless-I, the RNAi lines were crossed with a nanos-GAL4 driver which drives in early embryonic development. It was expected that crossing nanos-GAL-4 virgin females to RNAi-line males would not lead to viable offspring. This expectation was not fulfilled. Larval offspring developed and hatched normally with no obvious abnormality or lethality.

IV.3.3. Control Cross of RNAi Lines and *flightless-I* Overexpression Line

In a control experiment for RNAi activity, the RNAi lines were crossed with a fly line that overexpresses *flightless-I*. As was previously described, overexpression of *flightless-I* in bristles leads to a singed-like bristle phenotype. A fly line that carries flightless-I-GFP and bristle-GAL4 on the same allele (this allele is named *B11-Fli*) exists in our stock selection (figure 18). If the RNAi construct were active and effective in downregulating *flightless-I* expression, it was postulated that a cross of this overexpression line with the RNAi lines led to a reduction of the bristle phenotype.

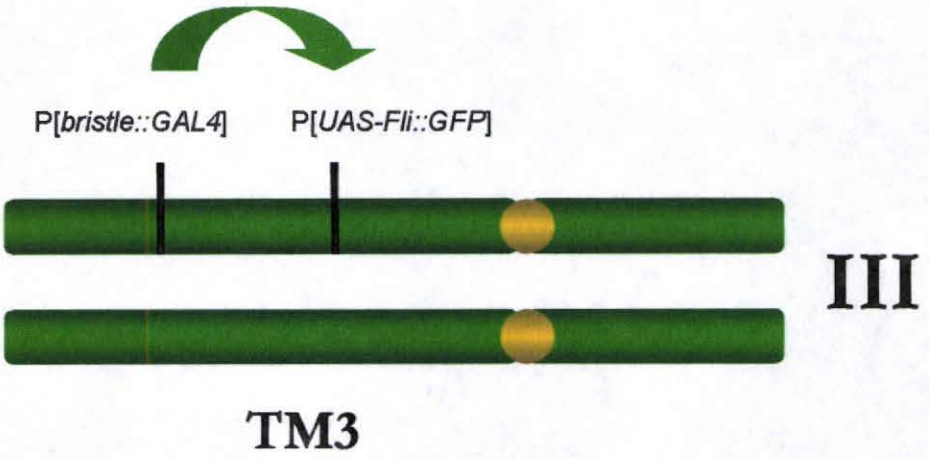


Figure 18. Model of the third chromosome of the *flightless-I* overexpression line. This line carries a recombinant allele with both a bristle GAL4 driver and a *flightless-I*-GFP construct fused to the UAS response element. The allele is balanced with the TM3 balancer, which carries the *stubble* bristle marker. The allele that carries a copy of bristle-GAL4 and UAS-Fli-GFP is called *B11-Fli*.

This overexpression line was originally balanced with a TM6 balancer chromosome (B11-Fli/TM6), which carries the *ubx* marker characterized by large halteres. However, this haltere phenotype was not readily recognizable in that line, and the line was therefore

balanced with TM3 (B11-Fli/TM3). Crosses between the overexpression line balanced with TM3 and the RNAi lines 750 and 752 were performed (crosses with 751 and 753 were not successful due to problems with bacterial infection encountered in our laboratory). Each cross gave rise to 50% offspring carrying the overexpression allele and the RNAi construct, and 50% of offspring carrying the RNAi construct balanced with TM3 (figure 19). In the analysis of this cross, offspring carrying the overexpression allele and the RNAi construct (B11-Fli/RNAi) were compared to the original overexpression line balanced with TM6 (B11-Fli/TM6) to detect any changes or reductions of the bristle phenotype. A comparison with B11-Fli/TM3 was not advisable due to the presence of the *stubble* bristle marker on TM3.

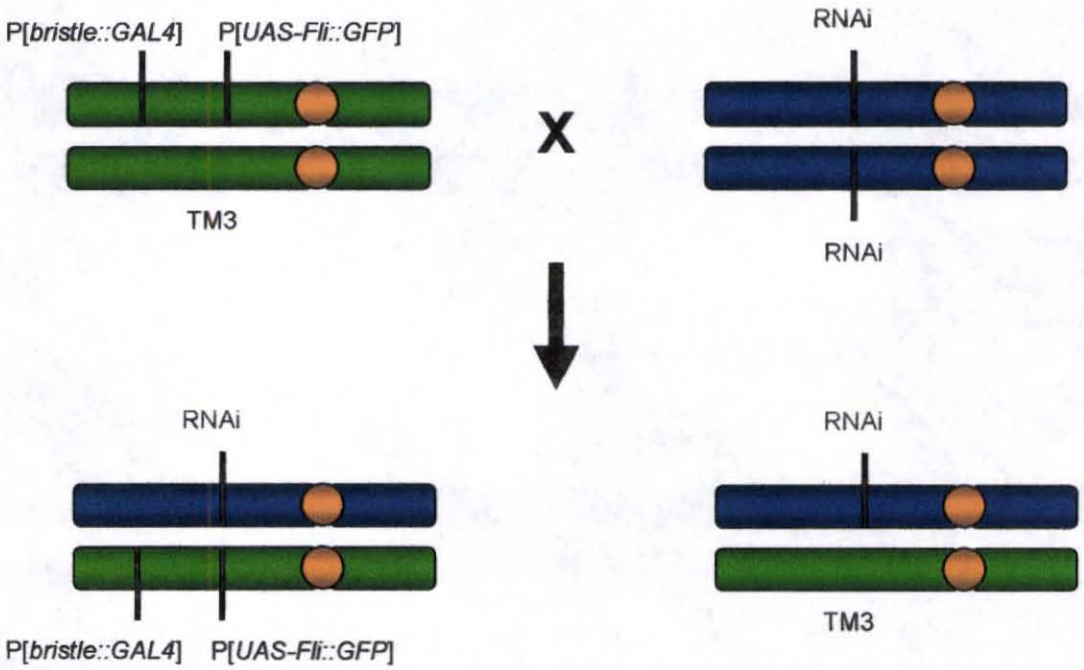


Figure 19. Cross of *flightless-I* overexpression line with a 3rd chromosome RNAi line gives rise to 50% offspring carrying the overexpression allele and an RNAi allele, and 50% offspring carrying an RNAi allele balanced with TM3. Flies carrying the overexpression allele and an RNAi allele were expected to show a reduction in the singed bristle phenotype.

As can be seen in figures 20 and 21, a reduction of the singed-like bristle phenotype appeared to have occurred. A reduction was particularly noticeable in the posterior scutellar bristles. In order to verify statistically significant differences, the lengths of the anterior scutellar bristles and posterior scutellar bristles of the B11-Fli/RNAi lines were measured and compared to those of the B11-Fli/TM6 line. Figures 22 and 23 summarize the obtained measurements for males and females of each line.

In the statistical analysis, the average lengths in μm of 15 anterior scutellar bristles and posterior scutellar bristles of males and females from each line were measured. These results were then incorporated into the Microsoft Excel software applying the t-Test with two samples assuming equal variances.

In was hypothesized that the differences in bristle lengths were significant. Thus, the null hypothesis would be that the differences were not significant. Four t-Tests were performed for each bristle type to test statistically significant differences in bristle length between males of the B11-Fli/TM6 line and males of the B11-Fli/RNAi lines, as well as females of the B11-Fli/TM6 line and females of the B11-Fli/RNAi lines (figure 24).

The probability of the differences in anterior and posterior scutellar bristle lengths to be not significant was in all of the eight cases less than 5%. Therefore, the null hypothesis had to be rejected and the differences in lengths of the anterior scutellar bristles and posterior scutellar bristles were determined to be statistically significant. Thus, a reduction of the singed-like bristle phenotype has in fact occurred.

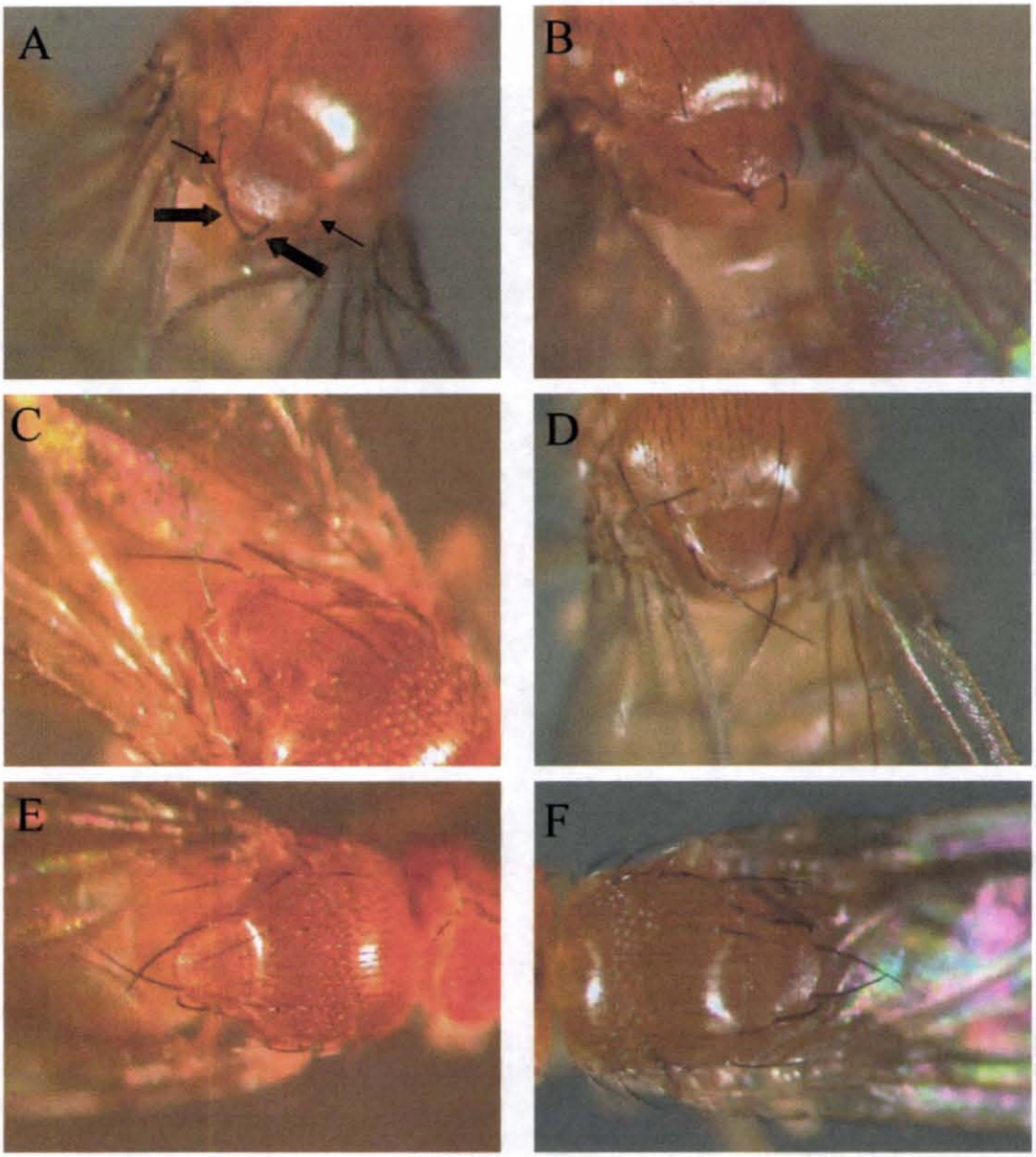


Figure 20. Differences in the length of anterior scutellar bristles (ASC, arrows in A) and posterior scutellar bristles (PSC, block arrows in A) in flies carrying B11-Fli and the RNAi construct and flies carrying B11-Fli balanced over TM6. A: B11-Fli/TM6 ♂. B: B11-Fli/TM6 ♀. C: B11-Fli/RNAi-750 ♂. D: B11-Fli/RNAi-750 ♀. E: B11-Fli/RNAi-752 ♂. F: B11-Fli/RNAi-752 ♀. Comparing the anterior and posterior scutellar bristles of the B11-Fli/TM6 line with those of the B11-Fli/RNAi lines a difference in length is noticeable. In A and B, bristles appear shorter and more singed-like.

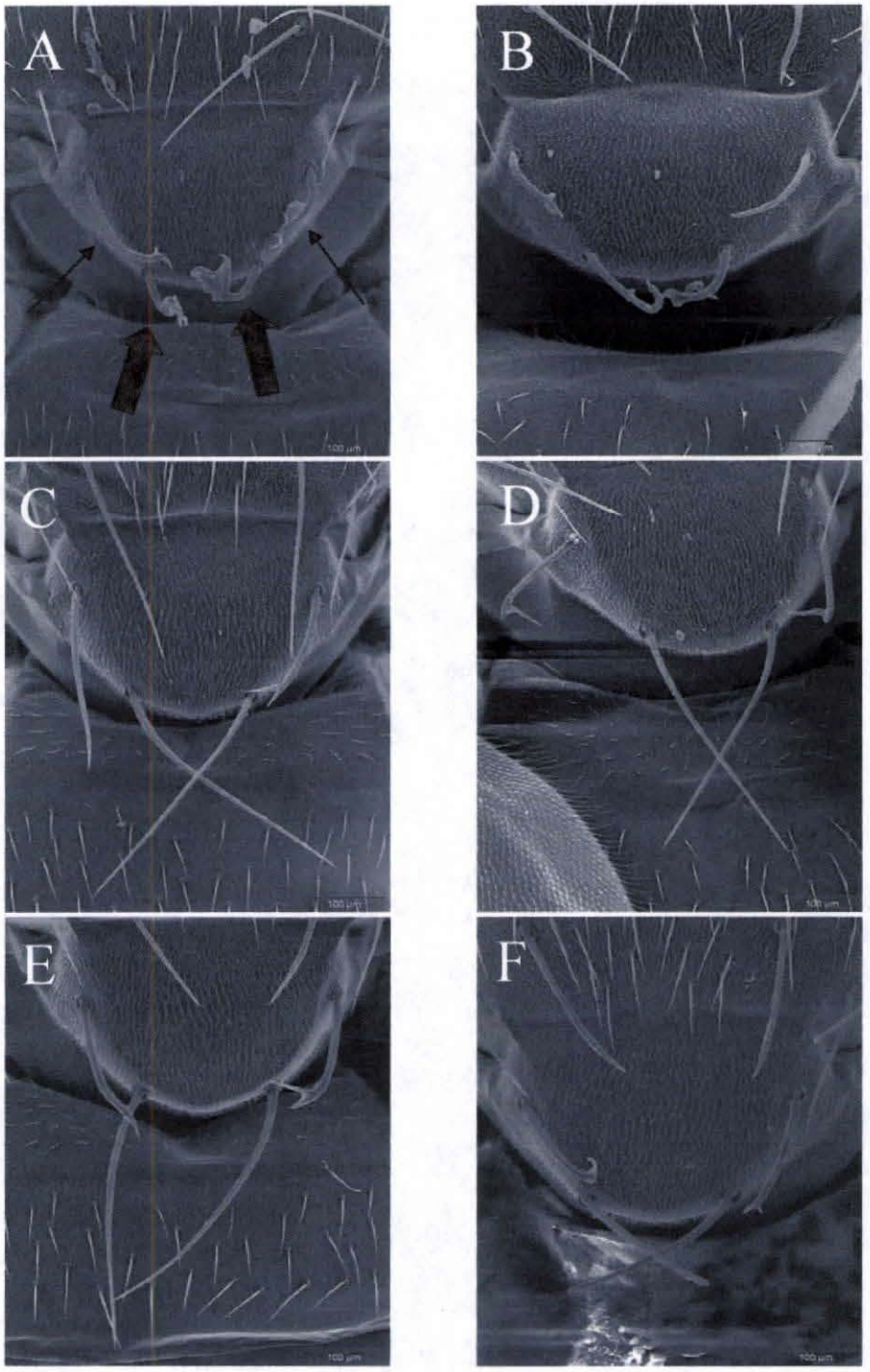


Figure 21. SEM Images (200x) of anterior scutellar bristles (arrows in A) and posterior scutellar bristles (block arrows in A) in flies carrying B11-Fli and the RNAi construct and flies carrying B11-Fli balanced over TM6.. A: B11-Fli/TM6 Males; B: B11-Fli/TM6 Females; C: B11-Fli/RNAi-750 Males; D: B11-Fli/RNAi-750 Females; E: B11-Fli/RNAi-752 Males; F: B11-Fli/RNAi-752 Females. As can be observed, the singed-like bristle phenotype in the B11-Fli/RNAi offspring was reduced.

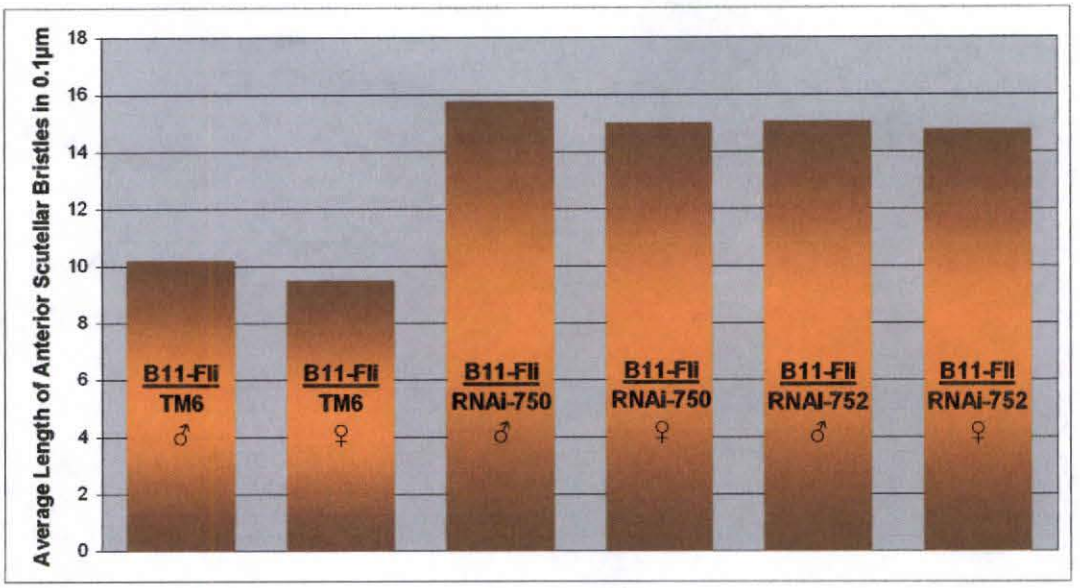


Figure 22. Average lengths of anterior scutellar bristles of B11-Fli/TM6 compared to the B11-Fli/RNAi lines. The average lengths of anterior scutellar bristles have increased in the B11-Fli/RNAi lines compared to the B11-Fli/TM6 line.

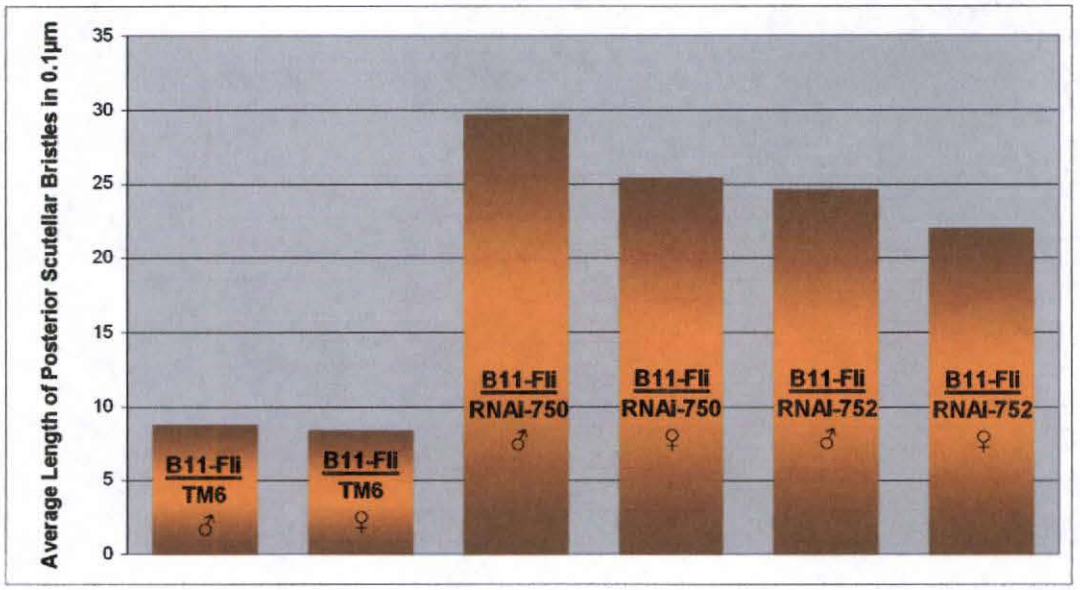


Figure 23. Average lengths of posterior scutellar bristles of B11-Fli/TM6 compared to the B11-Fli/RNAi lines. The average lengths of posterior scutellar bristles have increased in the B11-Fli/RNAi lines compared to the B11-Fli/TM6 line.

Anterior Scutellar Bristles				
	Mean [0.1µm]	Variance	t-value	p
B11-Fli/750 Males	15.76	13.85	4.53	9.97E-05
B11-Fli/TM6 Males	10.18	8.87		
B11-Fli/750 Females	15.02	10.62	4.84	4.24E-05
B11-Fli/TM6 Females	9.51	8.80		
B11-Fli/752 Males	15.03	18.77	3.57	1.30E-03
B11-Fli/TM6 Males	10.18	8.87		
B11-Fli/752 Females	14.75	7.18	5.08	2.25E-05
B11-Fli/TM6 Females	9.51	8.80		

Posterior Scutellar Bristles				
	Mean [0.1µm]	Variance	t-value	p
B11-Fli/750 Males	29.72	25.72	14.12	2.91E-14
B11-Fli/TM6 Males	8.74	7.40		
B11-Fli/750 Females	25.38	24.05	10.76	1.85E-11
B11-Fli/TM6 Females	8.32	13.60		
B11-Fli/752 Males	24.65	22.52	11.27	6.48E-12
B11-Fli/TM6 Males	8.74	7.40		
B11-Fli/752 Females	22.07	30.27	8.04	9.35E-09
B11-Fli/TM6 Females	8.32	13.60		

Figure 24. Statistically significant differences in average lengths of anterior scutellar bristles and posterior scutellar bristles. Males and females of the B11-Fli/RNAi offspring were compared to males and females, respectively, of the B11-Fli/TM6 line. In each case, fifteen pairs of bristles were measured to compute the mean. In all eight cases, the p value (two-tail) was less than 0.05.

IV.3.4. Level of *flightless-I* Knockdown

It was of importance to perform Western Blot analyses in order to determine variations in flightless-I protein levels due to activity of the RNAi construct. For that purpose, offspring of the crosses of the tub-GAL4 driver (balanced with TM3-*stubble*) with the RNAi lines 750 and 752 were used (figure 25). As mentioned, 50% of those offspring contained the tubulin-GAL4 allele and the allele carrying the RNAi construct. In this analysis, the 50% offspring carrying one copy of the RNAi allele and the TM3 marker were used as internal controls.

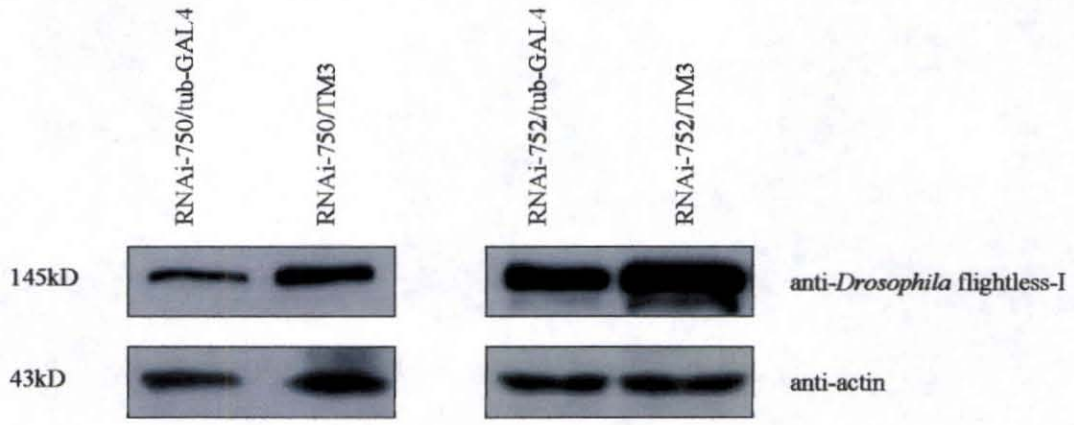


Figure 25. Western Blots of offspring of a cross of RNAi lines 750 (left) and 752 (right). The top panel shows the *Drosophila* flightless-I protein detected by monoclonal anti-*Drosophila* flightless-I antibody. The bottom panel shows *Drosophila* actin detected by anti-actin antibody.

As can be observed from figure 25, low-level downregulation of *flightless-I* expression appeared to have taken place in the cross of tubulin-GAL4 and the RNAi line 750. It is important to note that the overall amount of protein loaded on the left lane (RNAi-750/tub-GAL4) compared to the amount of protein loaded onto the lane adjacent to it (RNAi-750/TM3) was less, as seen in the difference in signal strength of the actin control. Nevertheless, the difference in flightless-I signal strength appeared to exceed the difference in signal strength of the actin control. Thus, a downregulation of flightless-I protein levels was implied. In the case of the cross between tubulin-GAL4 and RNAi line 752, low level downregulation of *flightless-I* expression seemed to have occurred as well. The appearance of a band below the flightless-I signal representing the RNAi-752/TM3 control could have been due to unspecific binding of the anti-*Drosophila* flightless-I antibody. Since the epitope of this antibody is the leucine-rich repeat domain, it was possible that the antibody bound to the leucine-rich repeat domain of another protein of a lower molecular weight. However, since the specificity of the antibody was shown (see below), it is also likely that this second band was due to too high concentrations of the secondary, HRP-conjugated antibody.

Considering the absence of a negative control in this Western blot analysis it is important to point out that the specificity of the anti-*Drosophila* flightless-I antibody had previously been shown (Maeda & de Couet, unpublished data). As a further control, the epitope of this antibody, the *Drosophila* flightless-I leucine rich-repeat domain, was used for BLAST searches to detect other possible interactions with proteins encoded by the *Drosophila* genome. These BLAST searches did not reveal any other proteins of similar molecular weight and, at the same time, high sequence identity (necessary for the

antibody to bind to its epitope), with which the anti-*Drosophila* flightless-I antibody could react. It could therefore be assumed that this antibody was specific for the *Drosophila* flightless-I protein.

V. Discussion

V.1. Summary and Interpretation of Results

As was outlined, *flightless-I* is a conserved, developmentally crucial gene and its protein product is hypothesized to be involved in actin regulation. Since null alleles of this gene are lethal, *flightless-I* was selected for investigation in order to gain further insight into the causes of lethality and thus conserved, developmentally pivotal mechanisms. The chosen approach was to utilize a combination of RNAi and UAS/GAL4 technology in *Drosophila melanogaster* to downregulate expression of the *flightless-I* gene. One construct was successfully designed, and the purpose of this thesis was to determine knockdown efficiency of that RNAi construct.

For that purpose, recombinant fly lines homozygous for the RNAi construct were initially crossed with a ubiquitous tubulin-GAL4 driver, a bristle-GAL4 driver, and an indirect flight muscle-GAL4 driver. Null-alleles of *flightless-I* were already known to be lethal, and the downregulation of *flightless-I* expression using the tubulin-GAL4 driver was expected to mimic the known null-alleles. Overexpression of *flightless-I* in bristles gave a singed-like bristle phenotype, and downregulation of *flightless-I* was thus expected to give rise to bristle abnormalities also. Flightlessness was already known in flies carrying *flightless-I* point mutation alleles, and downregulation of *flightless-I* in the indirect flight muscles was expected to cause flightlessness also.

However, offspring of these initial crosses did not show any changes in viability, changes in bristle structure, or flightlessness, respectively. These data therefore implied that the RNAi construct was either not present inside the flies' genomes, or not active due loss of the UAS, or otherwise ineffective in downregulating the *flightless-I* message.

The presence of the RNAi construct inside the flies' genomes was verified by PCR analysis, and the presence of the GAL4 response element was also verified by sequencing, indicating that the absence of mutant phenotypes was due to ineffectiveness of the RNAi construct. Therefore, additional crosses with a heat shock-GAL4 driver and a nanos-GAL4 driver were performed to downregulate the maternal *flightless-I* message. It was expected to observe lethality early in development during cellularization of the syncytial blastoderm. This expectation was not fulfilled, again suggesting that the RNAi construct was ineffective in targeting the *flightless-I* message.

As a control experiment, two of the RNAi lines (750 and 752) were crossed with a line that overexpresses *flightless-I* in bristles. This line displays the singed-like phenotype, and, if the RNAi construct were effective, a cross of this line with the RNAi lines was expected to give rise to a reduction of the singed-like bristle phenotype. Two pairs of bristles, the anterior scutellar bristles and the posterior scutellar bristles were chosen for this analysis. Offspring of these crosses that carried both a copy of the overexpression allele and the RNAi allele did show a statistically significant reduction of the singed-like bristle phenotype. Even though residual levels of the singed-like phenotype remained, these data indicated that the RNAi construct was indeed active and effective in downregulating the overexpressed *flightless-I* message.

In order to determine the level of *flightless-I* downregulation, Western blots were performed. In this analysis, offspring of the crosses between the homozygous RNAi lines 750 and 752 and the ubiquitous tubulin-GAL4 driver were used, were the 50% offspring that carried a copy of the RNAi construct and the TM3 balancer served as internal controls. In these Western blots, it was demonstrated that downregulation of the

flightless-I message has taken place, but occurred only on low levels. Relatively high levels of residual flightless-I protein remained, particularly in the offspring of the cross between RNAi line 752 and the tubulin-GAL4 line.

In conclusion, these data indicated that even though downregulation of the *flightless-I* message by the RNAi construct was achieved, this downregulation was not sufficient enough, as demonstrated by the lack of quantifiable phenotypes in the offspring of the initial crosses. Therefore, these experiments highlight the possible limitations of RNAi experiments, in which residual protein levels will remain that can rescue the wild type phenotype. This is important particularly in the case of the flightless-I protein. It has been shown by Campbell et al. (1993) that low levels of flightless-I were sufficient to restore viability. A single *flightless-I* transgene that was inserted into the heterochromatin of chromosome IV, were activity of the transgene was below normal, was able to rescue the lethal phenotype of null-alleles. Therefore, expression of *flightless-I* is difficult to downregulate, since only a near complete downregulation is expected to give a phenotype.

The low efficiency of downregulation by the RNAi construct used in these experiments could be explained by a possible inaccessibility of that construct to its target region of the *flightless-I* mRNA. That is, RISC might not be able to effectively access its target region due to the presence of unknown secondary factors binding to the mRNA or possible secondary structures of the mRNA itself. It is therefore of great importance to design several different RNAi constructs of different lengths targeting various regions of the *flightless-I* mRNA. In that context, no strict guidelines regarding knockdown efficiencies of RNAi constructs exist. However, it is suggested here that RNAi constructs

of lengths of several hundred base pairs targeting the 5'-UTR, the 3'-UTR, and various regions of the open reading frame can be made. These additional constructs possibly increase knockdown efficiencies and thus might give rise to quantifiable phenotypes which could be investigated to gain further insight into possible functions of the flightless-I protein. Recent research has demonstrated that these roles of the flightless-I protein can be greatly versatile. Several of these new findings are described in the last section of this thesis.

V.2. Recent Findings in Flightless-I Research

Given that flightless-I was hypothesized to be an actin regulator, and actin is involved in multiple cellular processes, flightless-I can also be postulated to indirectly influence those cellular pathways by regulating the underlying actin network. In the last section of this thesis, several of the new findings in flightless-I research are presented to demonstrate possible multiple facets of flightless-I function.

V.2.1. Flightless-I in Nuclear Receptor Signaling

Performing mammalian transfection assays, Lee et al. (2004) have shown that human flightless-I protein acts in nuclear receptor mediated transcription, which implied localization of flightless-I to the nucleus (figure 26). Nuclear hormone receptors (NRs) are a family of hormone-activated transcriptional activator proteins which bind directly or through other proteins to gene-regulatory elements and thereby activate or repress

transcription. In transcriptional activation, nuclear receptors mediate recruitment of coactivator protein complexes as well as methyltransferases and acetyltransferases that cause remodeling of the chromatin to enable access of RNA polymerase. One of the coactivator complexes is p160 which, upon activation, relays the nuclear receptor signal to the methyltransferase CARM1 and the acetyltransferase CBP/p300. Methylation and acetylation of lysine residues on histone tails then induces chromatin remodeling.

Lee et al. (2004) have shown that the leucine-rich repeat domain of human flightless-I protein binds CARM1, p160, and NRs. It was also demonstrated that human flightless-I protein cooperated synergistically with CARM1 and p160 to enhance NR function. As reviewed by Lee et al., actin and actin-like proteins (e.g. BAF53) had been found in the nucleus in association with ATP-dependent chromatin remodeling complex SWI/SNF. Since flightless-I had been suggested to bind actin, the authors therefore suggested that the flightless-I protein functions as a transcriptional regulator by linking different but complementary chromatin remodeling mechanisms. The flightless-I protein thus might have multiple roles as an actin regulator in the cytoskeleton and a transcriptional regulator in the nucleus.

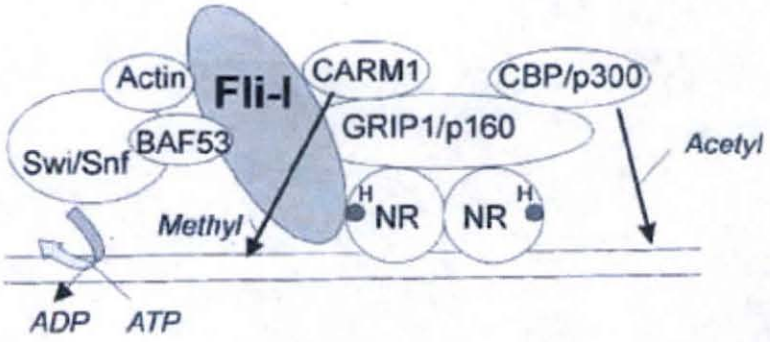


Figure 26. Localization of human flightless-I to the nucleus where it interacts with nuclear receptors (NRs), coactivators (GRIP1/p160), and chromatin remodeling complexes (CARM1, CBP/p300, SWI/SNF) to enhance nuclear receptor mediated transcription (image from Lee et al. 2004).

V.2.2. Flightless-I in β-catenin Signaling

The postulation of flightless-I performing multiple roles in the cytoskeleton and the nucleus was developed further in another study by Lee and Stallcup (2006). The authors have shown, also in mammalian transfection assays, that human flightless-I interacts with murine FLAP1, a protein previously found to be a binding partner for the mammalian flightless-I leucine-rich repeat domain (Liu & Yin 1998), to suppress β-catenin dependent transcription which also implied localization of human flightless-I to the nucleus (figure 27). β-catenin is a well known essential factor in multiple developmental processes across animal phyla. In the wnt signaling pathway, binding of wnt ligand to the frizzled receptor activates the disheveled protein and thereby the inactivation of the kinase activity of the GSK/Axin/APC complex. Inactivation of this complex prevents degradation of β-catenin, leading to accumulation of β-catenin in the nucleus. In the nucleus, β-catenin interacts with the LEF1/TCF protein complex to activate the transcription of target genes responsible for cellular proliferation and differentiation (e.g. in embryonic axis

formation). In the activation of target genes, additional regulators include coactivator complex p160 and acetyltransferase CBP/p300. In addition to transcriptional regulation, β-catenin also controls E-cadherin-mediated cell adhesion at the plasma membrane and mediates the interplay of adherens junction molecules with the actin cytoskeleton (Brembeck et al. 2006).

In their study, Lee and Stallcup found FLAP1 to bind to β-catenin and interact synergistically with the acetyltransferase p300 to enhance β-catenin dependent transcription. The authors demonstrated that human flightless-I protein suppressed this enhancing effect by binding to FLAP1 (see figure 27). In this interaction, both the leucine-rich repeat domain and the gelsolin-like domains of flightless-I were required.

Lee and Stallcup thus proposed multiple roles of human flightless-I in the nucleus. In nuclear receptor mediated transcription, the human flightless-I protein might have an activating role. In β-catenin dependent transcription, human flightless-I could play a suppressing role.

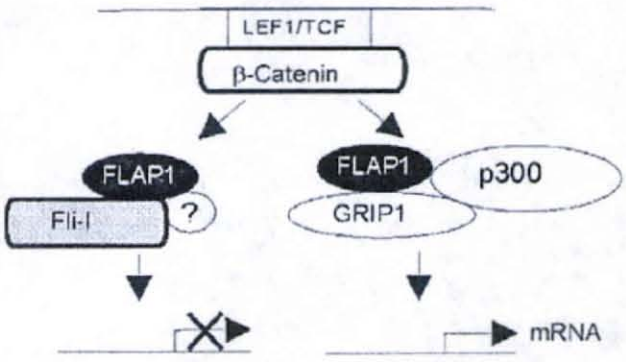


Figure 27. Interaction of human Fli-I and FLAP1 in β-catenin dependent transcription. FLAP1 interacts synergistically with coactivator GRIP1 (part of the p160 coactivator complex) and acetyltransferase p300 to enhance β-catenin dependent transcription. Binding of Fli-I to FLAP1 suppresses transcription. (Image from Lee & Stallcup 2006)

On a related note, Fong and de Couet (1999) identified previously unknown binding partners for the mammalian flightless-I leucine-rich repeat domain, which they called LRRFIP1 and LRRFIP2 (LRR FLI interacting protein 1 and 2), by the yeast two-hybrid system. Both LRRFIP1 and LRRFIP2 share significant sequence homology with murine FLAP1. The N-terminal domain of LRRFIP2 was later found in coimmunoprecipitation assays to be a binding partner for the disheveled protein of the wnt signaling pathway (Liu et al. 2005). Disheveled consists of three domains, the DIX domain at the N-terminus, the PDZ domain in the central region, and the DEP domain at the C-terminus, and LRRFIP2 was shown to bind the PDZ and DEP domains. Liu et al. (2005) have shown that by binding to disheveled, LRRFIP2 activated the wnt pathway in cultured mammalian cells, characterized by increasing levels of β-catenin in the nucleus. It was also demonstrated that injection of LRRFIP2 into the ventral pole of *Xenopus* embryos induced ectopic wnt target gene expression and double axis formation.

Furthermore, Capelluto et al. (2002) have illustrated by immunostaining of mammalian cell cultures and immonoprecipitation assays that the DIX domain mediated targeting of disheveled to actin stress fibers and cytoplasmic vesicles. The disheveled protein has thus been shown to interact with two proteins, actin and LRRFIP2, that are also known binding partners of flightless-I. Also, the flightless-I protein had already been implicated in indirectly influencing vesicle transport by regulating the underlying actin network (Straub et al. 1996). Therefore, the above described data further imply a possible indirect involvement of flightless-I in the developmentally important β-catenin pathway by mediating the fundamental actin network. Additional studies are needed to elucidate these potential roles of the flightless-I protein.

V.2.3. Flightless-I Overexpression and Loss of Intermediate Ocellar Bristles

The multiple facets of flightless-I function are furthermore demonstrated during neurogenesis of *Drosophila* sensory bristles. All cells of a bristle of the adult fly derive from a single sensory mother cell in the imaginal disc epithelium. Initially, clusters of proneural cells within the imaginal disc epithelium inhibit one another from differentiating into a mother cell via the Notch-Delta pathway (lateral inhibition). At first, all cells express Notch and its ligand Delta on equal levels, inhibiting each other to differentiate into sensory mother cells. Eventually, one cell increases Delta signaling (by increasing levels of the transcription factor achaete) to its neighboring cells, causing stronger (inhibitory) Notch signaling in those cells. This strong Notch signaling leads to activation of transcription factors suppressor of hairless and enhancer of split. These mediate suppression of *Delta* expression, thereby decreasing that cell's ability to fight back with a strong Delta signal. This leads to differential Delta levels among the proneural cells from which eventually the cell that produces the strongest Delta signal arises as the sensory mother cell (see Alberts et al. 2002 & Lodish et al. 2004 for review).

As was described in the introduction, overexpression of *flightless-I* in *Drosophila* bristles using a bristle-specific GAL4 driver (B11) led to a singed-like phenotype. Overexpressing *flightless-I* using a ubiquitous tubulin-GAL4 driver led to another distinct bristle phenotype, characterized by loss of intermediate ocellar bristles (figure 28.B) (Maeda, Guild & de Couet, unpublished data). Increased overexpression of *flightless-I* by a double FliI-GFP construct augmented the loss (figure 28.C). Interestingly, overexpressing *flightless-I* in bristles using the B11-GAL4 driver only gave rise to the singed-like phenotype, but not to a loss of ocellar bristles (figure 28.D), which implies

that the loss is not due to abnormalities in the bristles themselves but due to aberration in the tissue surrounding the bristles.

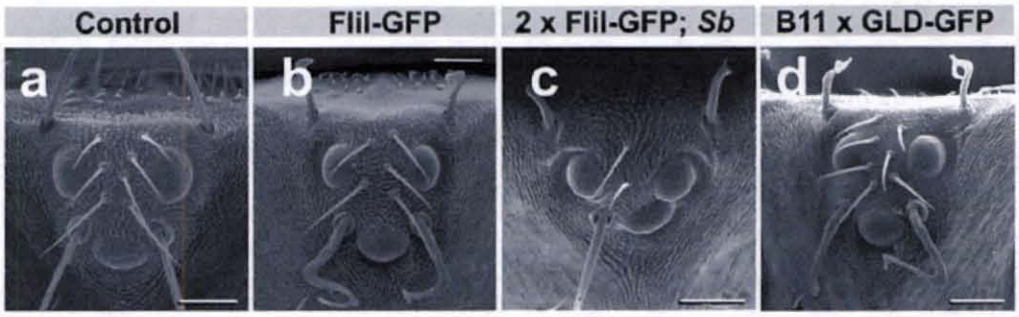


Figure 28. Loss of intermediate ocellar bristles due to overexpression of *flightless-I* utilizing the tubulin-GAL4 driver. Loss is augmented when two copies of *flightless-I* are overexpressed (C). No loss occurs utilizing the bristle-specific GAL driver B11 (D) (Maeda, Guild, and de Couet, unpublished results; image courtesy of H. G. de Couet).

More specifically, these data suggest that ubiquitous overexpression of *flightless-I* interfered with neurogenesis of intermediate ocellar bristles. Whether or not the overexpressed flightless-I protein interfered directly or indirectly with Notch-Delta signaling remains to be determined. However, as was described previously, interference of vesicular transport due to loss of flightless-I has been suggested by Straub et al. (1996) (also see Stamnes 2002 for review of actin involvement in vesicle transport). It could be possible that excess amounts of the flightless-I protein led to an abnormal actin filament network in the proneural cells of the imaginal disc epithelium, thereby somehow interfering with vesicular transport of proteins (e.g. delivery of Notch and Delta to the plasma membrane) necessary in the signaling pathway and thus distorting the lateral inhibition process.

V.2.4. Flightless-I Null-Alleles gave a Wing Phenotype

The *flightless-I* null alleles *fli-I*^{W2} and *fli-I*^{D44} have been shown to cause lethality at pupal stages (Perrimon et al. 1989, Miklos & de Couet 1990, de Couet et al. 1995). Recently, Kremer (personal communication 2007) has demonstrated that the loss of *flightless-I* gene expression in the *fli-I*^{W2} and *fli-I*^{D44} null-allele lines also led to the presence of additional cross veins in the wings. The fly lines were balanced with the green balancer FM7i (act5C-GFP) and mated according to the Punnett Square depicted in figure 29. The W-2/Y males were selected for analysis before lethality occurred. Figure 30 shows the structure of the wings of these males. As can be seen, additional cross veins emerged. In the development of crossveins an interplay of multiple signaling pathways, including BMP-like signaling, Map-kinase signaling, and Notch/Delta signaling, appear to take place (Marcus 2001, de Celis 2003). Thus, these data again imply that the flightless-I protein might play an indirect role in these pathways by regulating the actin filament network which in turn regulates intracellular protein transport crucial to relay signals in these pathways.

	W-2	**FM7i**
FM7i	W-2 / FM7i	FM7i / FM7i
Y	W-2 / Y	FM7i / Y

Figure 29. Punnett Square showing segregation ratios of a cross between W-2 females balanced with FM7i and the respective males. Fields shaded in red signify lethality. The W-2/Y males were selected for analysis.

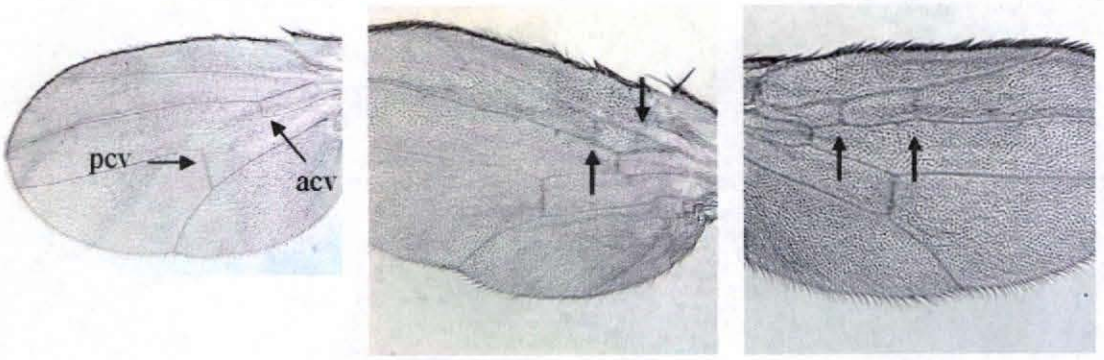


Figure 30. Additional cross veins in W-2/Y male wings. The picture on the left shows the anterior cross vein (acv) and the posterior cross vein (pcv) in wild type wings. The arrows in the two pictures on the right point to the additional cross veins that were present in wings of *fli-I*^{W2} pupae at 60-70h APF (images courtesy of Therese Kremer).

V.3. Future Objectives

In light of these recent implications of flightless-I function, particularly the postulated localization of flightless-I to the nucleus, our laboratory is currently in the process to conduct a yeast two-hybrid screen to detect possible binding partners of the flightless-I leucine-rich repeat domain in a *Drosophila* embryonic library. If in a yeast two-hybrid screen of a *Drosophila* embryonic library binding partners are found known to be involved in nuclear receptor and β-catenin signaling, as shown by Lee et al. (2004) and Lee and Stallcup (2006) in mammalian libraries, supportive data further substantiating postulated nuclear localization and transcriptional roles of flightless-I could be reached.

In the context of the RNAi knockdown experiments, since the low efficiency of the RNAi construct to downregulate *flightless-I* mRNA has been determined, it is of great importance to generate multiple RNAi constructs. As described earlier, these constructs should target the 5'-UTR, 3'-UTR, and regions of the open reading frame. Having

utilized the *E*-RNAi database of the German Cancer Research Center (DKFZ) in Heidelberg, Germany, our laboratory is in the process of cloning one 3'-UTR construct and one construct targeting the ORF. The database did not provide potential constructs for the 5'-UTR. Thus, one construct targeting that region is being designed using comparative BLAST analyses. As was described earlier, it is of importance to generate RNAi constructs that achieve a complete or near complete downregulation of *flightless-I* expression in order to induce mutant phenotypes.

Since crosses of the RNAi lines 751 and 753 with the B11-Fli/TM3 overexpression line have not been successful, it is of interest to repeat the experiment with these lines in order to detect possible differences in knockdown efficiencies. In that context, Western blots should be performed with offspring from these lines and the tubulin-GAL4 driver also. This is important since, even though each transformed line carries the same RNAi construct, it is inserted in different loci of the genome. These differences in localization might be represented by different knockdown efficiencies, detectable by Western blot analyses.

As a negative control for these Western blots, it is necessary to utilize a fly line that does not carry a copy of the *flightless-I* gene. It was described earlier that the *fli-I*^{D44} allele causes lethality at pupal stages. Males of the genotype D44/Y could be used shortly before or at the time lethality occurs, even though at this point it is not known whether or not flies of this genotype in fact have depleted flightless-I levels or whether a certain percentage of residual flightless-I protein remains. This question remains to be answered in future experiments.

A further interesting approach to possibly achieve greater knockdown efficiency is to design an RNAi line in the background of a heterozygous null mutation. The idea here is that such a line would only produce half of the wild type flightless-I protein levels. Since residual levels of flightless-I rescue the wild type, downregulation of flightless-I in the background of a null mutation might give rise to flightless-I levels below the rescue threshold and thus a *flightless-I* loss-of-function phenotype.

Another possibility to achieve greater levels of knockdown efficiency is to design a double recombinant RNAi line. That is, one allele that carries two copies of the RNAi construct. In lines 750, 751, and 752 the RNAi construct is located on the third chromosome. Depending on the precise location of the constructs in these lines it might be possible to generate a double recombinant allele by crossing over. Whether or not this is in fact achievable remains to be determined.

Furthermore, since some level of downregulation was detectable, it would be of interest to investigate offspring of crosses between the tubulin-GAL4 driver and the RNAi lines for possible changes in the number of intermediate oscellar bristles, which would substantiate the postulation that flightless-I is involved in neurogenesis of sensory bristles.

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VII. Appendix

VII.1. Crossing Scheme for Mapping and Generation of Homozygotes

In the following Punnett squares, *P* denotes the RNAi construct. Offspring with phenotype shaded in orange were collected for the inter se cross. The RNAi construct could have been on the 2nd or 3rd chromosome. TM3 was marked with stubble bristles. From the inter se cross homozygous flies were collected to generate homozygous stocks of the independent insertion lines.

Nomenclature: II. Chromosome ; III. Chromosome
II. Chromosome ; III. Chromosome

I. Initial Cross

Homozygotes

P ; +
P ; +
X
T(2;3)
CyO ; TM3

	P ; +	P ; +	P ; +	P ; +
T(2;3)	P ; + T(2;3)	P ; + T(2;3)	P ; + T(2;3)	P ; + T(2;3)
CyO ; TM3	P ; + CyO ; TM3	P ; + CyO ; TM3	P ; + CyO ; TM3	P ; + CyO ; TM3
T2 ; TM3	lethal	lethal	lethal	lethal
CyO ; T3	lethal	lethal	lethal	lethal

Heterozygotes

	$\frac{P\ ;\ +}{+\ ;\ +}$	X	$\frac{T(2;3)}{CyO\ ;\ TM3}$	
	P ; +	P ; +	+ ; +	+ ; +
T(2;3)	$\frac{P\ ;\ +}{T(2;3)}$	$\frac{P\ ;\ +}{T(2;3)}$	$\frac{+\ ;\ +}{T(2;3)}$	$\frac{+\ ;\ +}{T(2;3)}$
CyO ; TM3	$\frac{P\ ;\ +}{CyO\ ;\ TM3}$	$\frac{P\ ;\ +}{CyO\ ;\ TM3}$	$\frac{+\ ;\ +}{CyO\ ;\ TM3}$	$\frac{+\ ;\ +}{CyO\ ;\ TM3}$
T2 ; TM3	lethal	lethal	lethal	lethal
CyO ; T3	lethal	lethal	lethal	lethal

II. Inter Se Cross

2nd Chromosome

	$\frac{P\ ;\ +}{CyO\ ;\ TM3}$	X	$\frac{P\ ;\ +}{CyO\ ;\ TM3}$	
	P ; +	Cyo ; TM3	P ; TM3	CyO ; +
P ; +	$\frac{P\ ;\ +}{P\ ;\ +}$	$\frac{P\ ;\ +}{CyO\ ;\ TM3}$	$\frac{P\ ;\ +}{P\ ;\ TM3}$	$\frac{P\ ;\ +}{CyO\ ;\ +}$
CyO ; TM3	$\frac{P\ ;\ +}{CyO\ ;\ TM3}$	lethal	lethal	lethal
P ; TM3	$\frac{P\ ;\ +}{P\ ;\ TM3}$	lethal	lethal	$\frac{P\ ;\ TM3}{CyO\ ;\ +}$
CyO ; +	$\frac{P\ ;\ +}{CyO\ ;\ +}$	lethal	$\frac{P\ ;\ TM3}{CyO\ ;\ +}$	lethal

3rd Chromosome

	+ ; P / CyO ; TM3	X	+ ; P / CyO ; TM3	
	+ ; P	CyO ; TM3	+ ; TM3	CyO ; P
+ ; P	+ ; P / + ; P	+ ; P / CyO ; TM3	+ ; P / + ; TM3	+ ; P / CyO ; P
CyO ; TM3	+ ; P / CyO ; TM3	lethal	lethal	lethal
+ ; TM3	+ ; P / + ; TM3	lethal	lethal	CyO ; P / + ; TM3
CyO ; P	+ ; P / CyO ; P	lethal	+ ; TM3 / CyO ; P	lethal

VII.2. Figure Index

VII.3. Abbreviations

ASC	**Anterior Scutallar Bristle**
ACV	**Anterior Cross Veins**
CARM1	Coactivator-associated Arginine Methyltransferase 1
CBP/p300	**CREB Binding Protein Histone Acetyltransferase**
FLAP1	**FLI Associated Protein 1**
FLP/FRT	FLP Recombinase/FLP Recombinase Target
GFP	**Green Fluorescent Protein**
GLD	**Gelsolin-like Domain**
GSK/Axin/APC	Glycogen Synthase Kinase 3β/Axin/Adenomatous Polyposis Coli
LEF1/TCF	Lymphocyte Enhancer-Binding Factor/T Cell Factor
LRR	**Leucine-rich Repeat Domain**
LRRFIP1+2	Leucine-Rich Repeat in Flightless Interacting Protein 1 and 2
NR	**Nuclear Receptor**
PIP_2	Phosphatidylinositol 4,5 bisphosphate
PSC	**Posterior Scutellar Bristle**
PCV	**Posterior Cross vein**
RISC	RNA-inducing Silencing Complex
RNAi	**RNA Interference**
siRNA	**Short Interfering RNA**
UAS	Upstream Activation Sequence
UTR	**Untranslated Region**

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