

RNAi Induced Wing Modification in Leon Mutant *Drosophila* Females: Exploring the Tissue Specificity and Physiological Interference

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Abstract

The precision of growth of an animal is meticulously regulated by extrinsic and intrinsic factors, with focus on maintenance of organismal homeostasis. The clue to change in physiology or metabolism of an organism, at times, can be derived from the changes in phenotypes. In the *Drosophila melanogaster* model system, GAL 4-overexpressed RNAi driver males (Mini-White Marker), targeted against specific genes, when crossed with Leon mutant (19-2/TM6B) females, yield progeny of different wing types. Different RNAi lines expressing the phenotypes in a gradient of sodden, mid to normal, explains the varying severity of the wing phenotypes. The comparison of flies co-expressed RNAi and Leon mutant with wild type or Leon mutant females, show changes in wing venation, anterior cortical vein (acv) position, posterior cortical vein (pcv) position, bristles on the wing margins and the inter-segmental distance. There is a distinct evidence of both rescue and deterioration phenotype observed at various levels, with the varying levels of RNAi expression in sodden, mid and normal type. A correlational study of these modified wing phenotypes to the physiological and metabolic functionalities, reveals the expression of most of these genes targeted by RNAi, mainly in the brain, heart, thoracic-abdominal ganglion, salivary gland, ovary and testis. Therefore, it can be hypothesized that the Leon mutant can be correlated with the RNAi.

Keywords: Leon mutant; Hedgehog; Wing imaginal disc; Crossveins; Segmental distance; RNAi; Gal4 ms-1096

Introduction

In *Drosophila melanogaster*, the finest genetic toolbox and the feasibility of use of balancer chromosomes, makes it viable for use as an efficient model system for study of genetic and molecular mechanisms associated with development. The central importance of body size and shape in evolutionary biology has been further established by distinct external wing phenotypes. The patterning of wing veins, bristles and the shape of the wing, eventually attempt to decipher the molecular and chemical intricacies of sequential development of organism. Leon, the protein of interest is involved in dendritic arborization shortening in neurons and also higher puncta formation, enabling in faster short-range neuron impulse transmission. The wing, being one of the distinctly viewed phenotype, is an ectodermal structure with well-defined compartmentalization of the wing contents and portions into two distinct groups of boundary classifications; one being anterior-posterior and the other being dorsal-ventral [1-4]. The dorsal and ventral epithelial sheets are considered to superimpose on each other, to form what we see as an ectodermal wing blade in its mature form. The mature wing blade is an outcome of fate decision of different group of cells present in the larval imaginal disc. The final outcome however is seen at the end of 4-5 days after pupariation (AP). Several researches have proved the effect of natural variation on wing development, and also the studied the effect of wing patterning in several *Drosophila* mutant lines. Although the aerodynamics associated with the wing blades in insects and smaller organisms stands out to be clearly understood, but the consequential effect of variation on wing morphology is still under research. The precise consensus of these environmental factors and the reception at the organismal level has been consensused by five basic intra-cellular signaling pathways; Hedgehog Signaling (Hh), Bone Morphogenetic Protein (BMP), Epidermal growth factor receptor (Egfr), Wnt and Notch Signaling [5]. Optimizing selection on wing shape in natural populations is a very distinct pattern [6]. The heritability (in higher degrees), specifically in *D. mediopunctata* species, has also proved genetic flow of information for this optimizing selection criteria [7]. Vein cells and intervein cells (found between veins) are the two basic groups of cells in an adult wing

blade determining the venation pattern, with the former being densely packed with higher cuticular pigments than the later one. It is known that the vein cells are the only living cells in a mature wing blade because of the loss of the intervein cells shortly after pupariation (AP). The most common nomenclature of wing blade includes; five longitudinal veins (LVs) ranging from L1- L5 with proximodistal directionality. Two small rudimentary veins, L0 and L6 are found at the proximal end of wing blade. Three Crossveins (CVs) are anterior crossvein (acv) (L3-L4), posterior crossvein (pcv) (L4-L5) and humeral cross vein (L0 and wing margin) [5,8]. The dorsal and ventral differentiation of wings arise out of the differential protrusion of veins on either side of the wing blade w.r.t each other (Figure 1).

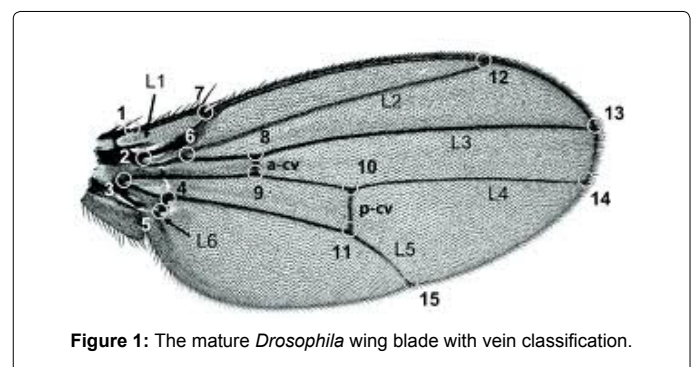


Figure 1: The mature *Drosophila* wing blade with vein classification.

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Evolutionary basis of wing vein morphology clearly suggests the possibility of existence of additional veins in primitive wing or the deletion loss of veins w.r.t what existed earlier [9,10].

Wing development

During embryonic development, 20-30 cells form a cluster, which invaginates from embryonic epithelium with still being adhered to the base with thin stalks. This is the earlier layout of wing development from the wing derived imaginal disc. The differentiation of the initial cluster of cells to a later folded disc epithelium (by the late third instar), is what actually forms the wing blades, hinges, and even the thorax of the fly [5] (initial stages of pupariation, there is an amalgamation of basal and dorsal wing epithelia and the longitudinal proveins forming gap). In contrary to the LV provein pattern in the developmental phase AP, the mature wing shows a narrower vein with fusion of L3 and L4 at the proximal end, forming acv. However, the important question underlying this nomenclature of cells what decides the cell's fate, i.e. either to be a vein cell or an intervein cell, and the time of onset and completion of this process of differentiation. This distinctive process actually starts in the late pupariation stage and continues till 4-5 days AP. This decision of cell after is not resultant of invasive or migratory cells during imaginal disc development [11-13]. The five basic intra-cellular signaling pathways and associated novel components play a pioneer role in positioning of veins in wing blade. These signaling show a predominant wing localized effect. The signaling guided wing venation pattern is classified as: Hedgehog (L3-L4), BMP (L2-L3) (L4-L5), Efg (Provein and Intervein development), Wnt and Notch signaling (L1-L2; L4-L5). The chemical morphogens that involves, the initial LV proveins being broader gets modified consequently, and thus gets refined to the normal narrow veins in the adult wing blade. The LV proveins of the dorsal and ventral side are formed separately on the wing imaginal disc, but in a later time of development, these get aligned to form the gap between the veins on the dorsal and ventral surfaces in a mature wing blade [7]. The developmental signaling of LVs and CVs are roughly same with exception being the precise positioning along the anterior-posterior part and proximo-distal part (Figure 2).

The GAL4 or Upstream Activator Sequence (UAS) is an exquisite genetic tool in GAL-mediated overexpression system for *Drosophila*

for targeted gene expression. The use of GAL overexpression has been most commonly for study of mis-expression. However the stereotype has been added with new dimensions for study of gene of interest via enhancers, cellular autonomy of a gene product analysis and analysis of loss of function phenotypes through targeted use of RNAi and dominant negative constructs [12,14]. ms-1096 GAL4 is a tissue specific GAL4 protein that overexpresses the target protein, and thus helps in studying the protein functionalities. The use of GAL4 driven RNAi flies has shown a temperature dependence mating efficiency with the maturation cycle time period variation. The most ambient temperature for a GAL driven fly is considered to be 28°C, with minimal activity being noted at 16°C. Post translational gene silencing (PTGS) or RNAi (interfering RNA), using siRNA (small interfering RNA) or miRNA (micro RNA), is best suited for studying the function of a particular gene by either knocking down or knocking out a particular gene coding for a specific product. The RNAi microinjected flies targeted against specific genes, in their downstream, manifest their effect in terms of the wing phenotype changes. The presence of the balancers is confirmed by a dominant mutation called Mini White, which coats for red eye color of the fly. Cyo balancers show a distinct curved wing and TM6B causes short-bodied flies with increased number of bristles on the shoulder part. Correlating modified wing phenotypes to the physiological and metabolic functionalities reveals the expression of most of these genes targeted by RNAi, mainly in the brain, heart, thoracic-abdominal ganglion, salivary gland, ovary and testis. Therefore, the clue to change in physiology or metabolism of an organism, at times can be derived from the changes in phenotypes. Even *Drosophila*, may retain some of the information, as the ectopic veins formed after genetic manipulations, can in some cases mimic aspects of the homologs pattern [6,14,15-18].

Materials and Methods

Drosophila stocks

The stocks used for analysis are collected from three stock centers; Vienna *Drosophila* RNAi center (VDRC), Bloomington RNAi *Drosophila* stock center (BL) and NIG-Fly. The stocks are described at <http://flybase.org> unless indicated otherwise. The stocks were also differentially overexpressed with RNAi and the Severity of over expression was arranged chronologically [18-21].

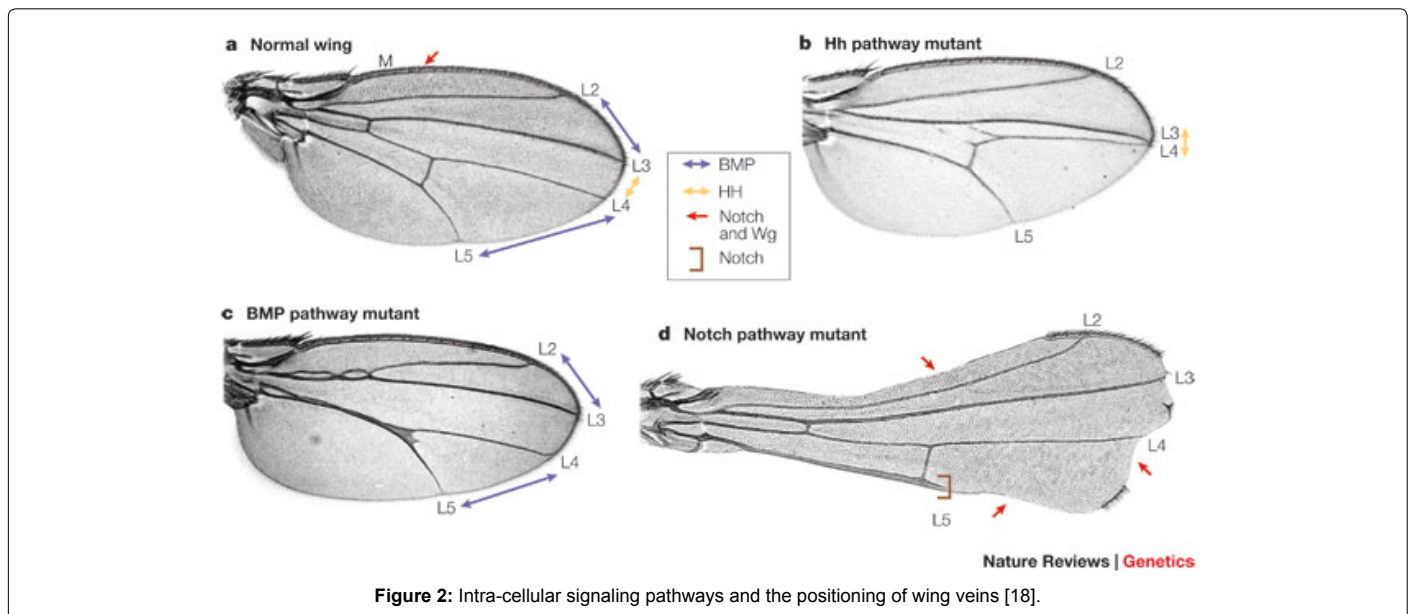


Figure 2: Intra-cellular signaling pathways and the positioning of wing veins [18].

Crossing of flies

The Leon mutant (19-2/TM6B) stocks were incubated at 28°C, and after 10 days of incubation were checked for the emerging pupae. Three virgin females (white-eyed), marked by whitish fat body, with black dot on their dorsal body, were collected and crossed with two RNAi driver male flies (red-eyed due to mini-white marker). The flies were initially looked regularly for checking health, and once the start laying eggs can be collected after ten days. Slides of dissected wings were made with controls as –

- Leon mutant females-W overexpressed by GAL 4 system.
- Leon mutant females-W/O GAL 4 overexpression.

Selection of flies for studying wing phenotypes

After 10 days of crossing, the progeny was collected and screened to eliminate all the male flies (white and red eyed) and female flies (white eyed with balancer phenotype like curved wings for *cyo* and short body with bristles for TM6B). The collected females for final wing dissection were straight-winged, red eyed, less bristles on the shoulder and longer body. Then, the collected flies were kept to freeze at 4°C for a day.

ms-gal4/Y;RNAi / *Cyo* ; +/ +

(Male)

X

+/ + ; +/ + ; Leon mutant 19-2/ TM6B

(Female)

Selected flies-

✓ ***ms-gal4* ; RNAi / + ; Leon mutant 19-2/+**

Rejected Flies:

ms-gal4 ; RNAi / + ; + / TM6B

ms-gal4 ; + / *Cyo* ; Leon mutant 19-2 / +

ms-gal4 ; + / *Cyo* ; + / TM6B

Wing dissection and microscopy

The frozen flies were used to cut the wings from the proximal end, and the dissected wings were collected in an eppendorf with 100 µL of PBST-Triton X buffer and left for mixing on a shaker for an hour. After though washing with PBST buffer, the wings were mounted on slides using 1:1 solution of Hoyer and Lactic acid (around 1-2 drops). The coverslip was fixed with acetone and later filmed under light microscope, and images were analyzed with the control images. The tissue specificity of the gene targeted by RNAi is checked by GFP tagging of the protein with GAL4 (GAL4-GFP-RNAi), which was later analyzed by fluorescence microscopy (Figures 3 and 4).

Parameterization

Each of these parameters is equally likely to occur, and thus given a score of 1. These were used to calculate the severity of the effect, if seen any. The size of the wing hasn't been considered as a parameter with a score of one, because of the fact that the size modifications were not equally likely, and was observed for very few of the fly lines which showed effect.

- Segmental distance between LCVs
- Anterior Cross vein
- Posterior Cross vein

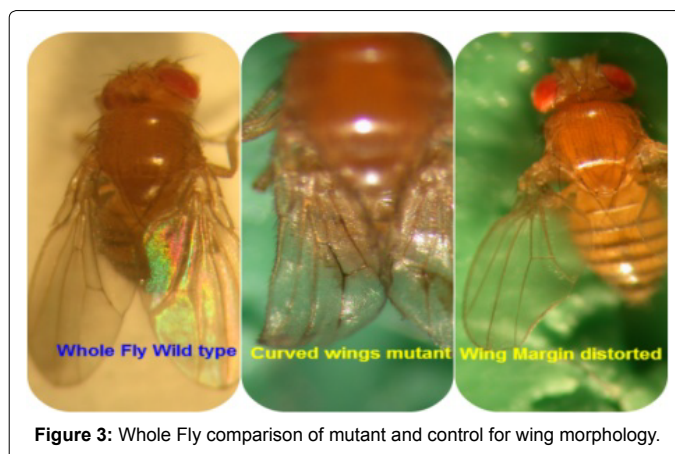


Figure 3: Whole Fly comparison of mutant and control for wing morphology.

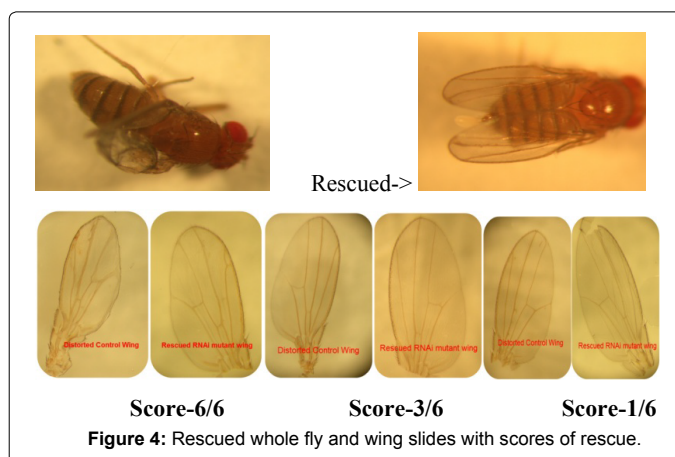


Figure 4: Rescued whole fly and wing slides with scores of rescue.

- Wing notching
- Vein nodes at the proximal and distal ends
- Bristles and serration to form lobe at L5.

Scoring the effect

$$\text{Percentage of Effect (Rescue / Distortion)} = \frac{\text{No of parameters affected}}{\text{Total possible parameters to be affected}} \times 100$$

Results and Discussion

Broadly the wing phenotype modifications can be classified into-

- **Rescue®** - Improved wing architecture in progeny vs. control.
- **Deteriorated (D)** - degraded wing architecture in progeny vs. control.
- **No Effect (N)** - wing architecture same as control.

Rescue®

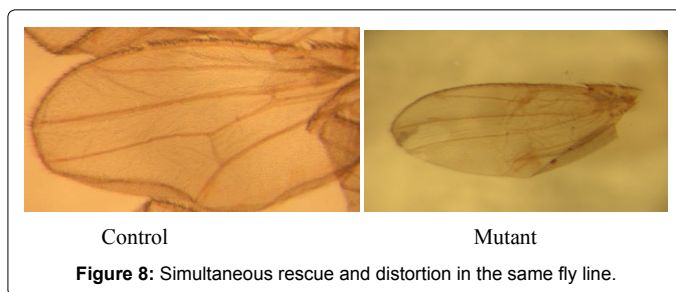
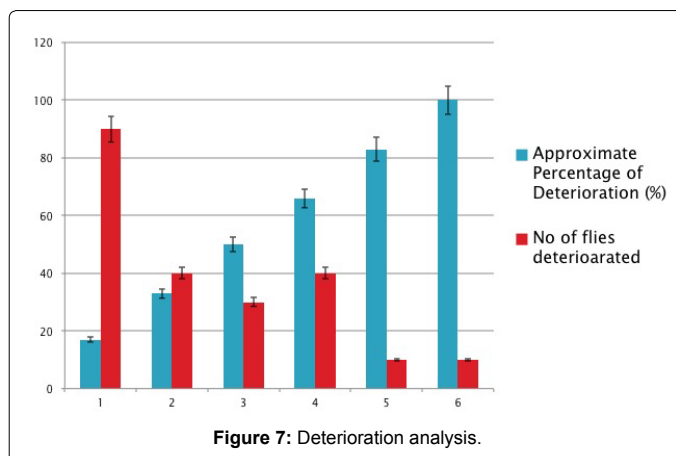
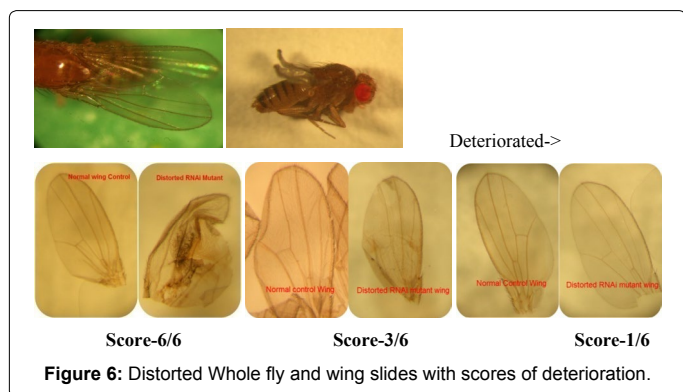
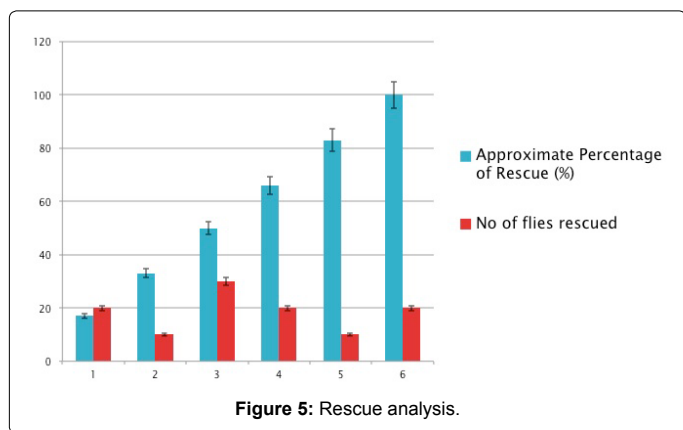
The rescue phenotype is an important analysis study in *Drosophila* genetics, considering the beneficial effects, that can be further extracted of it. However, there is a strict variation in the number of parameters rescued, or in other terms, the relative degree of development of wing blade in the co-expressed mutant in comparison to the control. A score of 6/6 sets out an extreme rescue event, in which all the parameters considered to be expressed, are actually rescued. A 3/6 rescue symbolises 50% rescue and a 1/6 sets out the lower extreme of rescue phenotype, i.e. minimum parameter rescued. The images seen above show the effect on wing margin bristles is a rare effect seen, whereas segmental

distance, acv and pcv are most distinctly effected or rescued parts. For a 3/6 score, acv, L4-L5 segmental distance and lobe formation at L5 is recued, and thus, taking into account three parameters. Similarly, the results for all the fly lines were computed (Figure 5).

The ability to resue the wing patterning features in *Drosophila* is seen to be mostly for a 50% rescue, i.e. a score of 3/6. In general, the fly homeostasis allows a 50% rescue of wing phenotypes. Evolutionarily, this seems top be convincong because of the possibility that the extreme rescue events (score of 5/6 or 6/6) might lead to cross talking of the basic intra-cellular signaling pathways, and thereby, negaqtively affecting fly vaibility. The least favorable rescue is observed to be 5/6 or 83% rescue, meaning the rescue percent 50, is an thershold in deciding teither the fly favors below 50% rescue (considering fly system efficiency). But an efficient fly system can actually favor very high degrees of rescue (score of 5/65 or 6/6). The Rescue percentage is also the differentially driven by the RNAi overexpression levels. The gradient of expression may also effect the percentage of rescue.

Deterioration

The deterioration of wing balde induced due to RNAi, is a very common effect observed with the severity of distortion varying with the fly linesw dependng on the fly system integrity and the RNAi overexpression levels. A 6/6 distortion shows complete loss of wing blade charecteristics features, and thus marks an extreme with a 1/6 being the lower extreme with one parameter loss, generally being the acv/pcv or the segmental distance variation. A 3/6 scored fly line had distorted in terms of acv, L4-L5 segmental loss and the lobe at l5 loss with loss of terminal L5 vein at the distal end. This system was used in classifying the fly lines as per the distortion percentage, a possible answer to the fly machinery efficiency (Figure 6).



The deterioration phenotype is commonly seen for a single parameter loss, i.e score of 1/6. The general loss being, loss of acv/pcv or segmental distance modifications. Considering the evolution signficiance, the distortion percentage mostly lying at 15%, also signifies the sensitivity of the fly machinery to the negative or deleterious effects of mutation on the physiology. The least favorable deterioration is for a 5/6 (83%) or 6/6 (100%) deterioration, confirming the earlier suggested possibility of the stircter sensitivity opf fly machinery to negative changes of the psihology, thereby affecting homoestasis. This distortion possible might have arised out of the difference in RNAi overexpression in the fly lines, but mostly 15% distortion is what lies in the favorable range of fly homeostasis (Figure 7).

Both rescued and deteriorated

A very distinctive effect is the same co expressed fly wing exhibiting both rescue and deterioration phenotypes together. However, the degree of rescue or distortion is different and mostly the fly lines, which showed this dual effect, had deterioration score outweighing the rescue. The possible factor accounting for such a phenotypic expression roots to the sensitivity of the signaling pathways guiding the positioning of wing patterns. The signaling pathways when simultaneously perceive both rescue and distortion the fly machinery favors deterioration of the wing phenotypes, mostly inhibiting the wing vein proteins in such cases, in an effort to maintain homeostasis (Figure 8).

Rescue^o-wing notching: Score-1/6

Distorted (D)-acv completely lost, pcv apparently exists, L5 lost, venation distorted; Score-4/6

RNAi gradient and effects correlation

The varying intensity of RNAi overexpression helps us to cross check whether the RNAi is showing desired effect. For the severely expressed RNAi, cases with no effect theoretically should be minimum and some affect either rescue or distortion should dominate and that is marked in

the analysis results (Figure 9). In the severely expressed RNAi rescue, phenotypes are most commonly seen whereas it is negligibly less in mid and normally expressed RNAi. For mid and normally expressed ones, there is a significant fly line population showing no effect, which

possibly is due to the non specificity of the genes targeted by RNAi, where such genes either show tissue specific expression in reproductive tissues or carcass, and are not linked to the wing development from imaginal disc (Table 1).

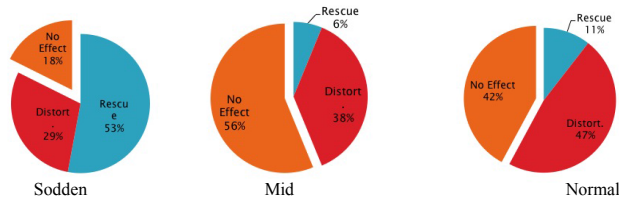
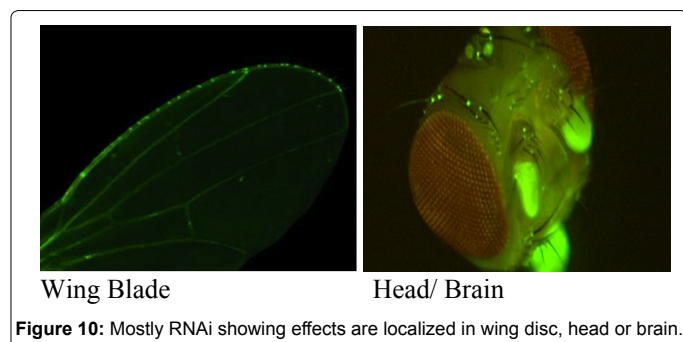


Figure 9: RNAi gradient with effect types.

Sl. No.	Tube Label	Targeted Gene	Related gene & function	Expression in adult Tissue	Observed Phenotype	Physiological/Metabolic Function
1	17260R-1/T6	CG17260	CG17259 (aminoacyl-t-RNA synthetase class II)	Testis and male reproductive tissue	Rescued-Proximal Wing vein and acv restored	Zinc ion binding, Protein features-RWD Domain.
2	106192/CyO	CG5794	at1 (guanyl ribonucleotide binding.)	Brain, Imaginal Disc	Rescued-wing notching lost Distorted-acv, pcv and wing vein lost	Ubiquitin thioesterase activity, regulation of gene silencing, protein ubiquitination.
3	100136/CyO	RhoGAP93B	CG7056 (specific DNA binding Transcription Factor)	Brain and Thoracic Abdominal ganglion	Distorted-acv,L3-L4 distance, proximal vein	Rho-GTPase activity, axon guidance activity, Myth4 domain in proteins.
4	102513/CyO	vito	Scny (Ubiquitin thioesterase)	Female reproductive parts	Distorted-pcv	Positive regulation of cell growth, acts as a nucleoprotein
5	100708/CyO	Src42A	mle (chromatin binding)	CNS, Imaginal DISC	Rescued-acv,pcv,L4,L5,L3-L4 distance widened	Protein tyrosine kinase activity, biogenesis regulator, cellular compartmentalization
6	38998 /CyO	Gint3	Sp2637 (asparagine activity)	Eye, Thoracic abdominal ganglion	Rescued-acv,pcv,vein architecture	Protein binding,PUB domain,PUG domain,UBX domain.
7	108832/CyO	ash1	CG9330(ATPase activity, transporter)	Ovary	Rescued-acv,proximal wing veins of L3 & L4	Histone lysine N-methyl transferase activity,ssRNA binding activity
8	101684/CyO	ru, rho3	---	Brain, Eye, head	Distorted-L2-L3 narrowed at proximal end	Serine-type peptidase activity
9	105883/CyO	Rbp1-like	Tim9a (P-P bond hydrolysis driven protein)	Brain	Distorted-Notching,acv lost,vein distorted	Nucleotide binding, nucleic acid binding,RNA splicing
10	23263 /CyO	DN-Rab14	---	---	Distorted-acv lost,L3-L4 segment widened	---
11	9379R-2/T6	By	mura (zinc ion binding)	Crop and salivary gland	Distorted-Wing venation	Actin binding, wing morphogenesis
12	100809/CyO	Vap-33-1	Lva (actin/microtubule binding)	Brain, Eye, Heart	Distorted-pcv shifted,L4-L5 widened	Structural molecule activity, innate immune response
13	101669/CyO	Hsp27	Hsp 23(hypoxia, cold acclimation)	Malpighian tubules,Ovary	Distorted-L3-L54 widened	Protein binding, defense response
14	11152/CyO	scny	Vito (cell growth regulator)	Brain, eye, head,	Distorted-L4-L5 widened	Ubiquitin thioesterase activity
15	105534/CyO	Rabex-5	Scf(topoisomerase activity)	Salivary gland	Distorted-L4-L5 narrowed	Imaginal disc development
16	5562R-3/CyO	gbb	eIF6 (translation elongation factor)	Salivary gland, male accessory gland	Rescued-acv, pcv, wing vein restored	Transformation growth factor beta receptor binding, neurotransmitter regulator,NMJs growth
17	105946/CyO	e(y)3	MKP4 (phosphatase activity)	Ovary, Eye	Distorted-L5, L4-L5 widened	Chromatin binding, genesilencing, Imaginal disc development
18	108814/CyO	TNF (egr)	CG2269	Eye, brain, heart	Distorted-acv,pcv,L5	Protein binding,innate immunity
19	110618/CyO	MRG15	CG4338	Eye, brain, Ovary	Distorted-L4-L5 widened	Methylated histone binding, chromatin silencing
20	6637R-2/CyO	Isn, Vps22	Eby (spermatid development)	Head, ovary, eye Brain	Distorted-acv,pcv,veins	Apoptosis & notch signalling regulator
21	3998R-1/CyO	zf30C	Taf11 (transcription factor binding)	Brain, Thoracic abdominal ganglion	Rescued-acv	Neuron development,ZINC finger C2H2 domain
22	51977/T6	fng	CG10589	Thoracic abdominal ganglion	Distorted-pcv, L4-L5 widened	Single organism development,biogenesis
23	10079R-1 (II)	EGFR	---	---	Distorted-L4-L5 widened	---

The database of the fly lines showing effect has been tabulated to compare and analyze the possible.

Table 1: RNAi –Leon Co expressed mutant and physiological interference.



Organ/Tissue localization

Upon analysis, the most common tissue specific expression is seen in wing disc and brain or head. The expression was confirmed from the fly base data of fly anatomy of expression at adult stage. The GFP tagged fluorescent expression of these genes targeted showed the most RNAi lines, showing effect to be either expressed in brain and head. The expression in ovary, testis, fat body, salivary gland and thoracic abdominal ganglion were tissues which show relatively less expression in cases of fly showing either rescue or distortion effects. Mostly flies showing a distortion effect were involved in neurological functions and in development of sensory organs (Figure 10).

Conclusion

In general, rescue and deterioration phenotypes were seen in flies where genes targeted by RNAi are expressed at higher percentage, specifically in wing tissues or the imaginal disc (coding for wing development). Most common tissue specific expression in adult fly with change in wing pattern is observed in brain, head, imaginal Disc, thoracic abdominal ganglion and eye. The RNAi gradient of over expression also influenced the phenotypic effect.

- Severely overexpressed RNAi→Predominantly Rescue phenotype.
- Mid overexpressed RNAi→Mostly No effect with few cases of distortion.
- Normally Overexpressed RNAi→Predominantly Distortion

The further analysis of these phenotypic changes can give an idea of the intracellular signaling pathways specifically targeted by these RNAi fly lines co expressed with Leon mutant. Information on position and morphogens are of prime importance in studying developmental biology. In *Drosophila* system, there are several diffusible proteins along the anterior-posterior compartments, leading to the nested expression of certain specific genes, which regulate the wing venation and cell positioning. Still even after 15 years of intense research about wing patterning, the scaled presence of morphogens and fate decision of wing disc cells in response to the five basic intracellular signaling pathways still remains not completely understood. However, the stereotyped use of wing pattern analysis in understanding the possible effect on physiology and developmental sequence still holds to be of significance.

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