Role of MADF-BESS Domain Protein Family in Gene Regulation and Wing Development in *Drosophila melanogaster*

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Certificate

This is to certify that this dissertation entitled '*Study of Role of MADF-BESS Domain Protein family in Gene Regulation and Wing Development in Drosophila melanogaster'* towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by Apurv Kulkarni at IISER Pune under the supervision of Dr. Girish Ratnaparkhi, Assistant Professor, Biology Division, IISER Pune during the academic year 2012-2013.

Dr. Girish Ratnaparkhi

Assistant Professor

Biology Division, IISER Pune

Declaration

I hereby declare that the matter embodied in the thesis entitled '*Study of Role of MADF-BESS Domain Protein Family in Gene Regulation and Wing Development in Drosophila melanogaster'* are the results of the investigations carried out by me at the Biology Division, IISER Pune under the supervision of Dr. Girish Ratnaparkhi, Assistant Professor, Biology Division, IISER Pune and the same has not been submitted elsewhere for any other degree.

Apurv Kulkarni

BS-MS Dual Degree Student

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Abstract

In *Drosophila melanogaster*, there are 16 genes listed in the MADF-BESS domain family. Three out of these sixteen genes -- *hinge1* (*CG9437*), *hinge2 (CG13897)* and *hinge3 (CG8359*) -- have been shown to give a bent wing phenotype when knocked down by RNA interference. I have characterized the phenotype that results from knock down of *hinge1* using flight assays, light microscopy and scanning electron microscopy. I found out that *hinge1, hinge2 and hinge3* are negative regulators of *wingless*, the best characterized *wnt* gene in *Drosophila melanogaster*.

Acknowledgements

I would like to thank Dr. Girish Ratnaparkhi for giving me the opportunity to work in his laboratory and mentoring this project, Vallari Shukla, for initiating this project and putting a lot of hard work in it, Dr. Richa Rikhy and Prof. L S Shashidhara for their invaluable suggestions, Dr Sanjay Sane for allowing us to use the facilities in his lab at NCBS to study insect flight, Sufia for helping me in performing the flight assays. Vijay (IISER microscopy facility) and Ketan (NCL SEM facility) for helping me with the microscopy part of my project and all the GR Lab members for their continuous help and support.

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INTRODUCTION

MADF-BESS Domain Family in *Drosophila melanogaster*

The proteins in the MADF-BESS domain family contain two conserved domains namely MADF and BESS. MADF stands for Myb/Sant like domain in Adf1 and BESS domain gets its name from the three proteins that defined the domain, BEAF, Suvar(3)7and Stonewall. The N terminal MADF domain is an eighty amino acid long DNA binding domain. The C terminal BESS domain is forty amino acid residues long and it's a protein-protein interaction domain (Figure one). In the genome of *Drosophila melanogaster* there are 16 proteins that contain both MADF and BESS domains (Figure1). These proteins share a very high sequence similarity with each other suggesting that they are a product of series of gene duplication events. Out of these sixteen, only five genes have been studied before. Coop, Dip3, Adf1, CTP Synthase and Stonewall are the proteins in the MADF- BESS family that have been shown to act as transcriptional regulators (Bhasker *et. al..*, 2002; Song *et. al*., 2010; Cutler *et. al.*,1998). In our lab, Vallari Shukla has shown that 3 out of these 16 genes, *CG9437*, *CG13897* and *CG8359* when knocked down one at a time by RNA interference by means of UAS-Gal4 system, using a wing specific Gal4, MS1096 Gal4, give a bent wing phenotype. We have decided to name these genes *hinge1*, *hinge2* and *hinge3.* The *hinge* gene knockdown phenotypes are dose dependent (Figure4). Knocking two out of these three genes simultaneously, enhances the phenotype, suggesting a functional overlap in these three genes. She has also shown genetic interaction of these genes with genes like *teashirt*, *homothorax* and *extradenticle* which play roles in development of the wing hinge.

Structure of *Drosophila* wing hinge

In flies, the larval precursors of the adult organs are called imaginal discs. The adult wing arises from larval wing disc (Figure two). The adult wing can be divided in two regions the wing blade and the hinge. The hinge is the region of the wing that connects the wing blade to the thorax of the fly. The hinge can further be divided in two regions, the distal hinge and the proximal hinge. The proximal hinge consists of

structures like tegula, a mechano-sensory organ, various plates like the humeral plate (hp), unnamed plate (UP) and the axillary sclerites (AS), which bring about the flexion of the wing. The distal hinge consists of structures like ‘costa’, which gives rise to the anterior most vein of the wing blade, ‘radius’, the strongest vein, which gives rise to some longitudinal veins like L2 and L3 in the wing blade.

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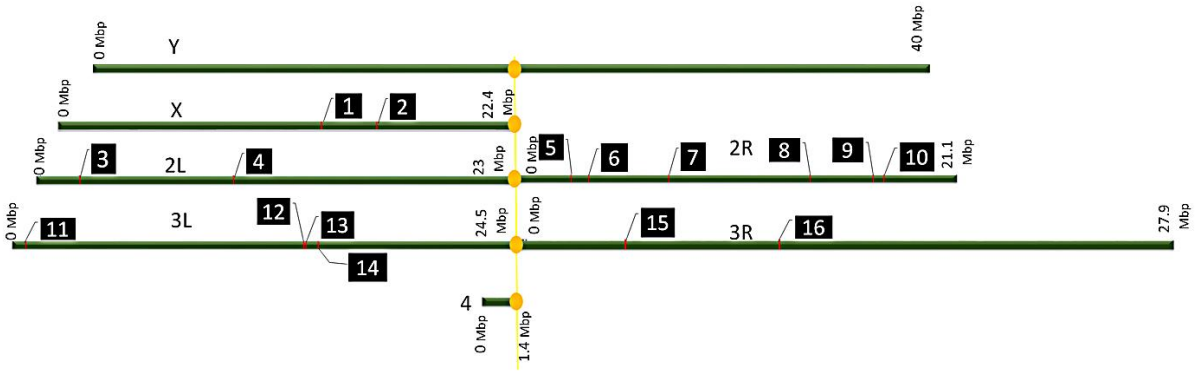

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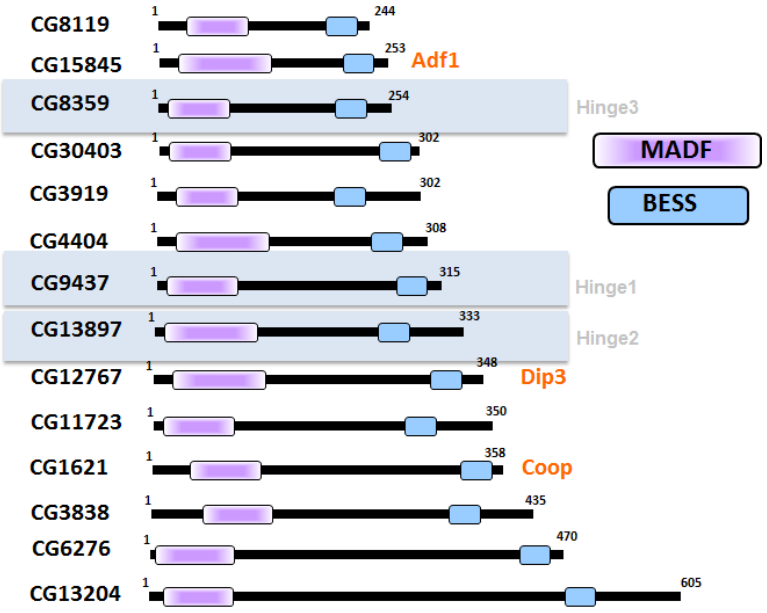

Figure one A: Distribution of MADF-BESS genes in the *Drosophila melanogaster* genome.

Horizontal green bars represent chromosomes in *Drosophila* genome. The vertical yellow line represents centromeres. The numbered boxes point to the position of MADF-BESS genes on the chromosomes. *1. CG4404, 2. CG8119, 3. CG11723, 4. CG3838, 5. adf1, 6.coop, 7. CG13204, 8.dip3, 9.hing1, 10.CG30403, 11.hinge2, 12.stonewall, 13.CG3919, 14. CTP Synthase, 15.CG6276, 16. hinge3*

Figure one B: MADF-BESS domain protein family in *Drosophila*: Figure shows MADF-BESS domain protein family in *Drosophila.* Hinge1, Hinge2 and Hinge3 have been highlighted. All the proteins have a N-terminal MADF and C-terminal BESS domain.

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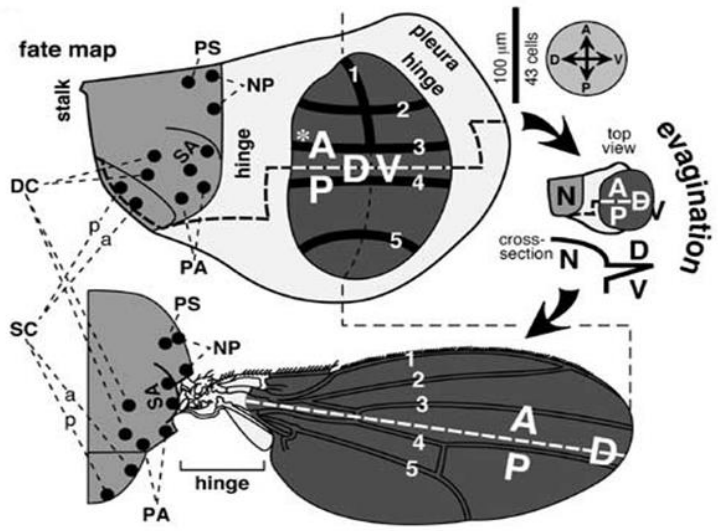

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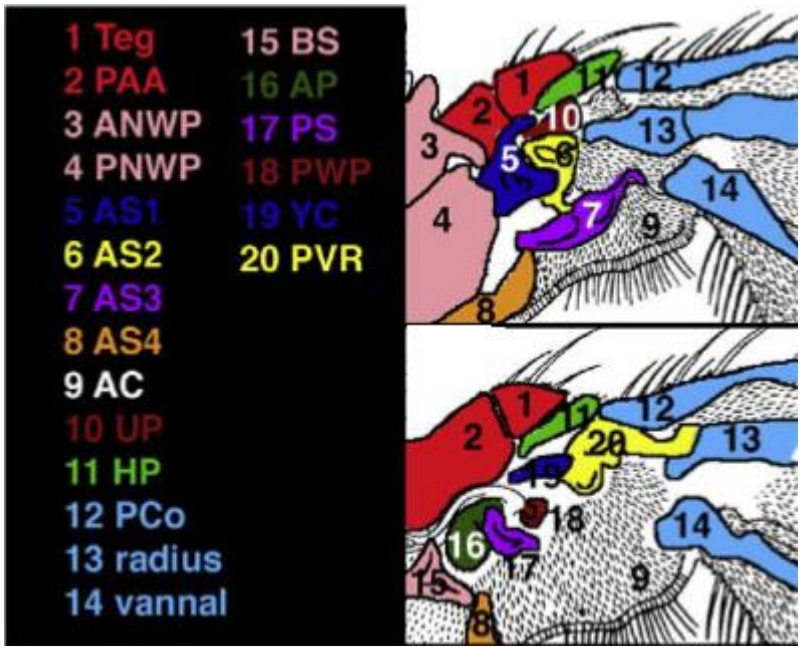

Figure two: Structure of the adult drosophila wing

A. Image taken from the book 'Imaginal Discs'. The image shows the fate map of wing disc and adult wing. The central dark gray region in the wing disc gives rise to wing blade and the light gray colored region in the wing disc gives rise to the wing hinge.

B. Image taken from Perea *et al.*, shows the structures in the dorsal proximal hinge (top) and ventral proximal hinge (bottom) .

Gene regulatory network in hinge development

In *Drosophila melanogaster* the wnt family gene (figure three) , *wingless*, acts as a master regulator of wing development (Grba *et. al.*, 2000). It forms long distance morphogenic gradients that pattern the wing disc (Strigini *et. al.*, 2000). Loss of *wingless* expression results in duplication of notum and absence of wings (Sharma and Chopra.,1976). In the canonical wingless pathway, Wingless is the secreted ligand. Along with some co-receptors, the G-protein coupled receptor, Frizzled, is the receptor for wingless. A protein called Dishevelled, acts like primary a cyptoplasmic messenger. In the absence of *wingless* signaling, the cytoplasmic protein, beta-Catenin is ubiquitinated by the so called 'destruction complex' and is subsequently degraded by protiosomic digestion. After binding of Wingless to Frizzled, the destruction complex function is disrupted and beta-Catenin starts accumulating in the cytoplasm and is subsequently nuclearized. Nuclear Beta-Catenin binds to Pangolin (TCF homolog in *Drosophila*), which in turn, with other co-activators activates *wingless* target genes. In *Drosophila* wing disc, *wingless* target genes include *vestigial, acheate, sensless and homothorax* (Neumann *et. al.*, 1996, Skeath *et. al.*, 1991, Azpiazu and Morata 2000). Apart from *wingless* the other important pathway involved in wing hinge development consists of *teashirt*, *homothorax*, *extradenticle*. *Teashirt* acts like an activator of *homothorax* and binding of Homothorax is necessary for nuclear localization of Extradenticle. The Homothorax-Extradenticle complex then activates its downstream targets. Loss of *teashirt* in wing disc results in so called 'aero plane' mutants, which due to lack of proper development of proximal hinge has held out wings (Soanes *et. a.*, 2001). On the other hand over expression of either *teashirt* or *homothorax* throughout the wing disc results in proximalization of the wing (Wu and Cohen. 2002). In first two larval instars of the wing development, both *teashirt* and *homothorax* are expressed throughout the wing disc. However in third larval instar, expression of *teashirt* is limited only to the proximal hinge. In late third larval instar *homothorax* is co-expressed in proximal hinge with *teashirt*. In the hinge *wingless* is expressed in two concentric rings, inner ring (IR) and outer ring (OR). The outer ring abuts the region of expression of *teashirt* (Perea *et. al.*, 2009). Apart from its expression in proximal

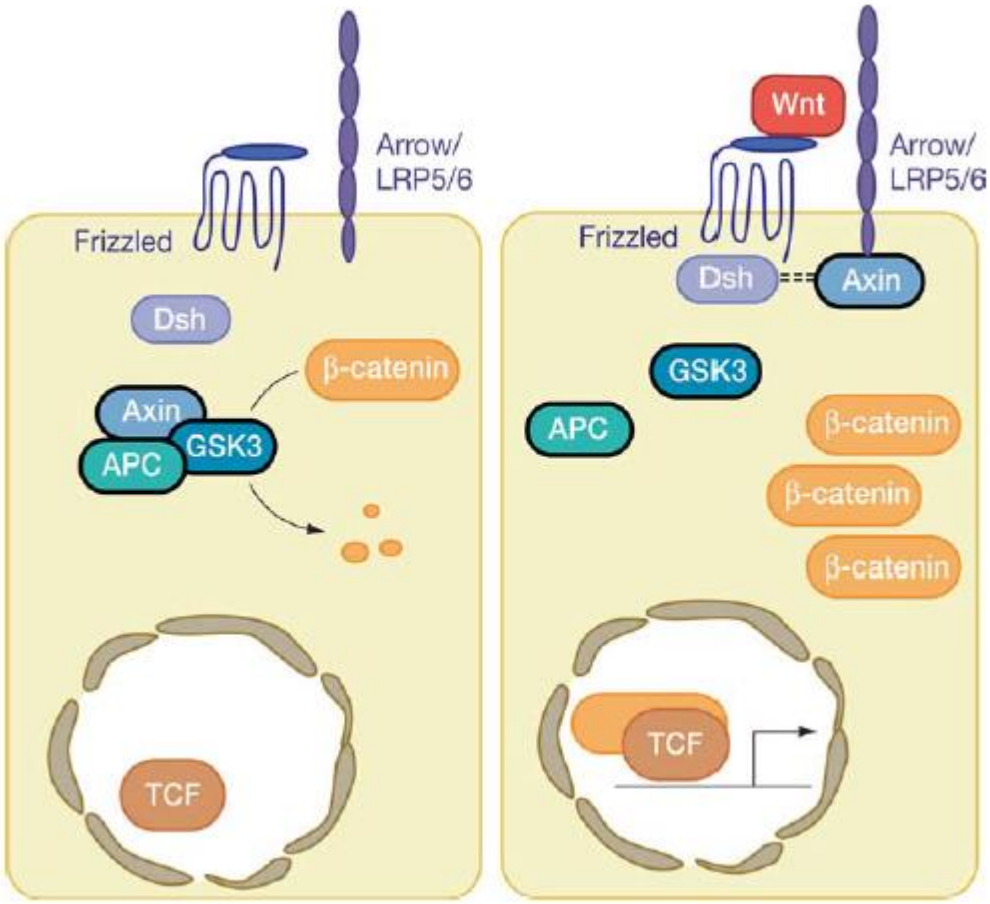

Figure Three: Canonical Wnt singling pathway

Figure taken from Logan et al., 2004. Figure shows the canonical Wnt Signaling pathway. In this pathway Wnt is the secreted ligand. Frizzled is the trans-membrane receptor. Dishivlled and Beta-Catenin are primary and secondary messenger. Nuclearized Beta-Catenin binds to TCF to activate downstream targets.

hinge, *homothorax* is strongly expressed in IR and OR and weakly expressed in the region between IR and OR. Throughout the wing development *teashirt* and *wingless* act like negative regulators of each other (figure four).

Drosophila **flight**

About 75% of all known animal species are insects. This makes insects the most successful of all animal species on this planet. Their ability to fly contributes a great deal to their success. Insects flap their wings at a very high speed, typically around hundreds of wing beats per second. Great speed, maneuverability and control are the features of the insect flight that make them the 'ultimate flying machines'. Fruit flies can complete the wing flapping cycle two hundred and fifty times in a single second (which is about the same frequency at which turbines in a commercial jet planes rotate). Wing flapping cycle of flies can be divided in four strokes. The dorsal reversal, down stroke, the ventral reversal and the up stroke (Dickinson *et. al.*, 1996). To maintain balance during flight, flies use their balancing organs called halteres. To maintain a stable position of the center of mass, with respect to the body during flight, halteres move with exactly the same frequency of that of the wing beats but are exactly out of phase with the wings.

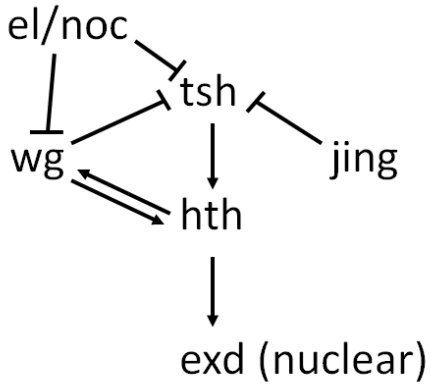

Figure four: Gene regulatory network in drosophila wing hinge development. Relationship between seven genes *elbow*, *no ocelli*, *wingless*, *jing*, *teashirt*, *homothorax* and *extradenticle* in the GRN that patterns wing hinge. *Wingless*, *elbow, no ocelli* and *jing* are negative regulators of *teashirt*. *Wingless* and *homothorax* form a positive feedback loop. *teashirt* positively regulates *homothorax*, which in turn is responsible for nuclear localization of Extradenticle.

Goals of my project

1. Characterization of phenotype given by knockdown of *hinge1* using:
    a. Flight assays
    b. Scanning electron microscopy and light microscopy
2. Enhancer suppressor screen to identify interactors of *hinge1*
3. Finding the molecular basis of the *hinge1* phenotype.

MATERIALS & METHODS

Light microscopy

Flies were anesthetized using CO_2 and then imaged. Wings were dissected and mounted on a glass slide in a drop of clove oil and a coverslip was placed and sealed using nail polish.

Scanning electron microscopy

The wings were dissected and mounted on stubs using conducting carbon tapes and the images were taken at medium low vacuum at a magnification of 800x.

Flight assays

The drum drop assay: A glass tube of 50 cm length and 8 cm diameter was used for the drum drop assay. A funnel was used to ensure that only one fly is dropped at a time. For the single flight assay and high speed filming, flies were tethered to tungsten needle using super glue and then filmed at 25 frames per seconds and 4000 frames per second respectively at NCBS in Dr. Sanjay Sane's lab (figure five).

Immunostaining of wing discs

Wing discs were dissected in PBS and fixed with 4% paraformaldehyde in 1X PBS for 60 minutes at room temperature. They were blocked in 1X PBS, 1% BSA, 0.3% Triton for 1 hour, incubated with the primary antibody (anti-Wg 1:1000) for sixteen hours at 4°C, washed 4 X 10' in blocking buffer and incubated with the appropriate fluorescent secondary antibody for 1 hour at room temperature in the dark. They were then washed and mounted in Antifade. Anti- Wg was purchased from Developmental Studies Hybridoma Center. Images were taken in a Zeiss 710 LSM Confocal microscope.

Enhancer suppressor screen:

All the crosses were set up at 25^{o} C

Fly lines used for the enhancer suppressor screen : Fly lines used in this project were procured from VDRC, NIG and Bloomington stock centers. Gal4 drivers used: MS1096, *patch*, *optomotorblind*, *daughterless*.

Table1. List of RNAi lines used:

	Gene knocked down	Line	Genotype	Source
1	*hinge1*	100101	P{KK103642}VIE-260B	VDRC
2	*hinge3*	105177	P{KK103584}VIE-260B	VDRC
3	*hinge2*	108487	P{KK111648}VIE-260B	VDRC
4	dip3	107803	P{KK111529}VIE-260B	VDRC
5	Noc	29370	y^1 v^1; P{TRiP.JF02534}attP2	Bloomington
6	CG3937	3937R-2		NIG
7	CG1577	1577R-2		NIG
9	CG13151	13151R-3		NIG
10	CG8550	8550R-3		NIG
11	CG1115	1115R-1		NIG
12	CG10900	10900R-1		NIG
13	CG3704	3704R-2		NIG
14	CG7854	7854R-3		NIG
15	CG9375	9375R-3		NIG
16	CG13345	13345RA		NIG
17	CG6345	6345R-2		NIG
18	CG12093	12093R-3		NIG
19	Atf6	BL26211	y^1 v^1; P{TRiP.JF02109}attP2	Bloomington
20	CG7254	7254R-1		NIG
21	CG11894	11894R-1		NIG
22	CG1763	1763R-2		NIG
23	CG3931	3931R-2		NIG
24	CG5086	5086R-2		NIG
25	CG8675	8675R-2		NIG
26	CG29389	29389R		NIG
27	CG6280	6280-5		NIG
28	CG3630	3630R-1		NIG
29	CG1059	1059R-4		NIG

30	CG3161	31617R-3		NIG
31	CG7338	7338R-1		NIG
32	CG6199	6199R-2		NIG
33	CG11101	11101R-2		NIG
34	CG5086	5086R-2		NIG
35	CG3940	3940R-1		NIG
36	CG6282	6282R-2		NIG
37	CG1338	1338R-1		NIG
38	CG10327	10327R-3		NIG
39	CG5874	5874R-1		NIG
40	CG85511	85511R-2		NIG
41	CG1130	1130R-3		NIG
42	CG11990	11990R-3		NIG
43	CG10523	10523R-2		NIG
44	CG3983	3983R-3		NIG
45	CG15444	15444R-1		NIG

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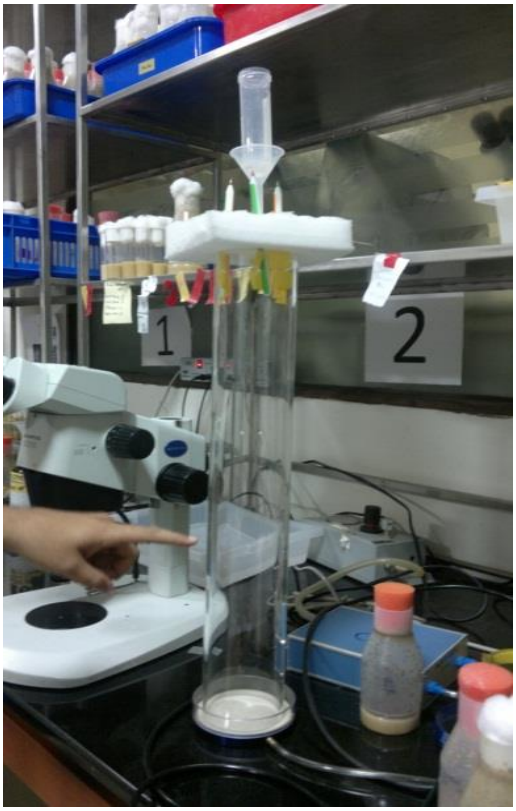

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Figure Five: Set up for the flight the assays

A. Figure shows the glass tube used for the drum drop assay
B. Figure shows a fly tethered to a tungsten needle for the single flight assay

RESULTS

The *hinge1* knockdown phenotype is dose dependent

Knocking down *hinge* genes using RNA interference by means of UAS-Gal4 system in the wing, results in a bent wing hinge phenotype. Figure six shows phenotype resulting from the knockdown of *hinge1* using MS1096 Gal4. This phenotype is dose dependent. MS1096 is a wing specific Gal4 driver. In females having only one copy of MS1096 Gal4 driver and one copy of dsRNA, the flies show phenotype where the wings are held out and bent. Since MS1096 Gal4 is present on the x chromosome, due to dosage compensation, males having one copy of Gal4 and one copy of dsRNA give a stronger phenotype than females having one copy of MS1096 Gal4 and one copy of dsRNA in terms of the angle at which the wings are bent. Flies having two copies of dsRNA, and a single copy of MS1096 Gal4 give an even stronger phenotype. This phenotype is so strong that the flies cannot expand their wings at all.

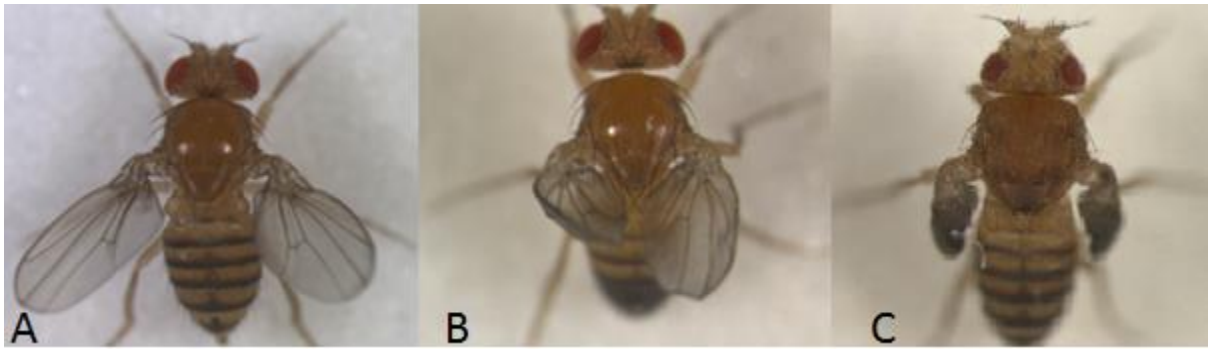


Figure six: Dose dependent phenotype given by *hinge1* knockdown

Images of adult flies showing the dose dependent wing phenotype of *hinge1* knockdown by RNA interference, driven by MS1096 Gal4. Genotypes of the flies are as following

A. MS1096/+; *hinge1*i/+ (female)

B. MS1096/y; *hinge1i*/+ (male)

C. MS1096/FM7a; *hinge1i/hinge1i* (female)

The *hinge1* animal cannot fly

Since the *hinge1* knockdown fly One of the aims of my fifth year project was to characterize the phenotype given by *hinge* gene knockdowns. We thought that studying the flight of these flies will tell us more about what is happening to the wing. In order to study the flight of these flies we did three experiments:

The single flight assay: In the single flight assays the flies are tethered to tungsten needles and are given an air puff. A gentle air puff acts as a stimulus to initiate the insect flight. This experiment tells whether or not the fly is able to flap wings in response to the air puff stimulus. When given a gentle air puff under the tethered condition:

a. All wild type flies flap wings (n=5) (positive control)
b. All females of the genotype MS1096/+; *hinge1*i/+ flap wings (n=5)
c. None of the males of the genotype MS1096/y; *hinge1*i/+ can flap wings (n=5)

The drum drop assay: In a drum drop assay flies are dropped one at a time in a transparent tube of 50cm length and 8cm diameter and the number of flies that can reach the walls of the tube before hitting the bottom of the tube are scored. This experiment tells us whether or not the flies are able to initiate and sustain flight.

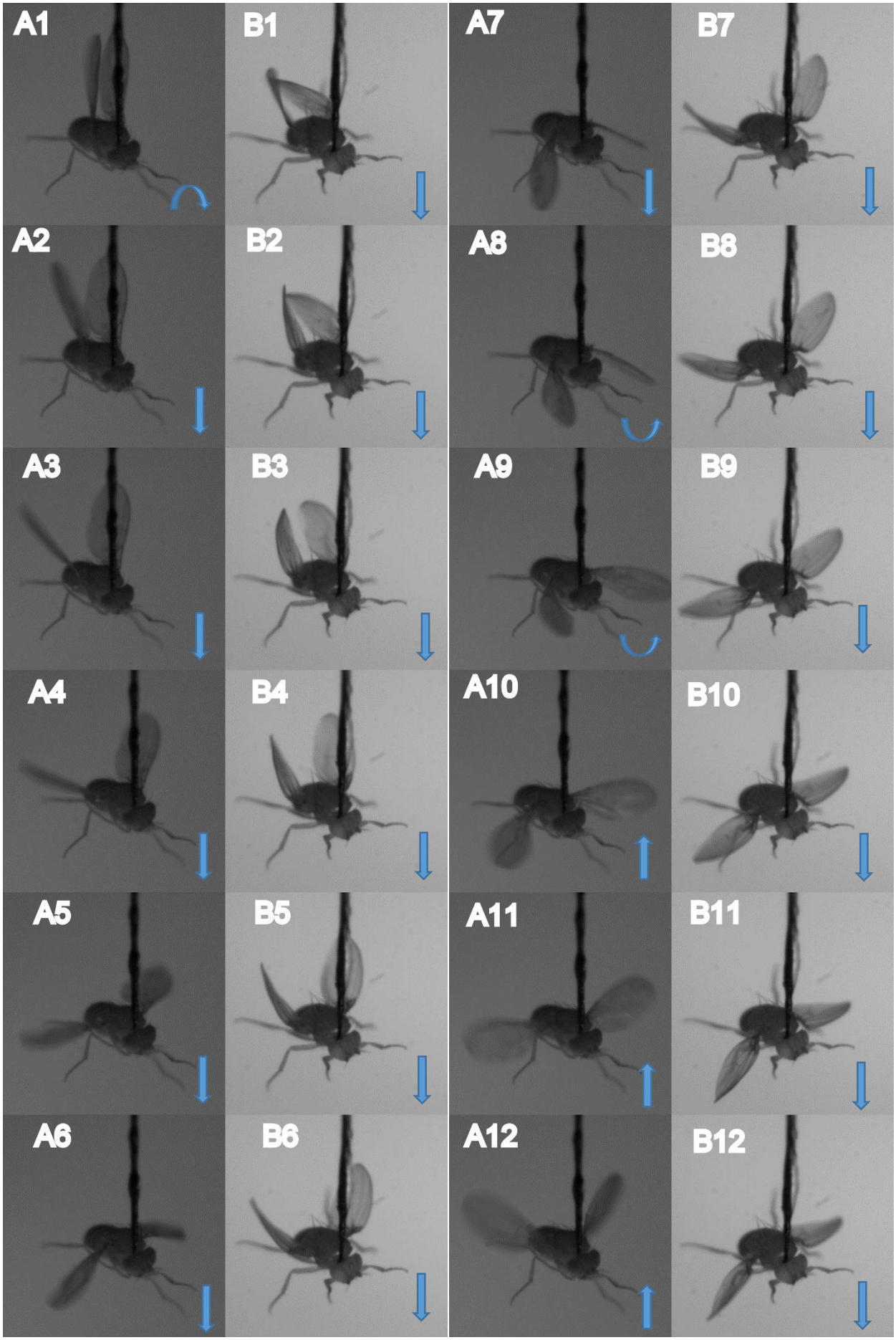
When dropped one at a time in the drum without giving a horizontal momentum: 95% of the wild type females tested after 2-4 days from the time of eclosion were able to reach the walls of the drum before hitting the bottom. Suggesting that most of them were able to initiate and sustain flight (n =40).

None of the flies of the genotype MS1096/+; *hinge1*i/+ tested 2-4 days after eclosion can reach the wall of the drum before hitting the bottom. Suggesting that none of them are able to fly (n=20).

High speed filming: In this experiment, flies were tethered to a tungsten needle and filmed using high speed cameras at four thousand frames per second so that we get four frames per stroke and then the flights of the mutant flies were compared with the flights of wild type flies (figure seven). One wing beat cycle of the *Drosophila* flight can be divided in four parts. The down stroke, ventral reversal, the up stroke and the dorsal reversal.

In females of the genotype MS1096/+; *hinge1*i/+ both the dorsal and ventral reversals are greatly reduced (since males of this same genotype cannot flap wings, only females were tested in this experiment). The down stroke is slower than normal. The down stroke of mutant flies take 3 millisecond as compared to 2 millisecond in wild type flies. The up stroke is faster than normal. The up strokes of mutant flies takes 0.6 millisecond as compared to 1 millisecond in wild type flies. And the wings hinder the motion of halteres.

For wild type flies, the wing beat frequency in the tethered condition is around 250 Hz. In case of the mutant flies, MS1096/+; *hinge1*i/+, the wing beat frequency was unaffected (250Hz)(n=5).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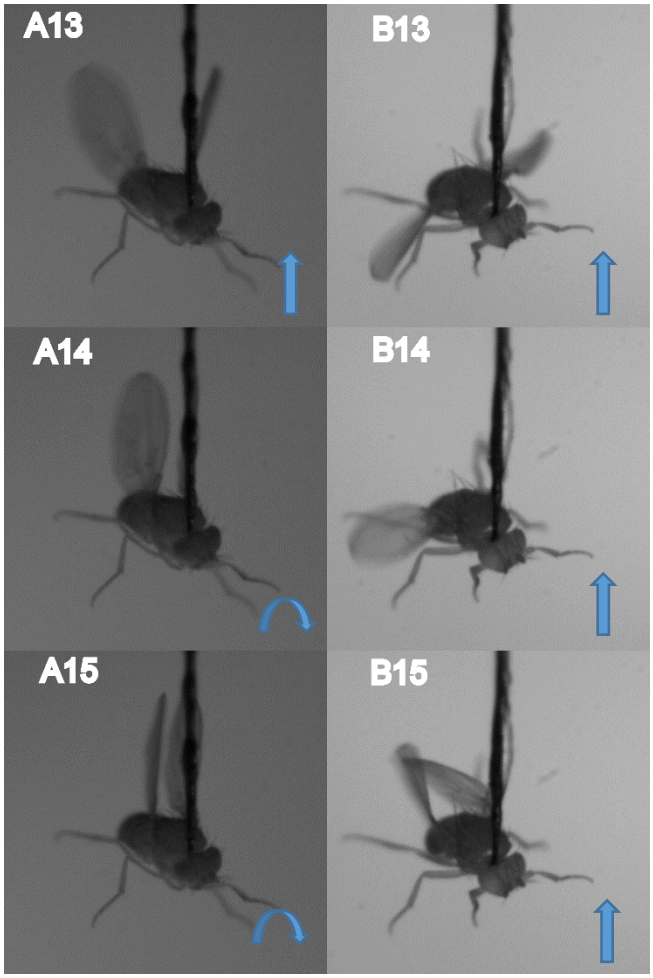


Figure seven: Wing beat cycle is severely affected in *hinge1* knockdown flies

hinge1 knockdown flies have a greatly reduced dorsal and ventral reversals and the wings hinder the motion of halteres.

A. Wing beat cycle of the wild type fly. A1 to A7 is down stroke, A8 to A10 is ventral reversal. A11-A13 is up stroke and A14 to A15 is dorsal reversal.

B. Wing beat cycle of MS1096/+; hinge1i/+ fly. B1-B12 is down stroke, and B13 to B15 is up stroke.

= Down stroke

= Up stroke

= Ventral reversal

= Dorsal reversal

Characterization of *hinge1* knockdown phenotype using electron and light microscopy

To see whether the bent wing phenotype given by knockdown of *hinge1* using RNAi was a result of any abnormality in the structures of proximal hinge, we took scanning electron micrographs of the proximal hinge region. After comparing these images with the proximal hinge of wild type flies, we found that there is no significant difference between the two, suggesting that the bent wing phenotype was not caused by any abnormality in the proximal hinge (figure eight)

The structures in the distal hinge are large enough to be clearly seen using light microscopy. Light microscopy images taken of *hinge1* knockdown fly wings, using different Gal4 drivers like *daughterless*, *optomotorblind* and *patch* revealed one common interesting feature of this phenotype. The region between the *wingless* IR and OR is severely affected. The two important structures in this region ‘radius’ and ‘vannal’ are severely deformed (figure nine).

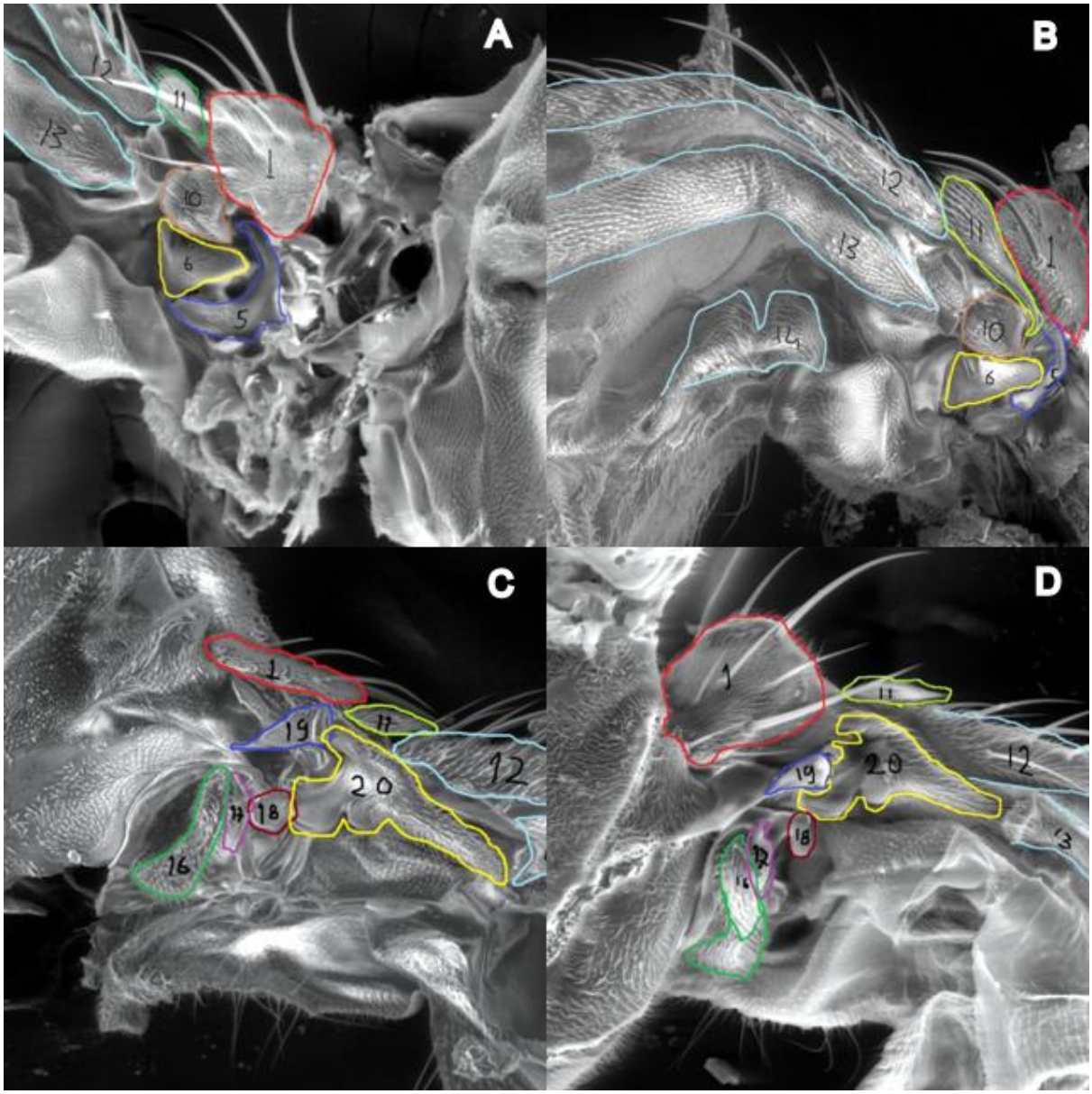


Figure eight: Scanning electron micrographs of proximal wing hinge showing that *hinge1* knockdown does not affect proximal hinge.

Scanning electron micrograph of adult drosophila proximal wing hinge taken at 800x magnification. Numbering of structures is according to Figure 2.

A. Wild type dorsal hinge

B. MS1096/+; *hinge1*i/+ dorsal hinge

C. Wild type ventral hinge

D. MS1096/+; *hinge1*i/+ ventral hinge

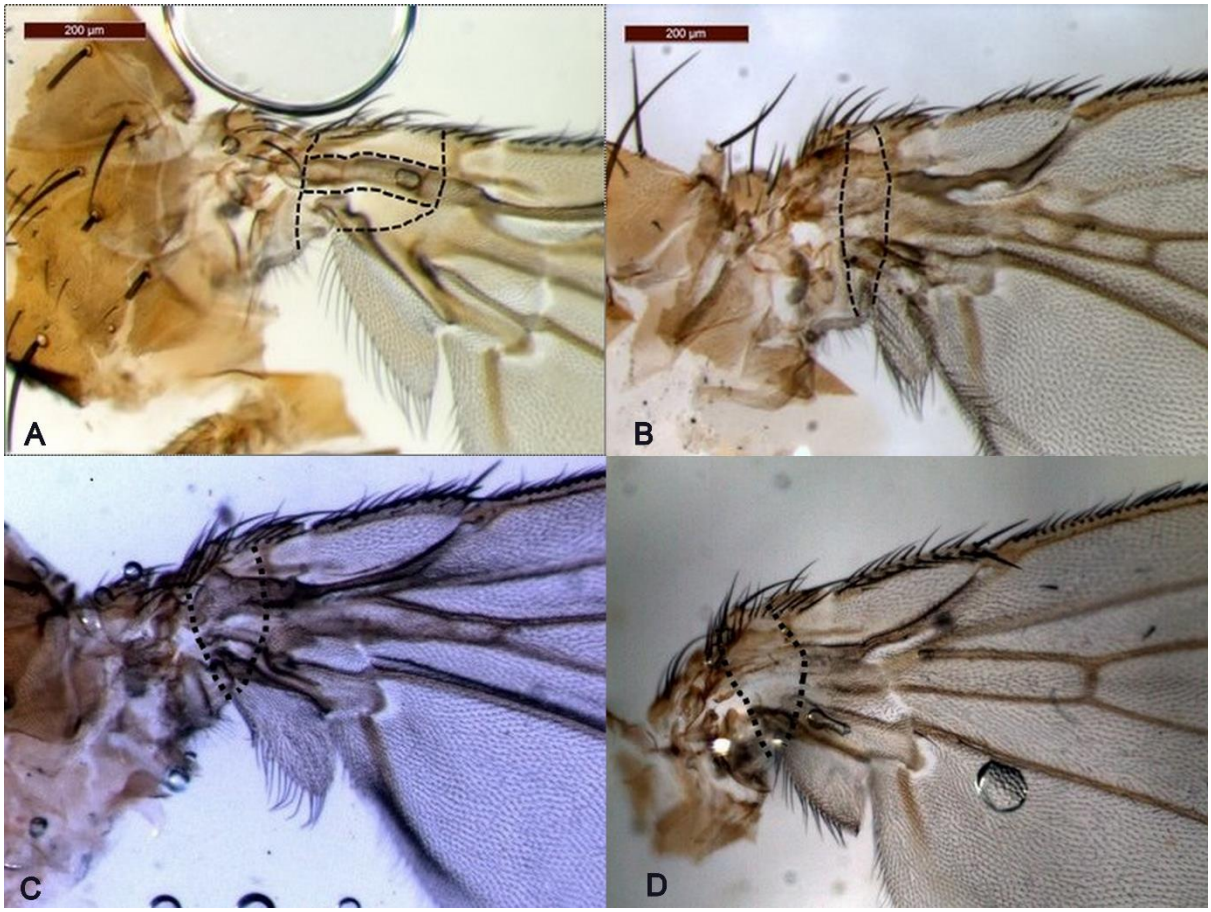


Figure nine: Knockdown of *hinge1* affects the region between IR and OR

Vertical dotted line on left in each image is Wg OR and on the right is Wg IR. In *hinge1* knockdowns using various Gal4 drivers, flies show a common phenotype. The region between Wg IR and Wg OR is severely affected

A. Wild type fly wing. The structure depicted by dotted horizontal lines is 'radius'

B. Wing hinge of *daughterless*Gal4/*hinge1*i fly

C. Wing hinge of *patch*Gal4/*hinge1*i fly

D. Wing hinge of *optomotorblind*Gal4/+; *hinge1*i/+

Ectopic *wingless* expression in *hinge1, hinge2* and *hinge3* knockdown flies

In order to find the interactors of *hinge1*, I looked into literature for mutants that have a similar phenotype to that of *hinge1* knockdown flies. One such mutant which looked very similar to *hinge1* knockdown flies was SoxF^{KG09145} which is a null mutation for the gene *soxF* (Danjoy *et. al.*., 2009). The wings of SoxF^{KG09145} flies are held out and bent backwards. Structures like alula, vennel and radius in the distal hinge are not properly developed, similar that of the *hinge1* knockdown flies. *soxF* is the only member of the sox family in drosophila. In vertebrates, proteins in the sox family have been have been shown to negatively regulate wnt signaling. *soxF* too has been recently shown to be a negative regulator of *wingless*, the major wnt protein in drosophila (Danjoy *et. al.*., 2009). In third instar larval wing disc, wingless is expressed in three regions, the dorso-ventral boundary, the inner ring, which gives rise to part of the distal hinge and the outer ring, which gives rise to part of the proximal hinge. *Wingless* expression in the inner and outer ring plays crucial roles in growth and patterning of wing hinge (Perea *et. al.*., 2009). Looking at all these facts we decided to look at wingless expression in *hinge1* knockdown flies. For this experiment I decided to use *patch*-Gal4 which is expressed in a patch at the anterio-posterior boundary in the wing disc and meets the inner ring, outer ring and D-V boundary wingless expression at right angles. The *patch*-Gal4 is also a good choice for this experiment because on both sides of the *patch*-Gal4 expressing cells there would be wild type cells which serve as internal control for this experiment. Immuno-staining of late third instar larvae of genotype *patch*-Gal4 /UAS-*hinge1*-dsRNA shows ectopic *wingless* expression in the gap region between the *wingless* inner and outer ring with an hundred percent penetrance (n=10) . Similar ectopic *wingless* staining was also seen in the gap region between *wingless* inner and outer ring of late third instar larvae of the genotype *patch*-Gal4 /UAS-*hinge2*-dsRNA and *patch*-Gal4 /UAS-*hinge3*-dsRNA (figure ten).

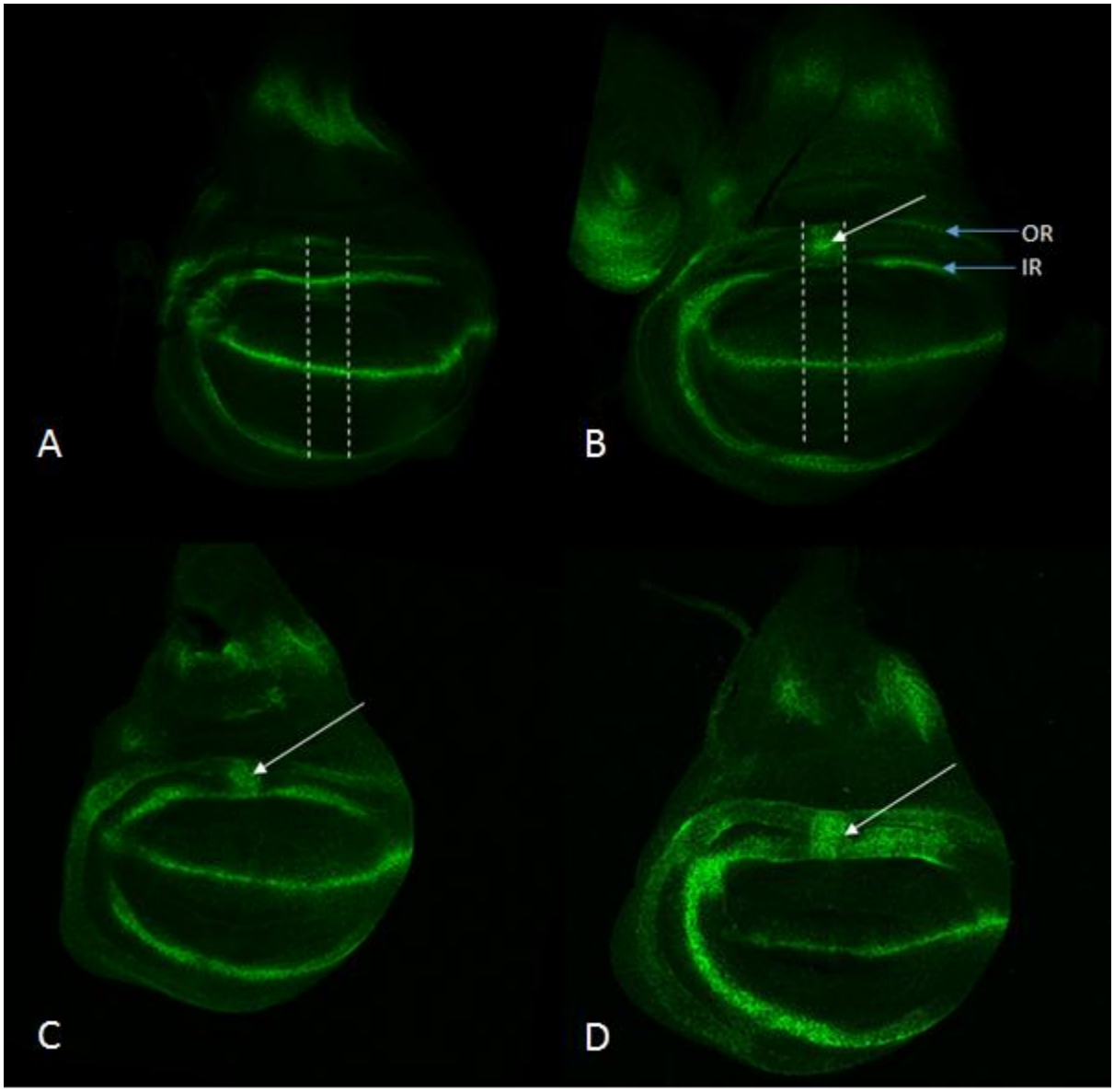


Figure ten: Ectopic *wingless* in *hinge1, hinge2 and hinge3* knockdowns

Figure shows ectopic Wingless staining in the dorsal hinge. White arrows point to the ectopic Wingless in hinge region of the wing. White dotted lines mark the region of expression of *patch*-Gal4 in the wing region of the wing disc.

A. Wing disc of wild type late third instar larva, stained with anti-Wingless antibody.

B. Wing disc of late third instar *patch*-Gal4/*hinge*1i larva, stained with anti-Wingless antibody

C. Wing disc of late third instar *patch*-Gal4/*hinge3*i larva, stained with anti-Wingless antibody

D. Wing disc of late third instar *patch*-Gal4/*hinge2*i larva, stained with anti-Wingless antibody

Noc and *dip3* interact with *hinge1*

The phenotype given by RNAi knockdown of *hinge1* using MS1096 Gal4 is very suitable for enhancer suppressor screen. The major features of this phenotype, reduced area of the alula and angle at which the wing hinge is bent are easily quantifiable. There is also scope for further reduction or increase in both these parameters, so that enhancers and suppressors could be found with equal ease. For the enhancer-suppressor screen a second mutation was introduced in the background of RNAi knockdown of *hinge1* and the mutations that rescued the phenotype i.e. the phenotype of the progeny looked more like the wild type flies, were called suppressors and the mutations that enhanced the phenotype were called enhancers of the phenotype. The list of targets tested is given in table1. Out of these targets, two targets, *noc* and *dip3* gave a clear interaction with *hinge1*. Knocking down *noc* (*no ocilli*) in the background of *hinge1* knockdown using MS1096 Gal4 gives a rescue of phenotype whereas overexpressing *dip3* (one of the 16 MADF-BESS) genes gives an enhancement of the phenotype (figure eleven)

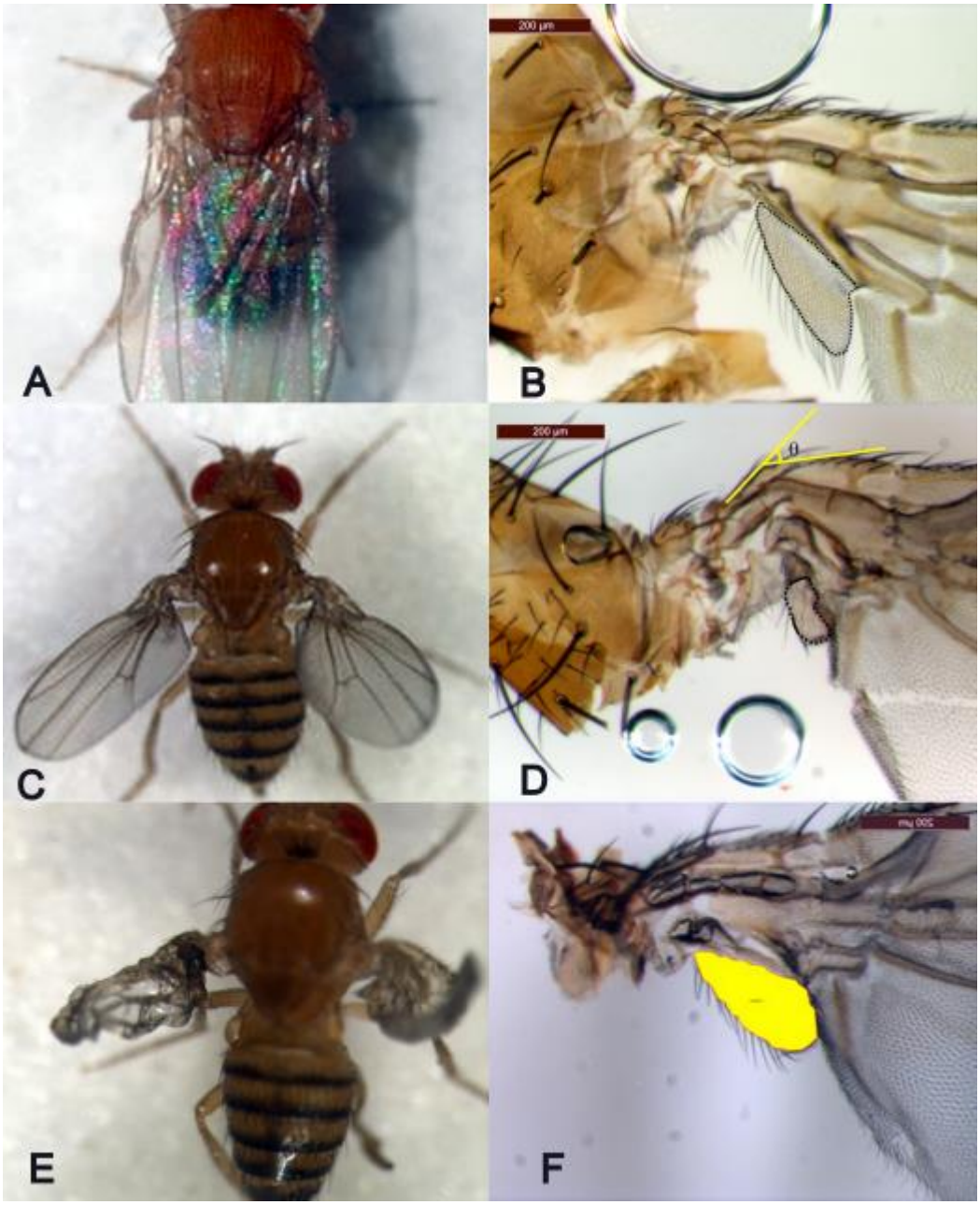


Figure eleven: A to F Enhancer suppressor screen using alula area and theta (angle at which the wing is bent) as parameters. EP dip3, an enhancer and *noc*RNAi, a suppressor if *hinge1* knockdown phenotype using MS1096 Gal4

A. Adult wild type fly

B. Wing of adult wild type fly with alula depicted by dotted line

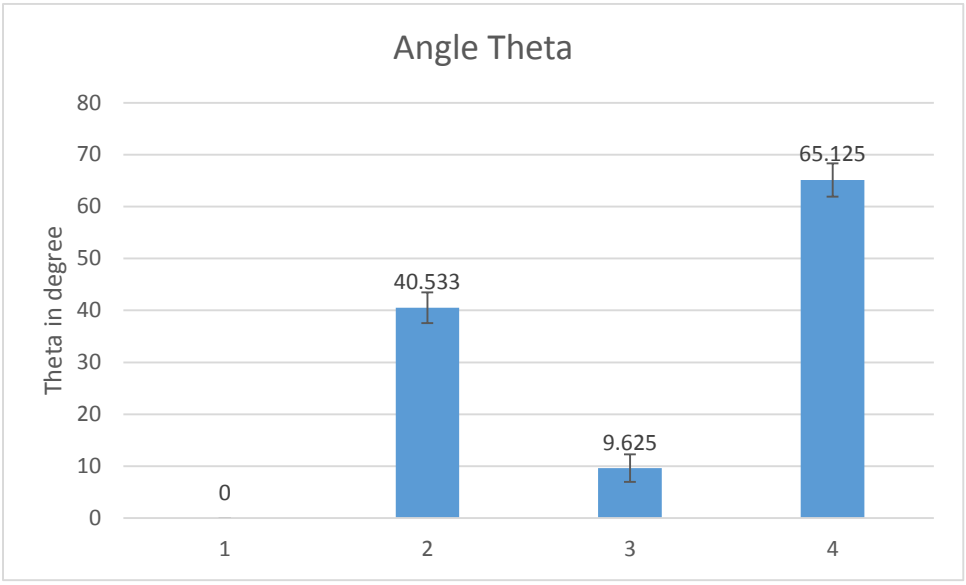
C. MS1096/+; *hinge1*i/+ adult fly

D. Wing of MS1096/+; *hinge1*i/+ fly. Alula depicted by dotted line

E. MS1096/+; EP-*dip3*/*hinge1*i adult fly showing enhancement of phenotype

F. Wing of MS1096/+; *noci*/*hinge1*i fly. Showing rescue of angle and alula area in yellow

G



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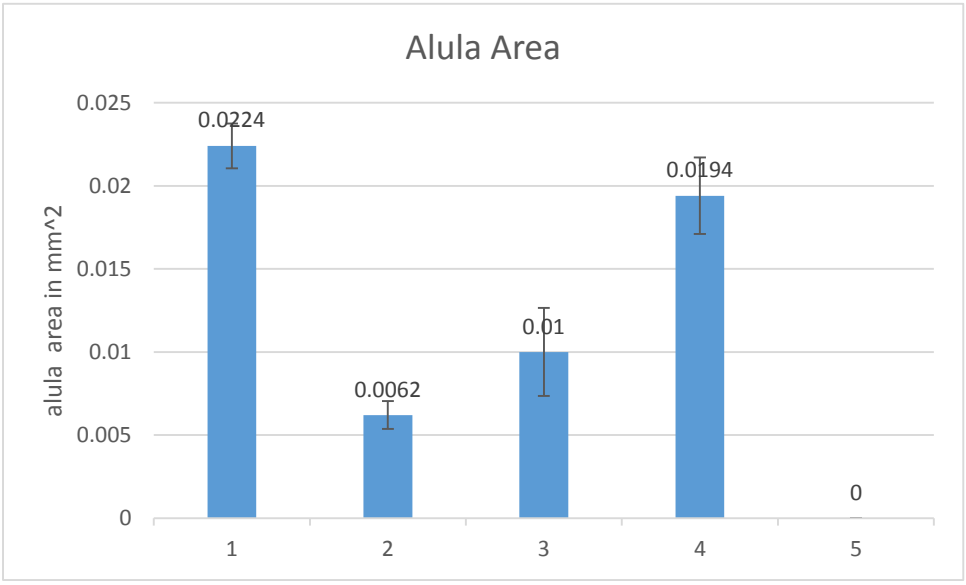

Figure eleven G & H: *Noc* knockdown rescues and *dip3* overexpression enhances the *hinge1* knockdown phenotype

G. Angle theta for
   1. Wild type fly
   2. MS1096/+; *hinge1*i/+ fly
   3. MS1096/+; *noci*/*hinge1*i fly
   4. MS1096/+; EP-*dip3*/*hinge1*i fly

H. Alula area for flies having following genotype:
   1. Wild type, 2. MS1096/+; *hinge1*i/+ 3.daGal/*hinge1*i

   4. MS1096/+; noci/*hinge1*i 5.MS1096/+; EP-*dip3*/*hinge1*i

DISCUSSION

Flight assays

The female flies of *hinge1* knockdown using MS1096 Gal4 are able to flap wings, as seen in the single flight assay. But the drum drop assay reveals that none of them can fly. Suggesting that this could be due to their inability to sustain flights. The high speed videos make its clear why these flies are unable to sustain their flights. In flies, halteres act like balancing organs. In flight they move with the same frequency as that of the wings but are exactly out of phase. In MS1096>*hinge1*RNAi flies the wings are bent backwards and they obstruct the motion of the halters making it impossible for the flies to maintain balance in flight. We had performed flight assays with a thought that it might shed some light on the physical cause of the bent wing phenotype. But apart from the fact that it showed us that these flies are flightless, it did not add much our knowledge.

Light microscopy of distal hinge

The important clue about what exactly is going on in the wings of *hinge1* knockdown flies, came from the analysis of distal hinge structures. For this experiment we had used three different Gal4 drivers, *daughterless*-Gal4, *optomotorblind*-Gal4 and *patch*-Gal4, one at a time to knockdown *hinge1* in the wing. These Gal4 drivers have diverse expression patterns in the wing. *daughterless*-Gal4 is ubiquitously expressed throughout the wing disc. *patch*-Gal4 and *optomotorblind*-Gal4 drivers are expressed around the A-P boundary in a narrow stripe and a broad band respectively. In spite of their different expression patterns the phenotypes given by these flies have a common feature. The region between *wingless* IR and OR is strongly affected. Instead of being flat and properly patterned, this region looks thick and due to the lack of patterning the structures like ‘radius’ which is the most important wing vein and ‘vannal’ are not formed properly or are completely disrupted.

Ectopic *wingless* expression between IR and OR

The fact that the region between the *wingless* IR and OR being strongly affected, helped us focus on the genes that are expressed in this region. Apart from *wingless*, *homothorax*, *extradenticle*, *routed* and *nubbin* are some of the gene that

are known to play important roles in growth and patterning of the wing hinge. We started by looking at *wingless* expression pattern in this region. *Wingless* is strongly expressed in two concentric rings IR and OR. In these two rings *wingless* forms a positive feedback loop with *homothorax*, which is also strongly expressed in these two rings. Between these two rings is a gap region where *wingless* is not expressed at all but *homothorax* is weekly expressed. In *hinge1*, *hinge2* and *hinge3* knockdown flies, *wingless* is ectopically expressed in this gap region. Suggesting that these three genes are negative regulator of *wingless* in this region. It would be interesting to see which enhancer, the OR enhancer or the IR enhancer which is also known as the spade-flag enhancer is responsible for the ectopic *wingless*. This is important because different sets of genes regulate these enhancers and knowing which enhancer is getting ectopically activated, will help us narrow down in finding the mode of action of these three genes (figure twelve). (Perea *et. al*., 2009).

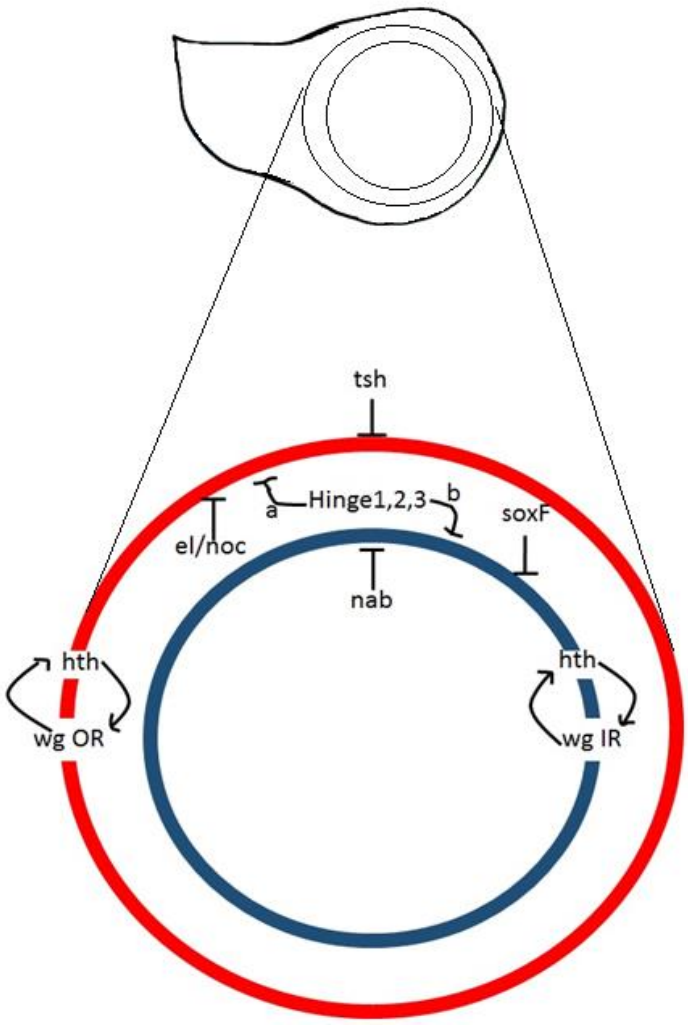

Figure twelve: Gene regulator network and potential position of *hinge1, hinge2* and *hinge3* in the gene regulatory network that regulates *wingless* expression in hinge.

Figure shows the late third instar larval wing disc and the two rings of expression of *wingless.* Different set of genes regulate *wingless* expression in the IR and OR region of the wing disc. Possible role of hinge genes. *Hinge1*,2,3 either block the spread of OR distally (a) or the spread of IR proximally (b)

Model for function equivalence and lack of redundancy in hinge genes

The interesting fact here is that all the three genes are necessary for the repression of *wingless* in the gap region, knocking down any one of these three gives the same phenotype. Suggesting that they are functionally equivalent and not redundant. There are two possible hypothetical models about the mechanism of action of these proteins that can explain this observation. One is that each of the *hinge1*, *hinge2* and *hinge3* proteins is unique and these three proteins form a heterotrimmeric complex and the proteins are functional only in the form of this complex. Absence of any one would result in non-formation of the functional complex, which in turn causes the phenotype. The second one is that the individual protein is functional and all the three are functionally equivalent and expression of each gene contributes approximately one third to the total number of functional proteins required (figure thirteen).

Future Directions

Expression pattern of *hinge1* genes: One of our future plans is to find out the expression pattern of *hinge1*. For this our lab is in the process of making antibody against the Hinge1 protein. Knowing the expression pattern might explain why knockdown of *hinge1* results in ectopic Wingless only in the hinge region.

Physical interaction between Hinge proteins: One possible way in which the lack of redundancy and functional equivalence of Hinge genes can be explained is by hypothesizing that the three Hinge proteins form a complex. To test this hypothesis we would like to plan experiments to check whether the Hinge proteins interact physically to form a complex or not.

Finding out which enhancer of *wingless* is affected in *hinge* gene knockdowns: Expression of *winges*s is controlled by two enhancers in the wing hinge. The OR enhancer and the IR enhancer, also known as the spade-flag enhancer. We would like to plan experiments to find out which of these two enhancers gets derepressed in *hinge* gene knockdowns.

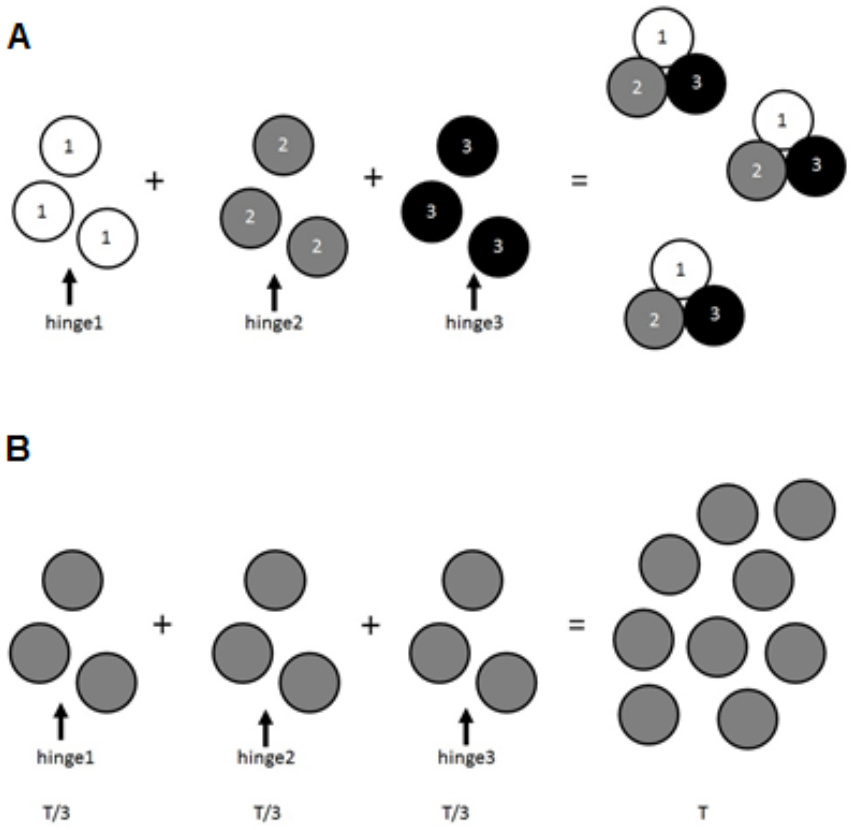

Figure thirteen: Two possible models that explain the equal importance and lack of redundancy in function of Hinge1, Hinge2 and Hinge3

a. In this model each Hinge protein could be unique. They form a hetero-trimmeric complex. Only the hetero trimmeric complex is functional and not the individual protein.
b. In this model all three Hinge proteins are functionally equivalent. Each protein is functional. Each gene contributes one third of the total proteins required for normal function.

APPENDIX I

Role of *dip3* in eye development

It has already been shown that overexpression of *dip3* (one of the sixteen MADF-BESS genes) in eye disc using an early expressing Gal4 driver, the eyeless Gal4 driver, perturbs the cell cycle and results in under proliferation of eye disc which is accompanied by over proliferation of antennal disc leading to reduced eye field and antennal duplication. Over expression of *dip3* also leads to down regulation of retinal determination genes and over expression of antennal determination genes causing eye to antennal transformation (Duong *et. al.*,2008). Over expression of *dip3* using a Gal4 driver that expresses in non-neuronal cells results in loss of photoreceptors. *dip3* null flies and mosaic patches having more than four photoreceptors mutant for *dip3* have an extra photoreceptor. Suggesting a role of *dip3* in blocking formation of extra photoreceptor (Duong *et. al.*, 2009). Recently, in our lab Shrivarsha Rajsheker has shown that *dip3* and *CTP Synthase* when knocked down individually by RNA interference using *eyeless*-Gal4, give a phenotype in which the ommatidial shape is either irregular or tetragonal instead of wild type hexagonal. This observation does not match with the previously published work since the *dip3* null flies do not show any abnormalities with the external ommatidial morphology. So we decided to further characterize the phenotype given by dip3 RNAi knockdown using *eyeless*-Gal4.

External morphology of *eyeless*-Gal4>*dip3il*CyO flies

The wild type *Drosophila* compound eye is made up of 750-800 hexagonal ommatidia. The *dip3* knock down flies show a range of phenotypes. The milder phenotype has slightly reduced eye field (around 600-700 ommatidia) to a severe phenotype where the entire eye field is missing. Each ommatidium gets its hexagonal shape because of the six secondary pigment cells, each of which becomes a side of the hexagon. The tertiary pigment cells and bristle cells occupy alternating vertices of this hexagon in the wild type fly. In *dip3* knock down flies, the shape of the ommatidia and the bristle pattern becomes irregular. The ommatidia that looked like tetragonal in light microscopy are not actually tetragonal. When

looked at 5000x in scanning electron microscopy it becomes clear that they are still hexagonal with two sides of the hexagon reduced in size.

Around 15% of the *dip3* knock down flies (n=300) have extra antennae (figure fourteen) And the flies that have extra antennae are mostly the ones that have a severely reduced eye field.

No extra photoreceptor were found in the *eyless*-Gal4>*dip3*i flies

In wild type flies all the ommatidia have eight photoreceptors. The photoreceptors one to six are called external photoreceptors and seventh and eighth photoreceptors are called inner photoreceptors. *dip3* null flies have an extra external photoreceptor. Imaging of eyes of *eyeless*-Gal4>*dip3*i /CyO flies using the revealed that these flies do not have an extra photoreceptor (figure fourteen).

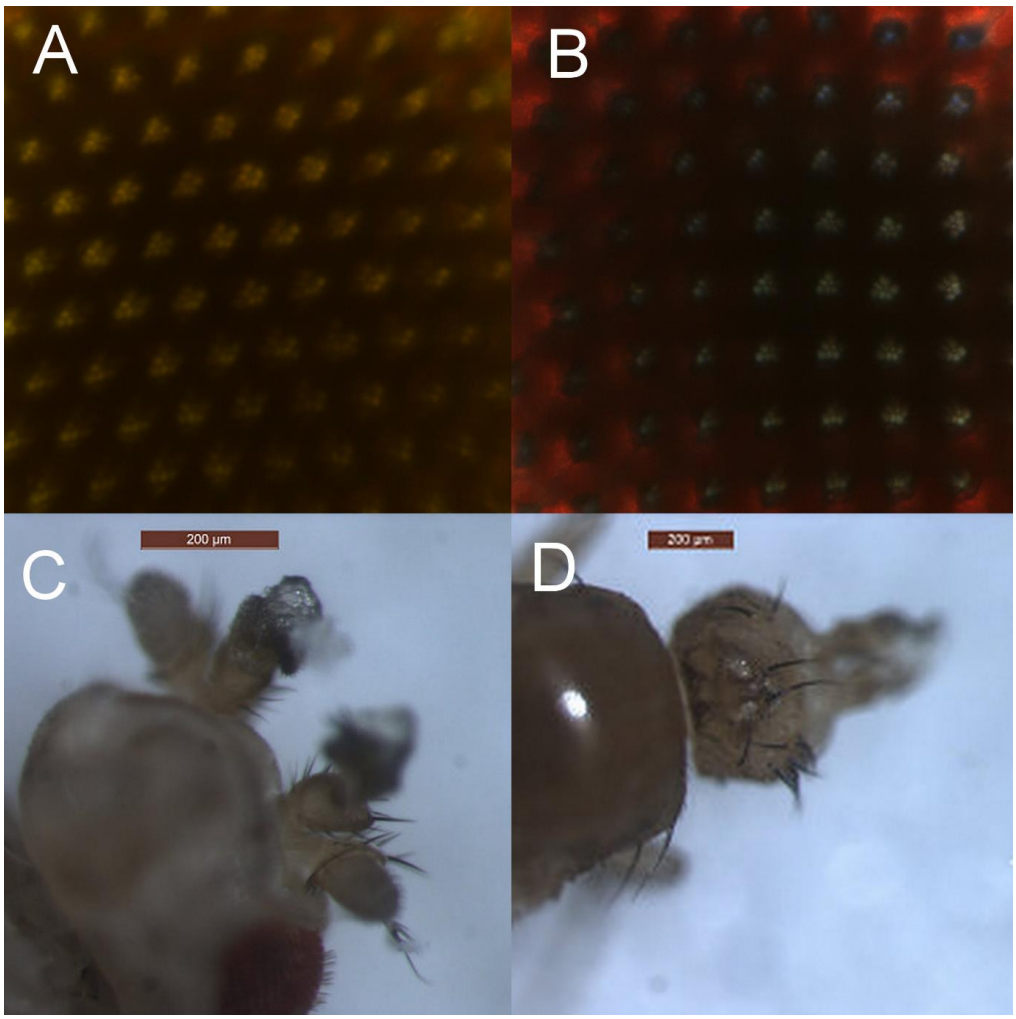

Figure fourteen: Phenotypes given by *dip3* RNAi knockdown
Various phenotypes given by *eyless*-Gal4>*dip3*i/+ flies, lack of extra photo receptor, antennal duplication, and irregular ommatidial arrangement
A. Confocal image of wild type compound eye. Seven photoreceptors are visible in each ommatidium.

B. Confocal image of *eyless*-Gal4>*dip3*i/+ fly compound eye. Similar to the wild type only seven photoreceptors are visible.

C. Antennal duplication in *eyless*-Gal4>*dip3*i/+ flies. Four instead of two antennae are present.

D. Antennal over growth and completely missing eye field in *eyless*-Gal4>*dip3*i/+

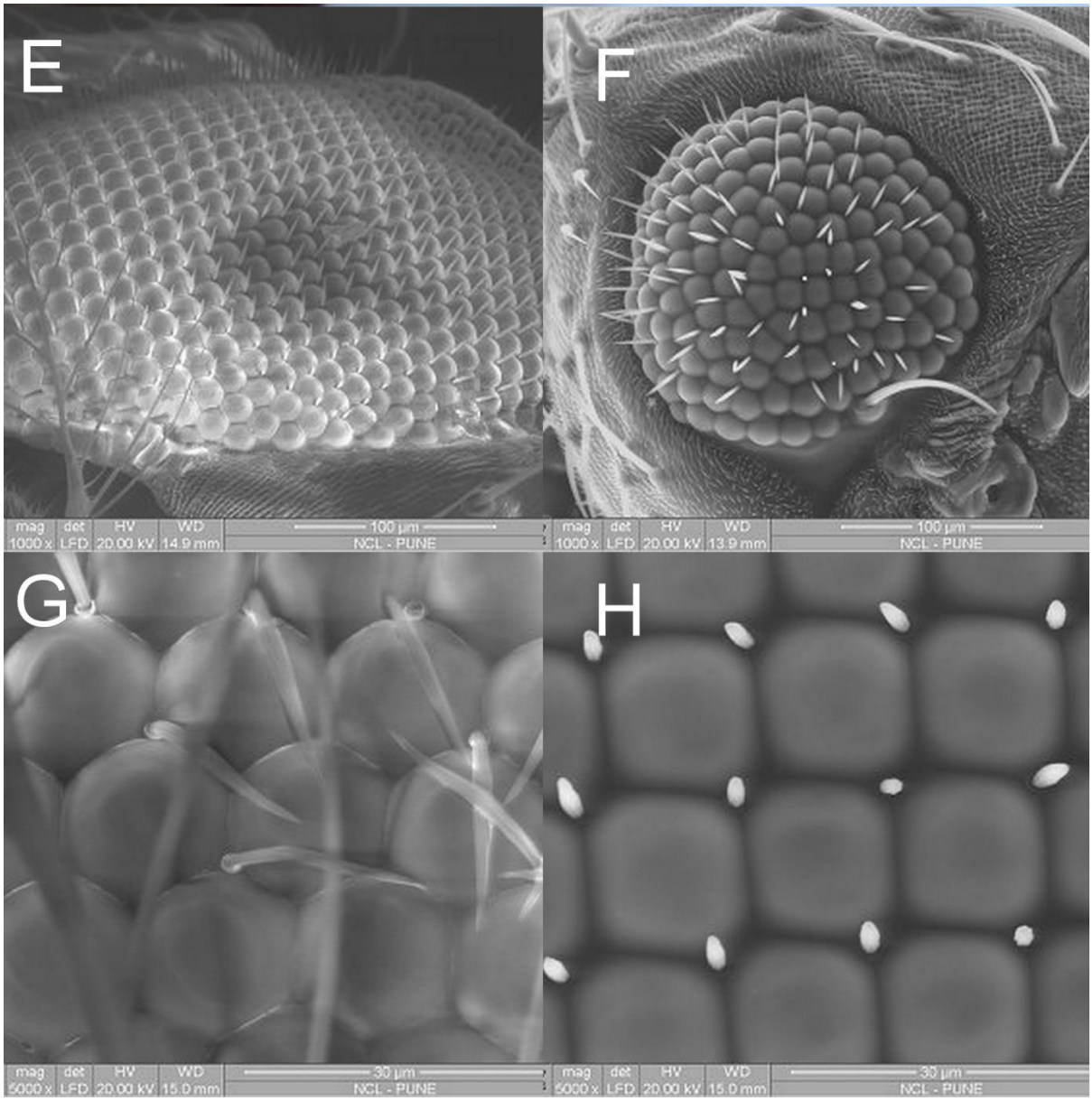

Figure fourteen: Phenotypes given by *dip3* RNAi knockdown, as monitored by SEM
Various phenotypes given by *eyless*-Gal4>*dip3*i/+ flies, lack of extra photo receptor, antennal duplication, and irregular ommatidial arrangement

E. Scanning electron micrograph of adult wild type compound eye having around 750-800 ommatidia (at 1000x magnification)

F. Scanning electron micrograph of adult *eyless*-Gal4>*dip3*i/+ fly's compound eye having around 130 ommatidia. Showing significant decrease in eye field

G. Scanning electron micrograph of adult wild type compound eye, showing hexagonal structure of each ommatidium

H. Scanning electron micrograph of adult *eyless*-Gal4>*dip3*i/+ fly's compound eye showing distorted hexagonal structure of each ommatidium.

Table 2: Phenotypes given by *dip3* RNAi knockdown, *dip3*^{1} and *dip3* overexpression

	***eyless*-Gal4>*dip3*i/+**	***dip3*^{1} (*dip3* null mutant)**	***dip3* overexpression**
Photoreceptors	No extra photoreceptor	An extra external photoreceptor	Loss of photoreceptors (late overexpression)
Antennal duplication	Yes	No	Yes (early overexpression)
External morphology	Affected	Not affected	Affected(early overexpression)

Discussion

It could be easily seen from the above table that there is a clear disparity between phenotypes given by *dip3* null flies and *eyless*-Gal4>*dip3*i/+flies. Since they give opposing phenotypes, It is hard to believe that this disparity arises purely from the differences in level of functional *dip3* in *dip3* null flies and *eyless*-Gal4>*dip3*i/+ flies. And the fact that *eyless*-Gal4>*dip3*i/+flies have a similar phenotype to that of *dip3* overexpressing flies, makes this even more complicated. As of now we do not have an explanation for the above mentioned observation.

Appendix II

Transcription Factor Binding Site Analysis of *hinge1* and *hinge3* proximal promoters

Proteins that regulate expression of other genes are called transcription factors. Some transcription factors achieve this by binding to regulatory DNA elements. Proximal promoters, 100-1000 base pairs long DNA sequences, mostly located upstream of origin of replication, are one such type of regulatory elements. In order to find out what transcription factors regulate the expression of *hinge1* and *hinge3*, transcription factor binding site analysis was performed on the proximal promoters of these genes. A tool based on 'Fly Factor Survey' (Noyes *et. al.*., 2008), a data base of transcription factor binding site sequences was used to check for the presence of clusters of these sequences in the proximal promoter of *hinge1* and *hinge3*.

Results

For transcription factor binding site analysis, the 500 base pair long DNA sequences, starting from transcription start sites of both *hinge1* and *hinge3* up stream, were chosen as proximal promoters. Both these sequences were analyzed for presence of all 310 transcription factor binding site sequences listed in the Fly Factor Survey database using the Genome Surveyor tool. Out of these 310 transcription factors respectively 65 and 73 transcription factors had high threshold (0.95) binding sites present in *hinge1* and *hinge3* proximal promoters. Out of all these transcription factors (65+73) only 20 had more than one high threshold binding sites present and were common to both *hinge1* and *hinge3* proximal promoters (figure fifteen)

Discussion

Since the transcription factor binding site sequences are very short typically 6-8 base pairs long and are degenerate, the probability of their presence without a real in vivo function is not insignificant. Hence just the presence of a transcription factor binding site in the promoter does not mean that the transcription factor is a regulator of that gene. Hence, the bioinformatic study of promoters can only predict the potential regulators of the genes and in vivo experiments need to be done to conform these predictions.

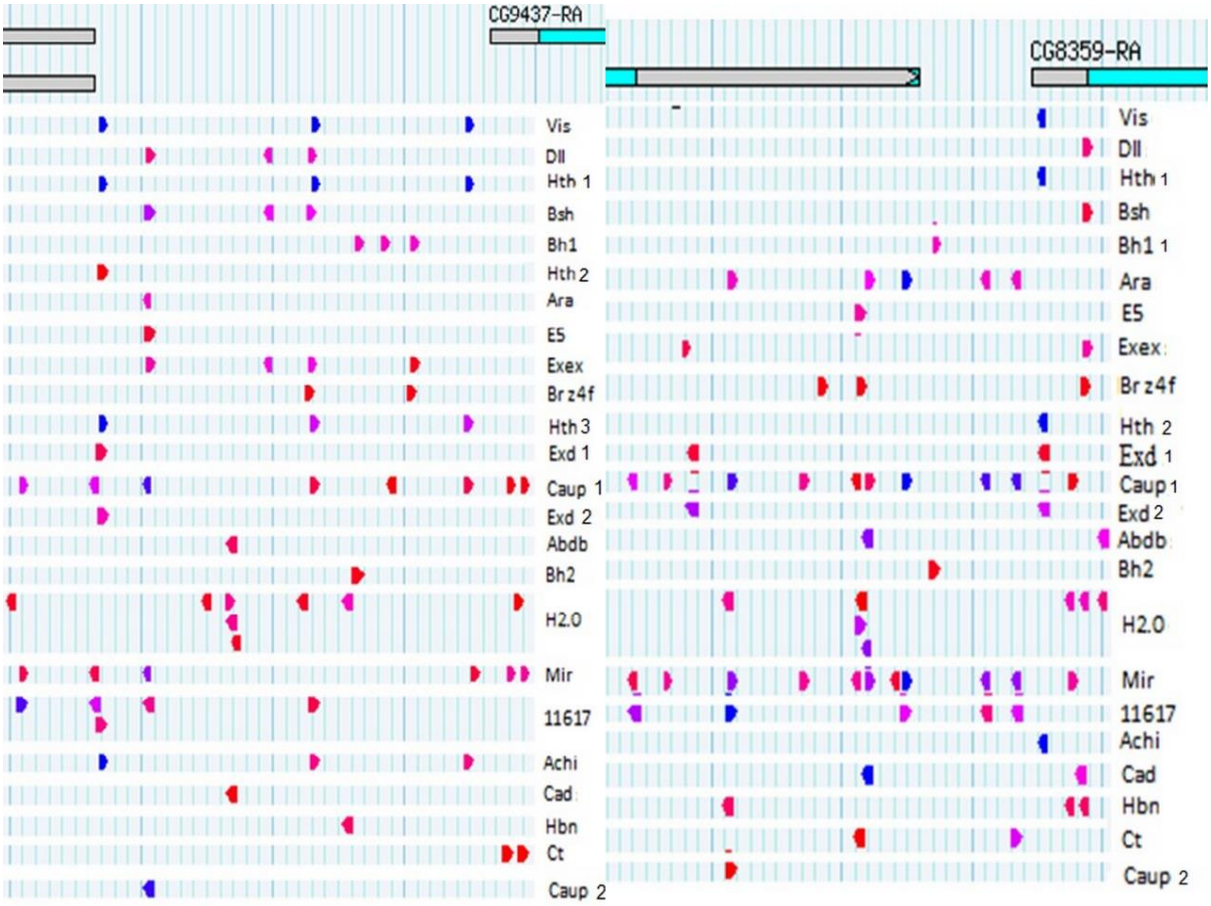

Figure fiteen: Binding sites of 20 potential regulators of *hinge1* and *hinge3* in their proximal promoters

Figure shows the location of high threshold (>0.95) binding sites of 20 transcription factors in proximal promoters of *hinge1* (CG9437) and *hinge3* (CG8359).Blue represents a perfect binding score of 1 and red represents a binding score of 0.95.

Table 3: List of potential transctiptional regulators of *hinge1* and *hinge3*.These are predictions, based on an iin-silico analysis and can be tested by experiments in the animal.

1	Caupolican
2	Mirror
3	Araucan
4	Homothorax
5	Extradenticle
6	Cut
7	Distaless
8	H2.0
9	Vismay
10	Achintya
11	Abd-B
12	Extra-extra
13	Brain specific homeobox
14	Caudal
15	Homeobrain
16	BarH-1
17	BarH-2
18	Brz-4
19	E5
20	CG11617

REFERENCES

1. Azpiazu N, Morata G. Function and regulation of homothorax in the wing imaginal disc of Drosophila. Development. 2000 Jun;127(12):2685-93.
2. Bhaskar V, Courey AJ: The MADF-BESS domain factor Dip3 potentiates synergistic activation by Dorsal and Twist. Gene 2002, 299:173–84.
3. Carl J. Neumann and Stephen M. Cohen. A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the Drosophila wing Development 122, 3477-3485 (1996)
4. Cutler G, Perry KM, Tjian R: Adf-1 is a nonmodular transcription factor that contains a TAF-binding Myb-like motif. Molecular and cellular biology 1998, 18:2252–61
5. Dichkinson, M.H, and Gotz K. G. (1996). The wake dynamics and flight forces of the fruit fly, *Drosophila melanogaster*. J. Exp. Biol., 199,2085-2104.w
6. Dichtel-Danjoy ML, Caldeira J, Casares F. SoxF is part of a novel negative-feedback loop in the wingless pathway that controls proliferation in the Drosophila wing disc. Development. 2009 Mar;136(5):761-9. doi: 10.1242/dev.032854.
7. Duong HA, Wang CW, Sun YH, Courey AJ. Transformation of eye to antenna by misexpression of a single gene. Mech Dev. 2008 Jan-Feb;125(1-2):130-41
8. Duong HA, Nagaraj R, Wang CW, Ratnaparkhi G, Henry Y, Courey AJ: Non-Cell- Autonomous Inhibition Of Photoreceptor Development by Dip3. Developmental Biology 2009, 323:105–113.
9. James B. Skeath and Sean B. Carroll. Regulation of achaete-scute gene expression and sensory organ pattern formation in the Drosophila wing Genes Dev. 1991 5: 984-995
10. Jonathan D. Zirin1 and Richard S. Mann2.,Differing strategies for the establishment and maintenance of teashirt and homothorax repression in the Drosophila wing Development 131, 5683-5693,2004
11. Noyes MB, Christensen RG, Wakabayashi A, Stormo GD, Brodsky MH, Wolfe SA. Analysis of homeodomain specificities allows the family-wide prediction of preferred recognition sites. Cell. 2008 Jun 27;133(7):1277-89. doi: 10.1016/j.cell.2008.05.023.

12. Perea D, Terriente J, Díaz-Benjumea FJ. Temporal and spatial windows delimit activation of the outer ring of wingless in the Drosophila wing. Dev Biol. 2009 Apr 15;328(2):445-55. doi: 10.1016/j.ydbio.2009.02.002. Epub 2009 Feb 13.
13. Sharma, R. P. and Chopra, V. L. (1976). Effect of wingless (wg1) mutation on wing and halter development in *Drosophila melanogaster*. Dev. Biol. 48,461-465.
14. Soanes KH, Bell JB. The drosophila aeroplane mutant is caused by an I-element insertion into a tissue-specific teashirt enhancer motif Genome. 2001 Oct;44(5):919-28.
15. Song H, Goetze S, Bischof J, Spichiger-haeusermann C, Kuster M, Brunner E, Basler K:Coop functions as a corepressor of Pangolin and antagonizes Wingless signaling. Genes & development 2010, 24:881–886.
16. Strigini M, Cohen SM. Wingless gradient formation in the Drosophila wing. Curr Biol. 2000 Mar 23;10(6):293-300.
17. Wu J, Cohen SM. Repression of Teashirt marks the initiation of wing development. Development. 2002 May;129(10):2411-8.
18. Yacine Graba, Denise Aragnol, Ute Rothbächer, and Jacques Pradel. Wnt/Wingless Signaling in Drosophila**. NCBI** resources**,** Bookshelf ID: NBK6231