CHAPTER 5

GAIN OF FUNCTION STUDIES OF CG32062

Ectopic expression of CG32062
Genetic Analysis
Regulation of knot Expression
Chapter 5

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5.1 INTRODUCTION

While much of advances in *Drosophila* genetics is due to number of ways loss of function studies can be carried out, this wonderful model system offers targeted gene expression or misexpression studies, which are equally informative to understand gene function. Using the targeted gene expression methods, it is possible to activate the gene of interest in a wide variety of tissue-specific and cell-specific ways (Brand and Perrimon, 1993). Ectopic expression or misexpression of a gene either induces a switch in the cell fate or assigns a new fate to the cell.

5.1.1. UAS-GAL4 Modulatory System

In the UAS-GAL4 system, an endogenous promoter (or enhancer) drives expression of the yeast transcriptional activator GAL4, which in turn activates the target gene (Brand and Perrimon, 1993). The GAL4 protein activates transcription of only those genes bearing GAL4 binding sites (Upstream Activation Sequence; UAS). The Promoter/Enhancer directs expression of GAL4 in a particular cell/tissue-specific manner, and GAL4 in turn directs transcription of the UAS-target gene in an identical cell-or tissue specific pattern. The key feature of the GAL4 system is that the GAL4 gene and UAS-target gene are initially separated into two distinct transgenic lines. In the GAL4 line, the activator protein is present, but has no target gene to activate. In UAS line, the target gene is silent in the absence of the activator. When the GAL4 and UAS lines are crossed, the target gene is turned on only in the progeny of the crosses (Fig 5.1).

Thus, a library of GAL4 lines can be built up, each line expressing GAL4 in a different spatiotemporal pattern. The UAS-target gene can then be ectopically expressed in a wide variety of patterns merely by crossing it to the library of GAL4 drivers. In a complementary fashion, a library of UAS-target genes can also be constructed and a large
number of different genes or combinations thereof, can be misexpressed in precisely the same domain by crossing to an appropriate GAL4 driver. Being bipartite system, generating and maintaining transgenic lines of interest is possible and easier.

The GAL4 system can be used to express any gene of interest ectopically, including one that might be lethal to the organism. In the absence of GAL4, the toxic target gene is silent and is activated only in the progeny arising from a cross to a GAL4-expressing line. If a protein is required in a number of developmental processes, or acts at several times in development, its separate roles can be conveniently studied by restricting ectopic expression to specific cells or tissues, or to a particular stage of development.

Fig. 5.1. GAL4/UAS MODULATORY SYSTEM.

P element carrying the GAL4 coding region drives the expression of GAL4 protein in a specific tissue on the basis of proximity of the P element to a tissue-specific enhancer. GAL4 protein (GAL4) then binds to its cognate UAS binding site and activates transcription of the downstream effector gene.

5.1.2. Advantages of Ectopic Gene Expression

Ectopic expression can be advantageous in the following ways (Brand and Perrimon 1993; review by Phelps and Brand, 1998). Ectopic expression gives unique functional
information in cases where no loss-of-function phenotypes are available due to genetic
redundancy (Halder et al., 1995), in addressing a new role of a gene in a cell by altering
the cell fate or altering the spatiotemporal expression domains (van Vactor et al., 1991), in
quantitative analysis of a gene function (Baylies and Bate, 1996) and analyzing different
mode of action of a gene (Manoukian and Krause, 1992). Similarly, it is also possible to
generate dominant phenotypes for a gene to position it within a genetic pathway by
epistasis analysis (Brand and Perrimon, 1994) and produce loss-of-function phenotypes
(rather than gain-of-function phenotypes) by driving the expression of endogenous
products by antagonizing agents (Drain et al, 1991; Griffith et al; 1993; Fitch et al., 1993).

In a classic experiment, Halder et al. (1995) showed that the ectopic expression of eyeless
is sufficient to generate extra eye structures that are not only morphologically normal but
also electrically active on illumination. In a study related to intercellular signaling, Van
Vactor et al., in 1991 used the mis-expression approach to assay whether all the cells that
express the sevenless receptor tyrosine kinase are competent to respond to the ligand Boss.
While Manoukian and Krause (1992) used the same approach to analyze different modes
of action of evenskipped that acts either as a positive or negative regulator of fuzhi-tarazu
transcription at different stages of development. Baylies and Bate (1996) used this
approach to find out the threshold level of a gene expression in a cell by overexpressing the
transcription factor twist within its wildtype mesoderm domain and found that ectopic
muscle is formed at the expense of other mesodermal derivatives.

When loss-of-function alleles for certain genes were not available, this strategy could be
used to analyze the gene function by introducing its inhibitors and antagonists. For
example, Drain et al. (1991) and Griffith et al. (1993) introduced protein kinase inhibitors
to block the endogenous gene function and Fitch et al. (1993) ectopically expressed
pertussis toxin to inactivate G_{i,a} subunit to dissect the gene function. Freeman (1994)
misexpressed Argos, a secreted regulator of cell determination in the developing eye. It
was gain-of-function studies that led to the conclusion that Argos is a soluble secretory
molecule and functions as a repressor of inductive signaling whose threshold level
determines the cell fate.
5.1.3. Wingless- A Morphogen And Wg Signaling Pathway

In holometabolous insects, such as *Drosophila*, the external adult structures like wings, legs are formed from internal larval structures, imaginal discs. These imaginal discs grow during larval stage, evert and differentiate during pupal stages, and finally develop into the body wall and other appendages (Bate and Martinez-Arias, 1991). Interaction between morphogens and other signaling molecules pattern these imaginal discs. Short-range interactions generate a subset of specialized cells called organizers that regulate and pattern the imaginal discs. Organizer, master control cells, in tum potentiates secretion of long-range morphogens, which not only provide positional information but also influence the cell-fate specification of the neighbouring cells (Crick and Lawrence, 1975; Blair, 1995; Brook et al., 1996). Wing discs are patterned along the three major axes, namely, Proximo-Distal (P/D), Anterio-Posterior (A/P) and Dorso-Ventral (D/V) axes.

The selector gene *apterous*, compartmentalizes the wing pouch into dorsal and ventral region by setting up organizing center at the DV boundary, wherein Notch (N) is activated. Consequently along the D/V boundary, Notch activates a morphogen, Wingless (Wg), which diffuses to both sides of the D/V boundary. In the Wg signaling pathway, stabilization of the signal transducer Armadillo (Arm) is the hallmark feature. In the absence of Wg signaling, Arm, recruited by APC to Axin/ GSK3β/Shaggy (Sgg) is further subjected to Ubiquitin-mediated degradation. In contrast, when active Wg binds to its receptor Frizzled (Fz), it recruits Dsh, an antagonist of Sgg, and thereby stabilizes Arm in the cytoplasm (Zecca et al., 1996; Neumann and Cohen, 1997; Winston et al., 1999). Subsequently, target genes like achaete (ac), distaless (dll) and vestigial (vg) are activated by high, moderate and low levels of Wg, in a concentration dependent manner, respectively (Fig.5.2).
Not only do N and Wg activate certain genes, but they also repress certain other genes. They repress the Hh target genes \( kn \) and \( ptc \) along the D/V boundary that becomes the future margin of the wing blade. Thus, along and close to the DV boundary, a fine-tuned regulation of signaling activity is maintained by the N and Wg signaling pathways. Interaction between Wg and Hh signaling has been found in a number of situations like vertebrate limb development, mammalian tooth development, hair follicle development in mice (Tavares et al., 2000; Sarkar et al., 2000; Gat et al., 1998). It is possible that there are common cis-regulatory regions in the transducers of these signaling pathways or there can be common proteins that are potential enough to regulate these signaling cascades.

### 5.1.4. Gain-of-function studies of CG32062

In the previous chapter, it has been observed that loss-of-function of CG32062 cell-autonomously affects intervein specification, a feature of A/P axis. However, its expression pattern is modulated along the D/V axis. To understand its molecular function in more detail, gain-of-function studies, particularly ectopic expression, were carried out on CG32062 using GAL4/UAS system.
5.2. RESULTS

5.2.1. Generation of UAS-CG32062 Transgenic line

The full-length cDNA LD15974 (cloned in pBluescript SK(+/-) plasmid) isolated from 0-
22h embryonic tissue, representing the CG32062-RE isoform, was obtained from BDGP
DGC EST library (1.0 Stapleton et al., 2002). The clone was verified both by restriction
digestion as well as by DNA sequencing using the primers spanning the entire length of the
cDNA. The sequencing result showed 100% homology to the published sequence (FlyBase
ID -FBcl0155624). The full-length cDNA LD15974 is 3457nt-long. This cDNA was sub­
cloned into the Drosophila transformation vector pUAST (Brand and Perrimon, 1993)
downstream to the UAS element between EcoRI and XhoI sites. The plasmid construct
pUAST-CG32062 was end sequenced prior to using the same to generate transgenic flies.
Independent transgenic flies with CG32062 integration on first second and third
chromosomes were obtained. Homozygous viable transgenic lines with second or third
chromosomal insertions were used in further gain-of-function studies.

5.2.2. Ectopic expression of CG32062

To characterize the gain-of-function phenotypes of CG32062, all the individual transgenic
lines were crossed to a GAL4 driver-library present in the laboratory (Table.5.1). Immunostaining of the wing imaginal discs of the progeny of C96-GAL4/UAS-CG32062
using anti-CG32062 antibodies showed an intense staining of the overexpressed protein
along the D/V boundary (Fig.5.4. (C'-C''), suggesting that the transgene indeed mis­
expresses CG32062. It was found that except vg-GAL4, C96-GAL4, MS1096-Gal4 and
sca-GAL4, ectopic expression of CG32062 using all other GAL4 drivers lead to
embryonic or early larval lethality. With MS1096-GAL4 the progenies survived upto early
pupal stages. With vg-GAL4, c96-GAL4 and sca-GAL4 the flies survived upto adult
stages.

Sca-GAL4 drives the expression in sensory organ progenitors (SOPs) of the wing disc. The
generation of these SOPs requires highest levels of Wg signaling, which in turn activates
the activity of the achaete (ac) and scute (sc). Mis-expression of CG32062 using Sca-
Fig. 5.3. Ectopic expression of CG32062 in sensory organ progenitors (SOPs) of the wing disc. (A-C) Adult flies of wildtype (A) and UAS-CG32062RNAi/Sca-GAL4 (B-C). Note loss of microchaetae on the mesothorax in B and C. (D'-D") Wing discs of wildtype (D') and UAS-CG32062RNAi/Sca-GAL4 (D") stained for Ac. Note loss of Ac expression in D".
Fig 5.4 Misexpression of CG32062 along the D/V boundary. (A-B) Adult wing blades of wildtype (A) and UAS-CG32062/vg-GAL4 (B) flies. Note serrted wing margin in B. (C'-C'') Expression pattern of CG32062 in wild type (C') and in UAS-CG32062/vg-GAL4 (C'') wing discs. (D'-D'') Expression pattern of Wg in wild type (D') and in UAS-CG32062/vg-GAL4 (D'') wing discs. At the disc level, ectopic CG32062 caused loss of Wg along the D/V boundary.
GAL4 led to the loss of Ac and Sc expression in the larval wing imaginal discs and adult flies showed loss of microchaete (Fig. 5.3 A-C, D'-D”).

vg-GAL4 or C96-GAL4 is expressed only in the D/V boundary, wherein endogenous CG32062 is absent. When CG32062 was misexpressed using vg-GAL4 or C96-GAL4, adult flies showed serrated wing margins (Fig. 5.4 (A-B)). At the disc level, ectopic CG32062 caused loss of Wg along the D/V boundary (Fig. 5.4(D'-D”)). However, there was no cell death associated with over-expression of CG32062.

Table 5.1.

Summary of Phenotypes induced by overexpression of CG32062

<table>
<thead>
<tr>
<th>DRIVERS</th>
<th>EXPRESSION (wing disc)</th>
<th>PHENOTYPE</th>
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<tbody>
<tr>
<td>omb-GAL4/dpp-GAL4/ptc-GAL4</td>
<td>Anterior-Posterior Axis (A/P axis)</td>
<td>Embryonic /Early larval lethality</td>
</tr>
<tr>
<td>en-GAL4</td>
<td>Posterior compartment</td>
<td>Embryonic /Early larval lethality</td>
</tr>
<tr>
<td>vg-GAL4/c96-GAL4</td>
<td>Dorso-Ventral Axis (D/V axis)</td>
<td>Serrate margined wings</td>
</tr>
<tr>
<td>403-AL4</td>
<td>Non D/V axis</td>
<td>Embryonic /Early larval lethality</td>
</tr>
<tr>
<td>sd-GAL4</td>
<td>Entire Pouch Region</td>
<td>Embryonic /Early larval lethality</td>
</tr>
<tr>
<td>ap-GAL4</td>
<td>Dorsal Pouch and Notum Region</td>
<td>Embryonic /Early larval lethality</td>
</tr>
<tr>
<td>pnr-GAL4</td>
<td>Notum Region</td>
<td>Embryonic /Early larval lethality</td>
</tr>
<tr>
<td>MS/096-GAL4</td>
<td>Dorsal Pouch</td>
<td>Early pupal lethality</td>
</tr>
<tr>
<td>408-GAL4</td>
<td>Leading edge cells</td>
<td>Embryonic /Early larval lethality</td>
</tr>
<tr>
<td>sca-GAL4</td>
<td>Proneural cells</td>
<td>Bristles on the thorax lost, Normal wings</td>
</tr>
<tr>
<td>ubx-GAL4</td>
<td>Peripodial cells</td>
<td>Embryonic /Early larval lethality</td>
</tr>
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<thead>
<tr>
<th>DRIVERS</th>
<th>EXPRESSION (eye disc)</th>
<th>PHENOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ey-GAL4</td>
<td>First and second Instar stages</td>
<td>Embryonic /Early larval lethality</td>
</tr>
<tr>
<td>gmr-GAL4</td>
<td>Morphogenetic furrow</td>
<td>Pupal lethality</td>
</tr>
<tr>
<td>elav-GAL4</td>
<td></td>
<td>Larval lethality</td>
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<table>
<thead>
<tr>
<th>DRIVERS</th>
<th>EXPRESSION (others)</th>
<th>PHENOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>tub-GAL4</td>
<td>Ubiquitous expression</td>
<td>Embryonic /Early larval lethality</td>
</tr>
<tr>
<td>actin-GAL4</td>
<td>Ubiquitous expression</td>
<td>Embryonic /Early larval lethality</td>
</tr>
</tbody>
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5.2.3. CG32062 Functions Upstream of Notch Signaling Pathway

As mentioned above, misexpression of CG32062 led to downregulation of Wg signaling and thereby serrated margin phenotype in adults. Epistasis experiment with different components of Wg signaling pathway was carried out to investigate at what level CG32062 affected Wg signaling pathway. Although Wg expression itself was down regulated, due to paracrine auto-regulation of Wg, it was not clear if CG32062 affected events upstream or downstream of Wg.

Different components of Wg signaling pathway were co-expressed with CG32062 along the D/V boundary. Dishevelled (Dsh) is a positive regulator of Wg signaling pathway. When both Dsh and CG32062 were misexpressed, serrated wing margin phenotype was suppressed (Fig 5.5 (A-C)) suggesting that CG32062 affected events upstream of Dsh in Wg signaling pathway.

Notch is the organizer that turns on Wg along the D/V boundary. Notch$^{intra}$, an activated form of Notch, was co-expressed with CG32062 along the D/V boundary. Both Wg expression in the wing disc and adult margin were restored (Fig 5.5 (D-F)) suggesting that CG32062 might function either upstream or at par with Notch.

5.2.4. Rescue of putative loss-of-function alleles

As described in the previous chapter, the EMS-induced allele R27.1 and the P-insertion P{EPgy2}EY01049 are both homozygous lethal. To rescue the lethality, CG32062 was overexpressed using hs-GAL4 by giving heat shock to the progenies at different stages of development for varying durations. Overexpression of CG32062 was not sufficient to rescue these mutant alleles. Either sufficient amount of right isoform of CG32062 is not expressed or lethality associated with those two alleles is not due to down regulation of CG32062. However, combined expression of UAS-CG32062 and UAS-CG32062$^{RNAi}$ transgenes showed partial suppression of RNAi-induced phenotypes (data not shown).
Fig. 5.5. CG32062 functions upstream of Notch signaling pathway. (A-F) Wing discs of UAS-CG32062/vg-GAL4 (A, D); UAS-Dsh/vg-GAL4 (B), UAS-CG32062; UAS-Dsh/vg-GAL4 (C); UAS-N\textsuperscript{intra}/vg-GAL4 (E) UAS-CG32062; UAS-N\textsuperscript{intra}/vg-GAL4. All discs are stained with anti-Wg antibodies. Over-expression of CG32062 downregulates Wg expression (A and D). Overexpression of Dsh or activated N (B and E respectively) causes increased levels of Wg expression. Co-expression of CG32062 with either Dsh (C) or activated N (F) restores the phenotype and activates Wg suggesting that CG32062 functions upstream or on par with Notch.
5.2.5. Temporal regulation of mis-expression

When the expression of CG32062 was driven using *omb-GAL4* along the A/P axis, the progeny were either embryonic lethal or early first instar larval lethal either at 18°C or 25°C. To compare the effect of over-expression of CG32062 in the same tissue where knock-down of *CG32062* induced phenotypes, strategy to temporally control the gene expression was employed. A temperature-sensitive GAL80 protein (GAL80ts), expressed ubiquitously from the *tubulin-1a* promoter, represses the transcriptional activity of GAL4 at permissive temperature (18°C), while it allows GAL4 activity at non-permissive temperature (30°C).

To regulate the gene expression spatio-temporally, *tubGAL80ts* allele was combined with CG32062. It was crossed to *omb-GAL4* flies, and the progenies were maintained at 18°C for 48 hrs and then shifted to 25°C for its development. The progeny in this case survived up to late pupal lethality (Fig 5.6 A-C). In the pharate adult wings, the width between L3-L4 were found be slightly broader than in the wild type (Fig 5.6D), an effect opposite to the loss-of-function phenotype suggesting that CG32062 is involved the specification of intervein fate.

5.2.6. Titration effect

Another strategy to regulate the gene expression is to titrate or adjust the number of GAL4 proteins binding to the Upstream Activated Sequences of the gene of interest that is overexpressed, by incorporating another non-specific UAS-target gene (for example, reporter gene UAS-GFP). This result in decreased binding of GAL4 to Upstream Activated Sequences (UAS) of the gene of interest and it thereby decreases the levels of over-expression.

When CG32062 expression is driven using *ptc-GAL4* along the A/P boundary, the progeny are embryonic or early larval lethal. When UAS-mcd8GFP was co-expressed along with CG32062, the progeny survived up to pharate adult stage. Adults were unable to eclose out of the pupal cases. When they were removed out of the pupal case forcibly, it was observed
Fig. 5.6 Temporal regulation of ectopic expression of CG32062. The embryonic lethality caused by overexpression of CG32062 driven by *omb*-GAL4 was rescued up to very late pupal stage in the presence of *tub*-GAL80<sup>ts</sup> (A,B,C). In the pharate adult wings, the width between L3-L4 is appeared to be slightly broader than in the wild type (D).

Fig 5.7 Ectopic expression of CG32062 by reducing the GAL4 activity. (A-B) Adult flies of UAS-CG32062/*ptc*-GAL4; UAS-mcd8::GFP. The embryonic lethality caused by overexpression of CG32062 driven by *ptc*-GAL4 was rescued up to adult stage by the co-expression of mcd8::GFP. In the adult wings, the distance between the L3-L4 veins is modulated (A). Legs also show deformations.
that the distance between L3-L4 veins were found to be disturbed (Fig 5.7 A) and the legs were deformed (Fig 5.7B).

5.2.7. Regulation of knot Expression

As mentioned in the previous chapter, knock-down of CG32062 causes reduction in \textit{kn} expression. Conversely, over-expression of CG32062 resulted in increased levels of \textit{kn} expression. Specificity and cell autonomy of this phenotype was evident when CG32062 levels were reduced or increased using \textit{MS1096-GAL4} driver. This GAL4 driver is expressed only in the dorsal compartment of the wing pouch (Fig 5.8(A'-A'')). An increase in \textit{kn} expression was observed only in the dorsal compartment when CG32062 levels was overexpressed using this GAL4 driver (Fig 5.8(B-D)). \textit{kn} expression in the ventral compartment remained unaffected.

5.3. DISCUSSION

Targeted gene expression is a technique complementary to the loss-of function analysis, wherein the levels of gene of interest is increased in the regions where it is already expressed or it is ectopically expressed in regions wherein it is normally not expressed. Using this strategy, role of CG32062 in wing development was analyzed in this study.

UAS-CG32062 transgene was generated by cloning the cDNA LD15974, which encodes the isoform CG32062-RE, which has all the predicted functional domains. Over-expression of CG32062 was early larval (driven by \textit{EN403-, omb-, ap-GAL4}) or early pupal (driven by \textit{MS1096-GAL4}) lethal. Combined expression of UAS-CG32062 and UAS-\textit{CG32062RNAI} transgenes showed partial suppression of RNAi-induced phenotypes. Its conditional expression after 48h of AEL with the help of temperature sensitive GAL80 and \textit{omb-GAL4} resulted in mild enlargement of L4-L5 intervein region. Ectopic expression of CG32062 in D/V cells (using \textit{vg-} or \textit{C96-GAL4} drivers), wherein endogenous gene expression is nil or very low, resulted in serrated wing margin. At the disc level, there was severe reduction in Wg expression. Wg expression was restored when
Fig 5.8 Up-regulation of Knot expression due to ectopic expression of CG32062. (A') Schematic showing the expression pattern of MS1096-GAL4, which is expressed only in the dorsal domain of the wing pouch. MS1096-GAL4/UAS-GFP. (B-D) wing discs of kn-lacZ (B), UAS-CG32062RNAi/MS1096-GAL4; kn-lacZ and UAS-CG32062/MS1096-GAL4; kn-lacZ (D) stained with anti-β-gal antibodies. Ectopic expression of CG32062 in the dorsal compartment of the wing disc upregulates the kn expression only in that domain (D) in comparison to the wild type expression (B). In contrast, loss of CG32062 downregulates kn expression.
CG32062 was co-expressed with Dsh or activated Notch, suggesting that CG32062 function is indeed upstream of Wg and Notch pathways.

Kn is required for the specification of intervein between L3 and L4. It functions by activating Notch pathway and repressing EGFR pathway in the intervein regions. Results of both loss-of-function and gain-of-function studies of CG32062 suggest that CG32062 is upstream of Kn. In the next chapter, the molecular mechanism by which CG32062 regulates kn expression in the presumptive L3-L4 intervein region is investigated.