CHAPTER 6

Role of CG32062 In Vein Development And Wing Patterning

CG32062 in Hh signaling pathway
Genetic and Biochemical interaction between CG32062 and Ci
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6.1. Introduction

Growth and development in a bilaterian animal is controlled by seven major signaling cascades, namely, Wnt, TGFβ, Hh, receptor tyrosine kinase (RTK), nuclear receptor, Jak/STAT, and Notch signaling pathways (Gerhart, 1999). Repeated use of these limited signaling cascades, with defined molecules, generate enormously variant destiny during different developmental contexts.

Generating and sending the information, receiving and responding to the signal clues between cells at the right amount, place and time is the prime mechanism for establishing cell fate determination, development and patterning of an organism. Cell-cell signaling between cells permits to influence each other while taking developmental decisions. Dynamic and complicated interplay between signaling pathways, multiple-level cross talk among molecules and tight feedback regulatory mechanisms occur during differentiation and function. Thus subtle signals are amplified elegantly into specific responses by such interactions (Fig 6.1).

The fly system offers a unique opportunity to study the interaction of all signaling pathways. Hh, Wg and Dpp are the morphogens mediating cell-cell interactions at a distance and Notch and EGFR signaling are two major signaling cascades involved in local cell-cell communications. Positional information generated by the morphogens provides clues for activating local signaling cascades such as Notch and EGFR in a defined way. These potent molecules involve other candidates to fine-tune their activity and thereby knit the circuitry system.
Fig. 6.1. Cell-cell signaling. Interaction between cells influences each other while taking developmental decisions. Dynamic and complicated interplay between signaling pathways, multiple-level cross talk among molecules and tight feedback regulatory mechanisms occur during differentiation and function. Thus, subtle signals are amplified elegantly into specific responses by such interactions.

Source: Neurosurg Focus 2004, American Association of Neurological surgeons
6.2. Major Signaling Pathways

Although all the seven signaling pathways are involved in many diverse roles during organogenesis, those signaling pathways implicated in wing development and vein development are discussed in this chapter (Fig. 6.2). An important criteria during development is that these pathways should not activate their targets randomly in an organism which otherwise would lead to abnormal consequences. Hence a tight regulation of these activation cascades is essential. As a consequence, they involve a number of regulatory proteins within and outside the signaling cascade.

6.2.1. Notch signaling

Notch signaling pathway, one of the most important mechanisms of cell signaling is mediated by Notch, a single-pass transmembrane receptor (Artavanis-Tsakonas et al. 1999; Kadesch 2004). Once ligand proteins of DSL (Delta/Serrate/LAG-2) family, binds to the receptor Notch, it gets proteolytically processed to form an activated C-terminal fragment, Notch intracellular domain (NICD) or Notchintra. This fragment translocates into the nucleus to activate its target genes. In both vertebrates and invertebrates, the CSL (CBF/Suppressor of Hairless/LAG-1) family of proteins are the transcriptional factors that mediate Notch signaling (Lai 2002). In the absence of Notch signaling, the CSL proteins collaborate with a co-repressor complex to repress the gene activation. In contrast, with the influx of active signals, NICD replaces this repressor complex with an activator complex containing Mastermind/LAG-3/SEL-8, thereby activating the gene expression (reviewed by Barolo and Posakony, 2002). A switch off mode of transcriptional repression to switch on mode of transcriptional activation of CSL target gene is mediated by Notch signaling.

6.2.2. Wg signaling

Wg signaling or Wnt signaling is another important signaling cascade that plays a major role in the development of an organism. In the canonical Wg signaling pathway, binding of the ligand Wg/Wnt to its receptor Frizzled (Fz) activates the downstream cascade. In the absence of the Wnt signaling, the negative regulator GSKβ/Shaggy degrades the
Fig. 6.2. Major Signaling pathways in Drosophila. (A) Wingless signaling pathway, (B) Hedgehog signaling pathway, (C) Notch Signaling pathway and (D) EGFR signaling pathway.

Source: (A) Worm Book; (B) Ingham PW, 1998; (C) Le Borgne, 2005; (D) Shilo BZ 2003.
accumulation of β-catenin/armadillo. Activation of Wg signaling recruits a positive regulator Dishevelled (Dsh) that inhibits the GSKβ/Shaggy activity and thereby aids in the stabilization of β-catenin/armadillo. Armadillo goes inside the nucleus to activate the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors. Inside the nucleus, the complex binds to LEF/TCF sites in promoters/enhancers and activates the expression of the target genes (Zecca et al., 1996; Neumann and Cohen, 1997; Winston et al., 1999).

6.2.3. EGFR signaling

One of the complex signaling pathways is the Receptor tyrosine Kinase (RTK) superfamily signaling pathways. There is only one member of epidermal growth factor receptor (EGFR) family in *Drosophila* and *C.elegans*, while there are four members in mammals. The *Drosophila* epidermal growth factor (EGF) receptor (DER/EGFR), has five types of ligands, namely, Spitz, Keren, Gurken, Vein all functioning in different contexts and a common feedback inhibitory ligand Argos. EGFR signaling is involved in the determination of embryonic ventral ectoderm fate, head development, wing and haltere development, notum differentiation, photoreceptor specification and development, induction of dorsal follicle cell fate and so on. The ligands undergo a series of trafficking and processing for tissue specific activation of the pathway. The Ras/Raf/MAP kinase cascade transduces the EGFR activated intracellular signaling, thereby activating the transcription factors such as Pointed. Both positive and negative regulation are involved within this pathway. Argos is the feedback negative inhibitor activated by EGFR signaling, binds to the receptor and inhibits further signaling. Eventually this pathway activates those genes, which have Ets binding site on their promoter/enhancer (Guichard et al., 1999; Freeman 1996; Zecca and Struhl, 2003; Ben-Zion Shilo, 2003).

6.2.4. Hedgehog signaling

Hedgehog signaling is one of the key signaling pathways that impinge on major developmental patterns both in *Drosophila* and vertebrates. In the absence of Hh signaling, the protein Patched (Ptc) inhibits the activity of another transmembrane
serpentine protein Smoothened (Smo). The secretory protein, Hh binds to its receptor Ptc, relieves the inhibition of Smo, by bringing about a conformational change in the latter. Activated Smo interacts intracellularly with a huge multimeric complex containing Cos2/Fused/Su(Fu)/Ci. This activates the signal transducer of Hh, namely Cubitus interruptus (Ci), a member of Gli family of transcriptional factors. In the absence of Hh signaling, Ci is proteolytically cleaved by protein kinase A (PKA) and Slimb, to form a C-terminal truncated fragment (Ci75) that represses the activation of Hh responsive genes. Hh signaling prevents the proteolytic cleavage of Ci and aids in the stabilization of the full-length Ci (Ci155), an activator form. This full-length fragment, Ci155, that functions as a transcriptional activator controls the transcription of Hh responsive genes such as ptc, dpp, en, kn etc. All these genes possess the Ci/Gli-binding sites on their promoter/enhancer region (Alexandre et al. 1996; Dominguez et al. 1996; Aza-Blanc et al. 1997; Wang et al., 1999; Lefers et al., 2001; Methot and Basler 2001; Jia et al., 2002; Lum and Beachy 2004; Sisson et al., 2006).

6.3. Interaction between signaling pathways

Repeated use of signaling molecules and pathways in different contexts of development within the cell requires dynamic and complicated cross-talk amongst them. This necessitates a tight control over the activity of these signaling pathways. Such a control is enforced either by recruiting different molecules outside these pathways or involving negative regulatory proteins within the pathway. In several cases these cascades take up the control over each other, thus leading to either synergistic action or antagonistic action amongst them. This higher level of exchange between signaling pathways make the system still more complicated, making every event of development in the system, a unique process. For example, specification and development of compound eye and growth and patterning of adult wing in Drosophila requires serial inputs from all major signaling pathways - Hedgehog, Wingless (Wg), Decapentaplegic (Dpp), EGFR, and Notch.
6.3.1. Notch-EGFR signaling pathways

A well-studied example of antagonism between Notch and EGFR signaling is the photoreceptor specification. During this event, while the repressor of EGFR signaling, Yan is activated by the Su(H) of Notch pathway, it is negatively regulated by Pointed of EGFR pathway. Direct competition between Pointed and Su(H) for binding to Yan enhancer exemplifies the Notch-EGFR antagonism (Rohrbaugh et al., 2002). Mesothorax bristle patterning is another example of antagonistic function of Notch-EGFR signaling networks. In contrast to the EGFR signaling that promotes macrochaetae development, Notch signaling permits development of many macrochaetae from a single cluster and inhibits additional cells of Proneural cluster to take up the role of SMC (sensory organ mother cell) (Culi et al., 2001).

Another interesting example of Notch-EGFR interaction is the specification of vein and intervein development (also refer to Fig.6.3.). Notch establishes a correct spacing of dorsal and ventral vein components. Asymmetrical distribution of Notch and its ligand, Delta in the intervein and vein region enables vein differentiation right from the late larval stage through the pupal stage. In the early larval stages, EGFR signaling activates the expression of rhomboid in the future vein competent cells. Rhomboid activates Delta expression while Notch represses rho transcription, thus confining Rhomboid to vein region where Notch is not active and thereby separating veins and interveins (de Celis 1997). Notch is also required for maintaining the separation between the vein and adjacent interveins by downregulating the EGFR signaling during the pupal stage (Martín-Blanco et al., 1999).

6.3.2. Hh-Wg signaling pathway

Strong interaction between Wg and Hh signaling occurs during the embryogenesis of Drosophila development. Similarly, Drosophila heart development is an example where Wg signaling functions epistatic to Hh signaling. In the presence of a homeodomain containing protein Tinman, both these networks function synergistically, to produce inductive signal to specify the dorsal midline and heart development (Park et al., 1996).
Fig. 6.3. Model I-Interaction between Notch and EGFR signaling to specify vein and intervein development in the wing imaginal disc. Ligands of both N and EGFR are distributed asymetrically. DI in the vein region and Vn in the intervein region. In the early larval stages, EGFR signaling activates the expression of Rho in the future vein competent cells. Rhomboid activates DI expression while Notch represses Rho transcription, thus confining Rho to vein region, where Notch is not active and thereby separating veins and interveins.
During wing development, Wg signaling down regulates targets of Hh signaling at the intersection of A/P and D/V boundaries (Glise et al., 2002). However, at the level of the activation of growth patterning gene Vg, both Wg and Hh pathways function synergistically (Kim et al., 1996).

6.3.3. Wg-EGFR signaling pathway

Specification of wing and notum fields during the second larval instar are specified by the complementary and mutually exclusive activities of Wingless (Wg) and EGFR signaling pathways (Baonza et al., 2000; Klein, 2001; Wang et al., 2000; Zecca and Struhl, 2002). These two signaling cascades are activated by Wg and Vn, respectively. While Wg is restricted to the subset of anterior distal cells (Ng et al., 1996; Williams et al., 1993), Vn is confined to a central line of proximal cells (Simcox et al., 1996). In contrary, downregulation of both Wg and EGFR signaling are important for the specification of the peripodial cells while Notch and Hh might retain the role in specifying such an identity (Baena-López et al., 2003)

6.3.4. Wg, Dpp and EGFR signaling pathways

Interaction between Wingless (Wg), Decapentaplegic (Dpp) and Drosophila EGF Receptor (DER) signaling during pupal stages are indispensable for the subdivision of adult abdominal segments along the DV axis into dorsal tergite, a ventral sternite and ventro-lateral pleural cuticle. Although Hh signaling activates the expression of Wg and Dpp, while Wg is restricted to sternite and medio-lateral tergite, Dpp is expressed to the pleura and the dorsal midline. Wg and EGFR signaling function synergistically, while Dpp functions antagonistically to specify the cell fates (Kopp et al., 1999).

6.3.5. Hh, Dpp and EGFR signaling pathways

Hh signaling in collaboration with other signaling networks patterns the medial region of the wing disc. Hh signaling activates Dpp in the A/P boundary, but inhibits Dpp signaling in those cells. Hh also activates knot in the A/P boundary cells. In response to Hh signaling, knot activates a repressor of Dpp signaling, namely master of thick veins (Mtv)
to downregulate Dpp signaling. In addition, kn inhibits EGFR activity and promotes Dl transcription to ensure a vein free zone. Thus, Hh signaling interacts with EGFR and Dpp signaling through knot to pattern the central region of the adult wing (Crozatier et al., 2002) (also refer Fig.6.4.).

Vein development is, thus, a classical example where five major signaling pathways N, Wg, EGFR, Hh and Dpp intersect. While the signaling pathways such as Notch, EGFR, Hh and Wg play a major role during the vein initiation stage that occurs in the larval period, Notch, EGFR and Dpp signaling pathways interact during vein differentiation, at the pupal stage. There are few proteins in the system, which interact with all these signaling pathways to specify the desired cell-fate.

Although these signaling pathways are very different in their organization and functional mechanism, the commonness among them lies in their final goal of activating the specific target genes by signal-regulated transcription factors. In major signaling pathways such as N, Wg and Hh respective transcriptional factors, namely, Su(H), TCF and Ci carry out both activation and repression role, thereby functioning as a switch. An interesting theme that has emerged out from a detailed study of these integrated signaling networks is that these potent transcriptional factors are not capable of activating these target genes alone at all, randomly and in inappropriate locations. Specificity of expression at appropriate places is made possible by the presence of certain local specific co-activators of the target gene. Thus this is a strong means of restricting the gene expression and preventing the spurious effects. For example, the Dpax2 gene expression in specification of cone cell development requires, expression of Pointed and Su(H), transcriptional factors of EGFR, and Notch signaling pathways in the presence of a protein, Lozenge (Flores et al., 2000). Such a combinatorial regulation permits the photoreceptor cell to gain competence in the appropriate location.

In the present study as well, it has been found that CG32062 is a protein that functions as a cofactor of Ci, the signal transducer of Hh signaling that regulates the kn expression and
Fig. 6.4. Model-II. Interactions amongst Hh, Notch, EGFR and Dpp signaling pathways to pattern the medial region of the wing. Hh activates Kn, which inhibits both EGFR signaling and Dpp signaling pathways, while it activates Notch and DSRF to specify vein-free zone in the medial region. This also ensures positioning of L3 and L4 veins to the adjacent regions.
thereby integrating Hh, Notch and EGFR signaling pathways to pattern the medial region of the adult wing.

6.4. RESULTS

From the results described in the previous chapters, it was concluded that CG32062 is required to activate intervein-specific genes such as \( kn \), \( DSRF \) and \( dl \) and to repress vein-specific genes \( dpERK1/2 \) and \( argos \). Epistasis experiments suggested that CG32062 is upstream of \( Kn \) and thereby suggesting that it could be a component of Hh pathway, at least in this region of the wing disc.

6.4.1. CG32062 in Hh signaling pathway

First, the effect of modulating CG32062 levels on genes upstream of \( kn \) such as \( Ci \), \( Ptc \) and \( Hh \) was examined. No effect on those genes was observed (Fig 6.5.(A'-A'')) and data not shown for \( Ci \) and \( Ptc \), suggesting that CG32062 may function downstream of or in parallel to \( Ci \).

It was compelling to know whether expression of CG32062 is dependent on Hedgehog signaling or not. To address this question, expression of CG32062 was analyzed in the background of loss of Hh (using \( hh^{15} \)). The \( hh^{15} \) allele is normal (homozygous viable) when maintained at 18 °C, while it behaves as a mutant (homozygous lethal) when shifted to higher temperature. The \( hh^{15} \) flies were maintained at 18°C for 48hrs and then shifted to 25°C. On the mutant larvae, when anti-CG32062 antibody staining was carried out, it was found that the levels CG32062 expression in the non-D/V cells were downregulated (Fig 6.5.(B'-B'')). This suggested that CG32062 is dependent on Hedgehog signaling for its expression.

6.4.2. Activation of CG32062 by Ci

Full-length activator form of \( Ci \) (Ci-155) turns on number of target genes such as \( ptc \), \( wg \), \( dpp \), \( hh \) in different tissues and stages during development. It was interesting to know whether \( Ci \) can also activate CG32062. Ci-155 was ectopically expressed in the dorsal
Fig. 6.5. CG32062 in Hh signaling pathway. (A'-A") hh-lacZ (A') and UAS-CG32062RNAi/omb-GAL4; hh-lacZ wingdiscs stained with anti-β-gal (green) and anti-CG32062 (red) antibodies. The Hh expression pattern is unaltered. (B'-B") ptc-lacZ (B') and ptc-lacZ; hhts/hhts (B") wing discs stained with anti-β-gal (green) and anti-CG32062 (red) antibodies. In the hh mutant background CG32062 is partially reduced, while Ptc expression is completely suppressed.
pouch of the wing disc using MS1096-GAL4, and anti-CG32062 immunostaining was carried out on the wing discs of the progeny. Although there was marginal increase in the intensity of CG32062 expression in the dorsal pouch alone, the results were not conclusive of the possibility of CG32062 being another target of Ci (Fig 6.6 (A'-A'')).

6.4.3. Genetic interaction between CG32062 and Ci

Results described so far suggest that CG32062 may function downstream of or in parallel to Ci. Over-expression of Ci using MS1096-GAL4 driver causes, ectopic expression of kn in the entire dorsal compartment of the wing pouch (Fig. 6.7(A'-A'')). As mentioned in Chapter 4, interestingly, when CG32062 was over-expressed in the entire dorsal compartment, it caused increase in Kn levels only along the A/P axis, wherein 155kDa Ci is stabilized. However, ectopic Ci was able to turn on kn expression in the entire dorsal compartment of the wing pouch. This effect of over-expressed Ci was suppressed, albeit partially, when UAS-Ci was combined with UAS- CG32062RNAi i.e. when Ci was over-expressed in the background of reduced levels of CG32062 (Fig. 6.7 (B'-B'')). These results suggest that although Ci is the prime regulator of kn expression, it is dependent on CG32062 to activate and/or to maintain Kn expression.

6.4.4. Ci and CG32062 are part of a single protein complex

Next, it was addressed whether CG32062 physically interacts with Ci. Immunoprecipitation of CG32062 from wildtype third instar larval wing discs using anti-CG32062 polyclonal antibodies resulted in co-precipitation of full-length (155kDa) Ci (Fig. 6.8(A)). As the anti-Ci antibodies used can detect only 155kDa protein, this experiment did not confirm if CG32062 can bind the 75kDa form of Ci protein. Several attempts to do reverse experiments i.e. using anti-Ci antibodies to immunoprecipitate Ci were failed, probably due to lower efficiency of the used antibodies for such experiments. Therefore, the transgene that expresses full-length Ci tagged with HA was used (Ci-HA; Wang et. al., 1999). Ci-HA was expressed in the wing pouch using MS1096-GAL4 driver. Cell lysate of third instar larval wing discs were subjected to immunoprecipitation using anti-HA monoclonal antibodies or anti-CG32062 polyclonal antibodies.
Fig. 6.6. Activation of CG32062 by Ci. Ectopic expression of Ci in the dorsal compartment of the wing pouch using MS1096-GAL4, probably activates CG32062 too, as shown by anti-CG32062 staining (A”). Wild type pattern of CG32062 (A’).

Fig. 6.7. Genetic interaction between CG32062 and Ci. All discs are stained for kn-lacZ (green) and A” and B” are also stained with DAPI. Ectopic expression of Ci in the dorsal compartment of the wing disc using MS1096-GAL4 activates knot expression in the entire domain (A’-A”). However, when Ci is overexpressed in the background where CG32062 is compromised, such an activation is considerably suppressed (B’-B”). This suggests that though Ci is the prime activator of Knot, it is dependent on CG32062 to activate or maintain knot expression in its domain.
Fig 6.8. CG32062 and Ci are part of a single protein complex. Interaction of CG32062 with Ci by Immunoprecipitation. Immunoprecipitation of CG32062 from wildtype third instar larval wing discs using anti-CG32062 polyclonal antibodies resulted in co-precipitation of full-length (155kDa) Ci (A). Ci-HA was expressed in the wing pouch using MS1096-GAL4 driver. Cell lysate of third instar larval wing discs were subjected to immunoprecipitation using anti-HA monoclonal antibodies or anti-CG32062 polyclonal antibodies. Immunoprecipitation of Ci-HA using anti-HA antibodies was confirmed on Western blot analysis using anti-Ci antibodies (B). Co-precipitation of CG32062 was also observed (C). Immunoprecipitation of CG32062 from Ci-HA expressing wing discs using anti-CG32062 polyclonal antibodies also resulted in co-precipitation of Ci-HA (D). Thus, it is likely that Ci and CG32062 are part of the same protein complex in the third instar wing discs.
Immunoprecipitation of Ci-HA using anti-HA antibodies was confirmed on Western blot analysis using anti-Ci antibodies (Fig. 6.8(B)). Co-precipitation of CG32062 was also observed (Fig. 6.8(C)). Immunoprecipitation of CG32062 from Ci-HA expressing wing discs using anti-CG32062 polyclonal antibodies also resulted in co-precipitation of Ci-HA (Fig. 6.8(D)). Thus, it is likely that Ci and CG32062 are part of the same protein complex in the third instar wing discs.

6.5. DISCUSSION

Mutual activation and repression between Notch and EGFR signaling pathways not only contributes to the generation of distinct cell fates but also reinforce the same. Specification between vein and intervein development is a good example for such an interaction.

Loss of CG32062 leads to fusion of L3 and L4 veins and loss of intervein between these two veins. Consistent with CG32062 functioning upstream of both N and EGFR pathways, markers specific to veins such as dpERK and Argos indicated an upregulated status, intervein specific markers such as Dl, E(Spl)mβ and more specifically dSRF indicated a downregulated status.

Earlier studies indicated that knot activates dSRF to assign intervein specificity. To do so, Knot functions upstream of EGFR signaling as well Notch signaling and from the present study, in agreement with both loss-of and gain-of function studies, it was observed that CG32062 functions upstream to Knot and regulates its expression.

Knot is a target of Hedgehog signaling and Ci regulates kn transcription (Mohler, 2000). Although mild downregulation of CG32062 in hh mutant background suggested that similar to knot, CG32062 is also dependent on Hh. Further experiments suggested that Ci and components upstream to Ci are unaffected by changes in the expression levels of
CG32062. Thus, it is concluded that CG32062 functions either by modifying Ci or together with Ci to specify the medial region of the wing disc.

Interestingly, loss of CG32062 did not affect either dpp or ptc, but only kn expression. Ectopic expression of CG32062 suggested that it can upregulate Knot expression only in the A/P boundary where full-length form of Ci is present, suggesting that CG32062 might collaborate with Ci to regulate kn expression.

As CG32062 possess both RRM type RNA-Binding Domain and polyQ stretches, CG32062 can possibly interact with either RNA molecules or protein molecules. Nuclear localization of CG32062 suggested it could regulate RNA molecules. Two candidates of interest in this study are Ci and Knot. There are two probable ways of regulation of kn by CG32062 at the transcript level: either CG32062 itself binds to kn RNA molecules to regulate its activity or it behaves as a co-factor to Ci, thereby binds to the promoter region of kn to regulate its expression. The first possibility is ruled out as down regulation of kn was observed at the level of its RNA as well as its reporter gene lacZ. As Ci expression itself is unaffected by CG32062, it may interact with Ci at the protein level to regulate kn expression.

Speculations and hypothesis appear to be correct from the immunoprecipitation experiments. Both CG32062 and Ci are found to interact with each other physically. Although there are two forms of Ci, namely 155kDa the activator form and 75 kDa the repressor form, from the present study interaction with 155kDa form is confirmed. Interaction with the activator form suggests that CG32062 might either be important for Ci stabilization, activation or nuclear export or a co-factor to Ci. Although ectopic expression of Ci is sufficient to turn on kn, its specific activity only along the A/P boundary appears to be possible only in the presence of CG32062. Hence it appears that CG2062 might function as a co-factor to Ci, specific to Knot regulation.
All these results collectively suggest that CG32062 is a potent and indispensable candidate in the A/P axis to mediate vein development during wing patterning of *Drosophila melanogaster*.