Studying the role of Decapentaplegic in regulating cell fate alteration in larval imaginal discs of *Drosophila*.

A thesis submitted for the degree of

Doctor of Philosophy

By

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DECLARATION

The work presented in this thesis has been carried out by me under the guidance of Dr. Sudip Mandal at Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, diploma or a fellowship to any other University or Institute. Whenever contributions of others are involved, every effort has been made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Place: Mohali

Date: 24/1/17

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In my capacity as the supervisor of the candidate's PhD thesis work, I certify that the above statements made by the candidate are true to the best of my knowledge.

Place: 24/1/17 ; Mohali .

Date:

Sudip Mandal

(Dr. Sudip Mandal)

[Supervisor's name and signature]

“To my parents…

…who have always loved me

unconditionally and taught me

to never give up ”

Words of thanks

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Aggarwal, P., Gera, J., Mandal, L., Mandal, S. 2016. Employment of Matrix Metalloproteinase-1 by Dpp to regulate Hedgehog activity during ectopic eye formation in *Drosophila melanogaster*.

Synopsis

Morphogens are signaling molecules that play a crucial role in cell fate specification and patterning during development in a concentration dependent manner. Morphogens regulate the expression of their target genes through canonical signaling pathways that are highly conserved across taxa. In spite of having conserved signaling cascades, the downstream target genes regulated by the same concentration of morphogen varies from one cell type to another. This pleotropic response evoked in different cell types is determined by the repertoire of transcription factors available as well as on the epigenetic landscape already set up during the course of development in that particular cell type.

Situation like cell fate alteration where a kind of cell completely gets converted into another cell type, however, poses a unique challenge to morphogen activity. During such conditions, a morphogen might need to activate genes that were otherwise kept epigenetically silent. This demands the removal of epigenetic repression on target genes apart from their transcriptional activation. Another intriguing aspect that needs to be reorganized during change in cell fate specification is the interplay between different morphogens. Cell fate determination in different tissues is established and maintained by the coordinated activities of different short and long-range morphogen signaling. It is intuitively obvious that when cells change their fate, depending upon the altered fate, the individual contribution of different morphogen activity on cell fate choice needs to be modified. The mechanisms underlying these processes are still elusive.

Drosophila larval imaginal discs serve as a system to study cell fate alteration. Although, the cells within the imaginal discs are naturally determined for a specific fate, their fate can be altered during regeneration associated with disc fragmentation and transplantation or through ectopic expression of certain selectors or homeotic genes. Interestingly, irrespective of the nature of transformation, morphogens are known to play an important role in imaginal discs cells undergoing fate alteration. Of the several morphogens studied in this context, Decapentaplegic (Dpp) seems to play a critical role. For instance, it has been demonstrated that induction of wing fate in developing leg discs by ectopic expression of *wingless* (*wg*) gets restricted specifically to the cells in the dorsal part of leg disc with high level of Dpp expression. Even cells undergoing wing to eye transdetermination upon ectopic expression of *eyeless* require high level of

Dpp and Hedgehog (Hh) activity. Furthermore, co-expression of *Dpp* with *eyeless* enhances the domain of ectopic photoreceptor differentiation while attenuation of Dpp signaling in *Mad* loss of function mutants reduces the frequency of leg to wing transdetermination mediated by ectopic *wg* expression.

Objectives:

Since the requirement of high levels of Dpp expression has been implicated with almost all kinds of cell fate alterations in imaginal discs of *Drosophila*, the general perception in the field is that Dpp signaling is critical for transcriptional activation of its target genes by activation of the canonical signaling pathway as it does during normal development. However, I argued that change in fate specification of imaginal discs cells during development is a more complicated process as compared to their normal differentiation. While during normal differentiation, Dpp needs to activate its target genes in the imaginal discs cells whose fate is already determined, during change in cell fate specification Dpp needs to activate a new set of target genes that are hitherto kept silent by epigenetic modifications. Furthermore, it is also essential that the interaction of Dpp signaling with other morphogen signaling pathways gets reorganized. Following this new conjecture, Iexplored for understanding non-canonical role of Dpp beyond its normal function during cell fate alteration in imaginal discs of *Drosophila*.

Outcome of my study:

The morphogen, Decapentaplegic (Dpp), employs a two-tier mechanism to activate its target retinal determining genes during ectopic eye formation in *Drosophila*.

For this study, ectopic eyes were generated by inducing the expression of *eyeless* employing UAS-Gal4 system in the larval wing imaginal discs of *Drosophila*. Two independent Gal4 driver lines *Dpp-Gal4,* that expresses along the anterior/posterior (A/P) boundary of the wing disc and *Ser-Gal4* that expresses majorly in the dorsal compartment of wing disc were used.

Previous studies have established that normal eye development in *Drosophila* is regulated by the coordinated activity of genes such as *eyeless* (*ey*), *sine oculis* (*So*), *eyesabsent* (*eya*) and *dachshund* (*dac*) that constitute the Retinal Determining (RD) gene network. These genes form a complex network regulating each other's expression

by various feedback loops and the proteins even interact with each other. Interestingly, induction of *eyeless* expression activates the genes of the RD network specifically in part of wing disc that expressed Dpp at high levels. Results of *in vivo* genetic analysis further reveals that during ectopic eye induction, along with activating the canonical signaling pathway responsible for transcriptional activation of RD genes, Dpp simultaneously triggers another cascade involving dTak1-mediated activation of JNK to regulate RD genes expression. In this pathway, upon activation JNK down regulates the expression of PcG genes to alleviate PcG mediated repression on its target RD genes in non-retinal tissue creating a condition permissive for change in cell fate specification. Upon attenuating the activities of members of this newly identified pathway, canonical Dpp signaling failed to trigger an optimal level of expression of RD genes which is critical for ectopic photoreceptor differentiation. Importantly, the drop in RD gene expression and subsequent reduction in ectopic photoreceptor differentiation resulting from inactivation of dTak1 get rescued by down regulating the expression of PcG group of genes. Put together, these results unravel a phenomenon in which the morphogen Dpp employs two independent pathways to elicit both instructive and permissive roles to regulate the expression of its target genes during eye formation in non-retinal tissue.

Decapentaplegic (Dpp), triggers dTak1-JNK signaling cascade to modulate the expression of PcG genes during different transdetermination systems in *Drosophila* imaginal discs.

Next, I was interested to know whether the two tier mechanism deployed by Dpp is only specifically involved during ectopic photoreceptor differentiation or is also associated with all other kinds of cell fate alteration. To address this, involvement of the Dpp mediated dTak1-JNK cascade was checked during two other independent transdetermination systems. In one case ectopic photoreceptor differentiation was induced in developing leg discs by ectopic expression of *eyeless* with *Dpp-Gal4*. Alternatively, for eye to wing conversion, *vestigial* (*vg*), one of the master regulators for wing formation, was expressed ectopically in the developing eye disc with *Dpp-Gal4*.

In both cases, change in cell fate specification was observed to be restricted in the domain of Dpp overexpression. Loss of function genetic analysis revealed that in a

manner similar to that observed during wing to eye transdetermination, during leg to eye as well as eye to wing transdetermination, Dpp employs dTak1-JNK signaling cascade to downregulate the activity of PcG proteins. In doing so, Dpp helps on creating a conducive condition that facilitates cell fate alteration.

Activation of Matrix Metalloproteinase-1 by Dpp to regulate Hedgehog activity during ectopic eye formation in developing wing discs

In the next section of the thesis, the role of *Drosophila* Matrix Metalloproteinase-1 was studied during ectopic eye induction in developing wing discs. Matrix metalloproteinase (MMPs) are a class of proteolytic enzymes that were earlier thought to degrade various extracellular matrix components to facilitate cell migration during development. But recent *in vivo* studies in various model organisms have revealed their roles in various other postnatal developmental, physiological and pathological processes.

Genetic analyses and reporter expression studies clearly established that during ectopic eye formation Dpp activates Mmp1 along the A/P boundary of the wing disc towards the posterior compartment that in turn limits the range of Hh signaling to restrict proliferation of the cells undergoing fate change. However, Dpp does not activate Mmp1 by its regular canonical signaling pathway that involves Mad and Med. Rather, it employs an alternate cascade involving dTak1 and JNK to transcriptionally activate the expression of Mmp1.

Attenuation of Mmp1 activity leads to a dramatic increase in the number of ectopic photoreceptors. Cell cycle analyses revealed that this happens due to an increase in the number of proliferating cells expressing Dac that eventually differentiate into ectopic photoreceptors suggesting the role of Mmp1 in controlling proliferation within the Dac expression domain. Analyzing the expression pattern of Cubitus interruptus (Ci), the transcription factor involved in the Hedgehog (Hh) signaling pathway, I found that Mmp1 attains this control over proliferation by restricting the range of Hh signaling. Importantly, the increase in proliferation observed in the Dac domain upon attenuating Mmp1 activity gets significantly rescued when the range of Hh signaling is reduced by generating ectopic eyes in wing discs that are heterozygous for the Hh mutant allele hh^{AC}. Together, these results provide evidence for a very interesting phenomenon where

the morphogen Dpp triggers the expression of matrix metalloproteinase to limit the activity of another morphogen, Hh and in the process brings a balance between proliferation and differentation.

Conclusion:

In this current study I wanted to address some aspects of morphogen activity associated with cell fate alteration that are fascinating but relatively unexplored. My investigation leads to the discovery of a novel signaling pathway induced by the morphogen Decapentaplegic in activating its target genes during change in cell fate specification. In its own merit, this mechanism unravels a novel phenomenon by which a morphogen can elicit both instructive and permissive roles to regulate the expression of its target genes. Furthermore, my thesis throws a new light in our understanding about how Dpp employs matrix metalloproteinase to limit the activity of another morphogen, Hh in establishing the homeostasis between proliferation and differentiation to regulate cellular plasticity. Given the conserved nature of morphogens and their signaling pathways between flies and vertebrates, coupled with the fact that cell fate switching of adult cells in vertebrates can be accomplished by modulating the expression levels of morphogens, it would be intriguing to determine whether morphogens play similar role in cellular plasticity even in higher vertebrates.

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Chapter: 1

Introduction

1.1. Cell fate specification

Development of multicellular organism from a single cell zygote proceeds in a sequential manner. To start with zygote divides to form a ball of cells called the blastula. The process of cell fate specification occurs in the blastula stage where the developmental potential of the cells get restricted. In subsequent stages of development, these cells divide and differentiate into the cells of particular lineage (Slack, 2002). The process of cell fate specification occurs in two steps (Gilbert, 2000).

(1) Specification: This is the first stage of commitment where the cell or tissue become capable of differentiating autonomously when placed in a neutral environment with respect to developmental signals. Cell fate can be reversed at this stage.

(2) Determination: Second stage of commitment, when a cell becomes capable of differentiating autonomously in to its original fate even after placing in totally different environment. Commitment at this stage is irreversible with respect to different developmental signals.

Determination arises as a result of specific stable changes in the expression of genes that is determined by the interplay of different environmental factors or morphogenetic determinants with intrinsic cell specific transcription factors (Goldberg et al., 2007). So, on the basis of different gene actions in different environment, a cell first gets determined to go into a specific lineage and finally under the action of certain genes, it gets differentiated into a specific fate. Therefore, the underlying concept for cell fate specification is the interaction between different sets of determinative factors that include various secreted or transmembrane extrinsic factors present in the cells microenvironment and the intrinsic signaling molecules that act in cell autonomous manner (Edlund and Jessell, 1999).

One of the important mechanism by which an extracellular signaling molecule brings about differential cell fate specification is by creating its concentration gradient. These soluble extracellular determinants that assign positional information to a cell in a concentration dependent manner are termed as morphogens (Wolpert, 1969).

1.2. Morphogen

Morphogens are long-range diffusible signaling molecules that act in a concentration dependent manner to evoke cell specific response in target cells (Fig. 1.1) (Rogers and Schier, 2011; Wolpert, 1969).

They can directly act as transcription factors as observed during early *Drosophila* embryonic development or as secreted molecules that bind to their receptors on cell surface to initiate their signaling cascade. Cells closer to morphogen source receive high concentration of it and express totally different set of genes in comparison to distant cells that receive low concentration of the morphogen (Affolter and Basler, 2007; Ashe and Briscoe, 2006; Rogers and Schier, 2011). In this way, the gradient of a morphogen brings about differential gene expression that forms the basis for patterning. Therefore, position of a cell in a morphogenetic field determines its gene activity and finally cell fate. As a result different types of cell fate patterning emerges in different tissue types during development (Stathopoulos and Iber, 2013; Tabata, 2001).

Major morphogen molecules that are known to play key role in cell fate specification and patterning in *Drosophila* are: Hedgehog (Hh), Decapentaplegic (Dpp), Wingless (Wg), fibroblast growth factor (FGF), Bicoid and Dorsal. Their respective homologs in vertebrates are Sonic hedgehog (SHH), Bone morphogenetic protein (BMP), Wnt and FGF. Hedgehog (Hh), Decapentaplegic (Dpp), fibroblast growth factor (FGF) are secreted by source cells and create concentration gradient across the field of target cellswhile Bicoid and Dorsal function as direct transcription factors (Perrimon et al., 2012).

1.3. Generation of morphogen concentration gradient:

Morphogens are generally released from the source cells and get distributed across the field of cells in a graded manner. Responding cells interpret different concentrations of morphogens in discrete ways by regulating differential gene expression. Morphogen

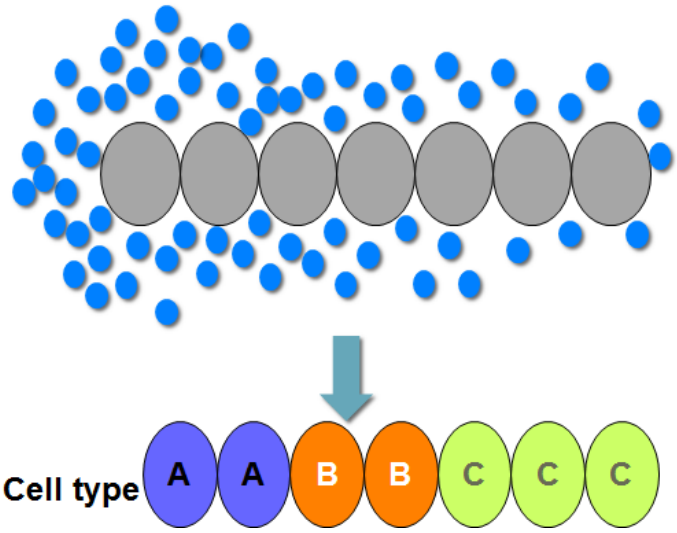

Figure 1.1 Morphogen gradient and pattern formation. Concentration gradient of morphogen molecules (shown in blue) instructs cells to adopt different cell fate (colored cells) depending upon the concentration of morphogen molecules they receive.

gradient across the field of cells is determined by a complex array of various regulatory mechanisms (Lander, 2007). Concentration gradient of a morphogen across a tissue can be generated by the following mechanisms.

1.3.1. Synthesis-Diffusion-Clearence model:

This is a simplest prevailing model for morphogens transport. According to this model, morphogens synthesized by a localized source and travel through a tissue by simple diffusion from higher concentration to lower concentration and get cleared (Rogers and Schier, 2011; Wartlick et al., 2009). Clearance removes morphogen from the morphogenetic field by endocytosis, immobilization or by degradation. Rate of diffusion and clearance determine the range of a morphogen signaling. High diffusivity and low clearance rate determine the longer range of morphogen gradient (Wartlick et al., 2009). Most of the morphogens show exponential decaying curve in the developing tissue that suggest that morhophogen clearance occur throughout the target tissue instead at a localized sink cells situated far away from the source cells.

1.3.2. Morphogen vehicle:

According to this model, morphogens interact with other proteins and lipids to form easily diffusible oligomeric complexes (Rogers and Schier, 2011). For instance, mature, secreted Hh molecules undergo modification by binding of cholesterol and palmitic acid moiety that facilitate their diffusion (Gallet, 2011). Modification of Hh facilitates its long range spread and signaling. Previous studies have suggested that lipidation of Hh and Wg might promote their packaging with lipo-protein particles. Lipidation enhances the interaction between morphogen and proteoglycans and thereby concentrate morphogen ligands at the cell surface (Eaton, 2008). Moreover, the packaging with lipo-protein particles promote their signaling inside the cells as lipo-protein receptor related proteins act as co-receptor for Hh and Wg (Fisher and Howie, 2006).

1.3.3. Extracellular and intracellular components:

Many extracellular factors and intracellular mechanisms also play an important role in defining gradient of a morphogen. Low affinity interactions with Heparan sulfate proteoglycans (HSPGs), components of extracellular matrix are known to modulate morphogens transport and signaling (Yan and Lin, 2009). HSPGs are composed of a

protein core attached with long chains of glycosaminoglcans. They can facilitate morphogen signaling locally by trapping ligands and can also acts as long range carrier of morphogens after cleavage of their protein core (Rogers and Schier, 2011). Therefore, HSPGs can enhance as well as decrease the morphogen diffusion. For example, HSPGs increases more tethering of Dpp to cell surface and as a result triggers more Dpp signaling. HSPGs mutant cells exhibit attenuation of Dpp signaling as compared to HSPGs expressing cells in the vicinity.

HSPGs can also act as co-receptors for morphogens. In *Drosophila*, HSPGs Dally like acts as co-receptor for Hh to facilitate its transcytosis inside the target cells (Gallet et al., 2008). Moreover, HSPGs binding to morphogen not only to restrict movement at the surface of cells but also near the morphogen source cells to limit range of morphogen. In contrast, HSPGs also facilitate long range distribution of some morphogens and might promote stabilization and spreading of morphogens by inhibiting clearance and internalization (Yan and Lin, 2009).

Though, extracellular diffusion play major role in morphogen spread, long filopodia like cellular extensions have also been reported to play important role in morphogen signaling (Kornberg, 2014). For instance, peripheral cells of the imaginal discs in *Drosophila* form actin based processes called cytonemes towards the Dpp producing cells along the A/P boundary (Kornberg, 2014; Roy and Kornberg, 2015; Shilo, 2016). Morphogens having high affinity for cell membranes also travel in the form of membranous vesicles called agrosomes (Strigini and Cohen, 1999). In *Drosophila* male germline stem cells, micro-tubulin based nanotubes are formed by the stem cells that extend in to the niche to make Dpp available for the self renewal of stem cells (Inaba et al., 2015).

Along with extracellular matrix components, intracellular mechanisms like planar transcytosis that includes repeated cycles of endocytosis and resecretion also play important role in morphogen spread in a tissue (Kicheva et al., 2007). Inhibition of endocytosis limits morphogen movement. For instance, clathrin mediated endocytosis participates in the movement of Dpp in wing disc (Gonzalez-Gaitan and Jackle, 1999). In contrast, in some cases, blocking of endocytosis enhances the range of morphogen signaling by lowering clearance rate or altering diffusion (Kicheva et al., 2007).

1.4. Activity gradient of the morphogen:

Though, morphogens are known to act by creating concentration gradient but the concentration gradient is not always directly proportional to the signaling activity of the morphogen in the responder cell. Activity gradient of the morphogen depends upon the following factors:

1.4.1. Distribution and feedback regulation of the receptors:

One important aspect that regulates the activity gradient is the relative abundance of receptors on the target cells. High frequency of receptors on the cell surface limits the distribution of morphogen by trapping it. Moreover, morphogens can also regulate the expression level of receptor to define their own activity either as a short range or long range morphogen (Chen and Struhl, 1996; Lecuit and Cohen, 1998). For instance, in wing imaginal discs in *Drosophila*, Hh activates the expression of its own receptor Patched (Ptc) in the cells adjacent to Hh producing cells along the anterior-posterior (A/P) border and resulting in trapping of more Hh near the source cells. That results in to the formation of short range morphogen gradient for Hh activity (Chen and Struhl, 1996). In contrast, Dpp downregulates the expression of its receptor *tkv* along the A/P border, as a result less ligands trapping happens close to the source and thereby facilitate its long range diffusion (Lecuit and Cohen, 1998).

1.4.2. Level of affinity for binding site:

The other factor that determines the activity gradient of the morphogen is the binding affinity of transcriptional effectors molecules of the signaling cascade triggered by the morphogen to the regulatory sequence of the target gene (Rogers and Schier, 2011). More concentration of a morphogen is required to evoke gene expression from an enhancer having low affinity for its transcriptional activator and vice-versa. For instance, both *Patch* and *Dpp* are Hedgehog target genes. But, Dpp expresses in a broader domain along the A/P axis while Patch expression gets restricted towards the region receiving more Hh in the A/P axis (Parker et al., 2011). Restriction in the expression is because of low affinity of activated Ci (transcriptional activator for Hh signaling) for *Patch* enhancer as compared to *Dpp*. Therefore, Patch activation requires more concentration of activated Ci as compared to *Dpp* and as a result restricted Patch expression only in the cells receiving high Hh.

Even the morphogens that directly act as transcription factors also work in the similar fashion. For instance, Bicoid (Bcd) in *Drosophila* embryo brings about patterning along the anterior-posterior axis on the basis of its differential affinity for different genes (Driever et al., 1989; Struhl et al., 1989). Low affinity for Bcd binding leads to expression of anterior most genes receiving high concentration of it. Conversely, genes expressed in the posterior most part of the embryo are activated by the low concentration of Bcd having high affinity for their regulatory elements.

1.4.3. Feedback regulators:

Morphogen can further regulate the extent of the expression of its target genes by creating a gradient of transcription factors that can include both activators and repressors (Hamaratoglu et al., 2011). For example, morphogen Dpp in wing disc establishes an inverse gradient of its own transcriptional repressor Brinker that is reciprocal to the gradient of its transcriptional effector and thereby sets an expression limit for their target genes. Brinker competes with pMad for their occupancy to Dpp responsive elements in Dpp target genes to bring about their repression (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). In turn, in the cells with high Dpp signaling, Mad and Medea form a complex with other transcription factor schnurri to directly inhibit Brinker (Pyrowolakis et al., 2004). Therefore, along with direct transcription of its target genes *optomotor blind* and *spalt* by Dpp signaling, their expressions also get regulated indirectly by transcriptional repressor Brinker. Interestingly, Dpp also activates another transcription factor *Pentagon* (*Pent*) in the lateral wing disc cells receiving low concentration of Dpp (Vuilleumier et al., 2010). Pent gets secreted out from the cells and interacts with HSPGs Dally to promote long range distribution of Dpp thereby positively regulates Dpp signaling in the cells receiving low Dpp ligands. Similar to *Brinker*, *Pent* transcription is also directly repressed by Dpp signaling in a concentration dependent manner. Therefore, high Dpp signaling negatively regulates Pent while in turn Pent positively regulate Dpp signaling in the cells receiving less concentration of Dpp (Vuilleumier et al., 2011).

1.5. Morphogens role in maginal discs patterning:

Although several tissues in diverse model organisms have been employed to understand the mechanisms underlying morphogen gradient formation and morphogen activity during

development, the larval imaginal discs of *Drosophila* have always been extensively explored for this purpose.

1.5.1. Imaginal discs:

Imaginal discs are sac of epithelial cells present within the larvae that are highly determined to form specific structures of the adult. These disc cells get determined for a specific fate from a very early embryonic stage on the basis of positional signaling cues they receive. During the larval stages, these imaginal disc cells proliferate, resulting in an increase in disc size. *Drosophila* larvae have total nineteen imaginal discs, with 9 bilateral pairs that includes labial, clypeolabial, eye-antennal discs, wing discs, haltere discs, 3 pairs of leg discs and a single genital disc that differentiate in to their respective adult cuticular structures (Fig. 1.2). Though, pattern formation in the majority of imaginal discs start from the larval stages but they mostly undergo differention during the pupal stages. However, in the eye disc, the process of differentiation initiates in the larval stages.

Imaginal discs consist of two layers of epithelial cells known as peripodial and disc proper. As shown in Fig. 1.3 peripodial layer is made up of large squamous epithelial cells and disc proper layer is made up of elongated columnar epithelial cells. Apical surfaces of both the layers face towards the lumen of the sac. Cells in the imaginal discs have apico-basolateral polarity that is very important for their function.

1.5.2. Compartment formation and patterning in imaginal discs by morphogens:

Molecular and genetic analyses of developing larval imaginal discs have established that proper functional coordination among several morphogens that include Decapentaplegic (Dpp), Wingless, Engrailed and Hedgehog is critical for attributing specific identity to different cell types of imaginal disc (Beira and Paro, 2016). Change in expression of any morphogen leads to various developmental defects. Positional identity provided by specific expression of these genes sub divide the imaginal discs into specific compartments. Interestingly, these compartments are separated by sharp boundaries and the cells of one compartment generally do not mix with the cells of other compartments (Baker, 2007).

As shown in Fig. 1.4B and C, in the wing imaginal discs, restricted expression of Engrailed, a homeodomain protein sub-divides the discs into posterior and anterior compartment

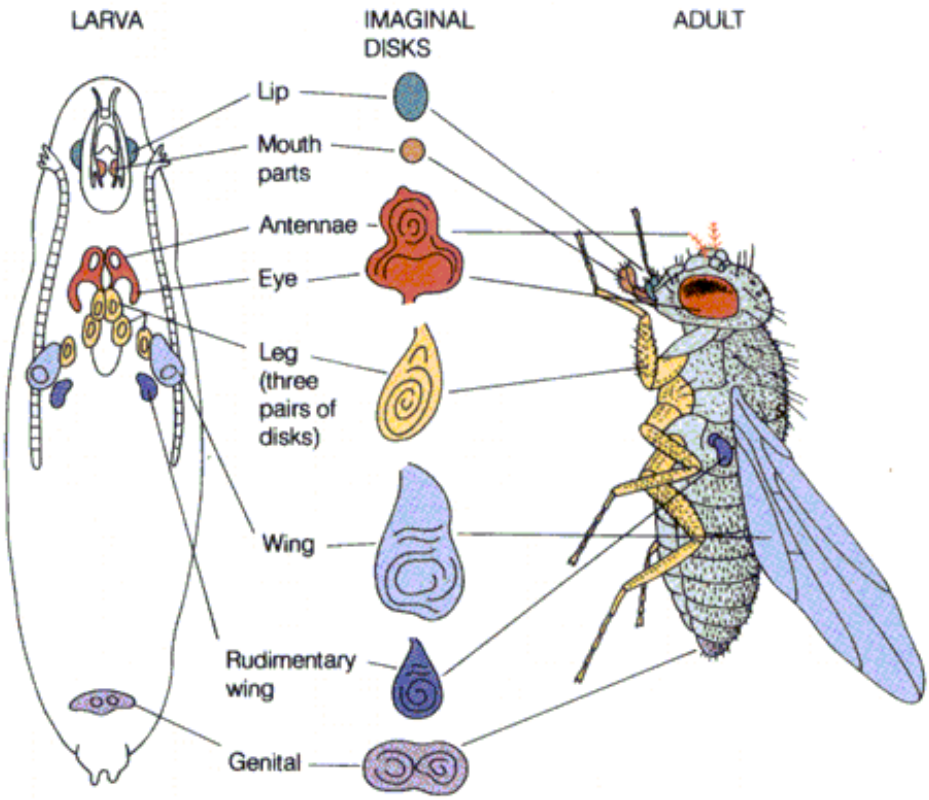

Figure 1.2. Imaginal discs in *Drosophila*. Imaginal discs present during the larval period that differentiate into their respective cuticular structures in adult fly (Mathews/Van Holde/Ahern 3rd Edition).

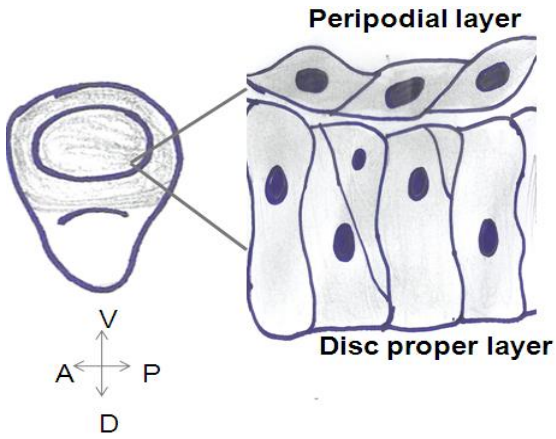

Figure 1.3. Crossection through wing imaginal disc shows two layered epithelium. Imaginal discs are flattened sac cosist of two layers peripodial and disc proper. Directions of anterior (A), posterior (P), dorsal (D), ventral (V) compartments in the wing disc are shown.

(Strigini and Cohen, 1999). Engrailed transcriptionally activates the expression of *hedgehog* (*hh*) in the posterior compartment that maintains sharp boundaries between anterior and posterior compartments. Hh acts as a short-range morphogen and induces the expression of *Dpp* along the anterior compartment cells adjacent to A/P border (Fig. 1.4C, D). Dpp in turn act as a long range morphogen to bring about proper patterning of both the anterior and posterior compartment cells. On the other hand, patterning along dorsal-ventral axis is achieved by another important morphogen Wingless (Wg). Wg is a member of Wnt family in *Drosophila*, act as a long-range morphogen to specify Dorsal/Ventral (D/V) axis (Fig. 1.4E). Notch signaling activates the expression of *wingless* (*wg*) and *vestigial* (*vg*) (Kim et al., 1995; Neumann and Cohen, 1997). Interestingly, Notch brings about proper specification of wing margin by interaction with its both the ligands Serrate and Delta. Serrate expresses in the dorsal compartment while Delta is required as ventral to dorsal signal (Diaz-Benjumea and Cohen, 1995). Dpp and Wg signaling act synergistically to regulate wing differentiation (Tabata and Takei, 2004).

Leg discs as shown in Fig. 1.5 are almost similar to wing discs in terms of compartments specification except that the induction of Dpp by Hh is restricted to the dorsal-anterior cells because in the ventral anterior cells wg represses the transcription of Dpp (Beira and Paro, 2016). Importantly, Dpp and wg transcriptionally represses the expression of each others in developing leg disc in contrast to their co-operative interaction during wing development

However, the developing eye disc is not divided into lineage specific compartments as observed in wing discs. Pattern formation in eye disc is achieved by a wave of morphogenesis that starts from the posterior margin of eye disc and progresses towards the anterior margin (shown by arrow in Fig. 1.5) (Desplan, 1997). The frontal edge of this wave is marked by an indentation termed as morphogenetic furrow (MF). Cells anterior to furrow remains undifferentiated while those posterior to it undergo differentiation. Hh is expressed in the differentiating ommatidial clusters posterior to morphogenetic furrow, and it activates Dpp expression along the morphogenetic furrow in the eye disc (Curtiss and Mlodzik, 2000). Previous studies have clearly established the role of Hh signaling in the initiation of morphogenetic furrow while Dpp plays important role in regulating the rate of furrow progression (Burke and Basler, 1996; Duman-Scheel et al., 2002).

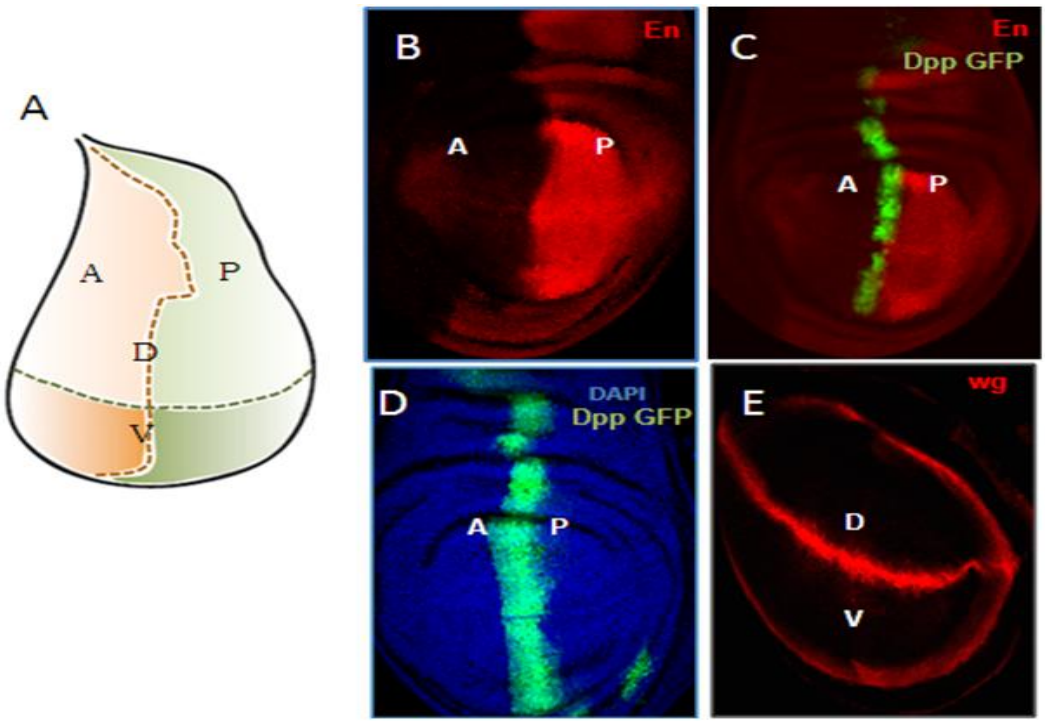

Figure 1.4. Spatial expression of different compartment specific proteins in third instar larval wing imaginal disc. (A) Schematic representation of wing imaginal disc showing different compartments **(B)** En expression in the posterior compartment **(C)** Reporter GFP expression for Dpp and En expression with intact sharp boundaries **(D)** Reporter GFP expression in the Dpp domain along the A/P boundary **(E)** Wg expression along the D/V boundary.

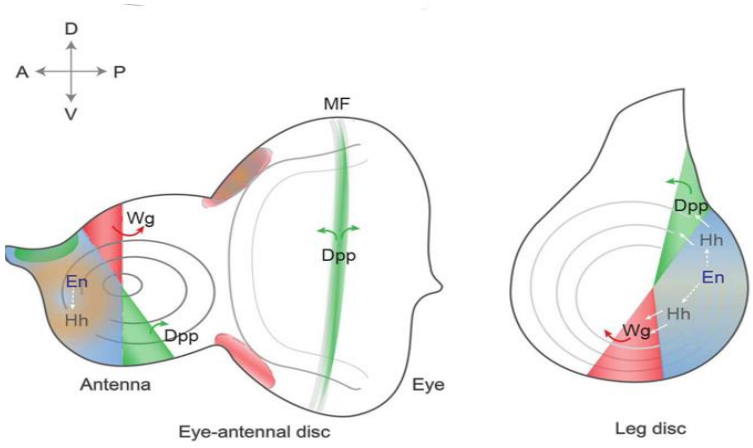

Figure 1.5. Cartoon of *Drosophila* third instar larval imaginal discs showing different compartments and expression of genes. (A) eye-antennal imaginal disc **(B)** leg imaginal disc.Directions of anterior (A), posterior (P), dorsal (D), ventral (V) compartments in the discs are shown (Beira and Paro, 2016).

Though, there is no specific compartmentalization, still morpogen Hh and Dpp interact in a way similar to wing disc. Here also, Hh induces the expression of Dpp along the morphogenetic furrow (Beira and Paro, 2016). While Dpp positively regulates differentiation in eye disc, but Wg signaling negatively regulates eye differentiation. Importantly, Dpp and wg represses the expression of each other in the eye disc and that is different from their interaction in the wing disc.

1.6. Signal transduction by morphogens:

Most of the morphogen molecules act as ligands that bind to their specific receptors present on the membranes of responder cells and activate a signaling cascade by regulating the activities of transcription factors and co-factors and thereby trigger the expression of their downstream target genes. Several studies have documented that the signaling pathway triggered by a morphogens are highly conserved across taxa (Ingham and McMahon, 2001; Weiss et al., 2010).

For my studies, it is important to understand the signaling cascades activated by the morphogen Dpp and Hh in *Drosophila*. Therefore, these signaling pathways are dealt in details.

Dpp, a fly homolog of vertebrate TGF-β is a key morphogen that plays very important role in development and homeostasis of tissues (Hamaratoglu et al., 2014). It regulates patterning of adult appendages and embryo (Neumann and Cohen, 1997). Dpp is a soluble secretory extracellular protein that binds to heteromeric complex of serine-threonine kinase receptor type-I and type-II that are Thickvein (Tkv) and Punt (Put) in *Drosophila*. Binding of Dpp to its receptors phosphorylates and activates its signal transducer Mad. Mad form heteromeric complex with another transcription factor Medea (Med) and goes in to the nucleus to activate Dpp target genes. Therefore, concentration of Dpp gets transduced in to the gradient of phosphorylated Mad (P-Mad) by the activation of its receptors Thickvein and Punt (Fig.1.6) (Hamaratoglu et al., 2014; Shen and Dahmann, 2005). P-Mad is sole signal transducer of Dpp canonical signaling (Sekelsky et al., 1995). Different concentrations of P-Mad activate different Dpp target genes (Kim et al., 1997). Importantly, signal transduction pathway opted by Dpp is conserved with TGF-β signaling cascade. As elucidated by studies in various mammalian cell lines, the signaling cascade is highly conserved as binding of members of TGF-β family activate serine-threonine kinase

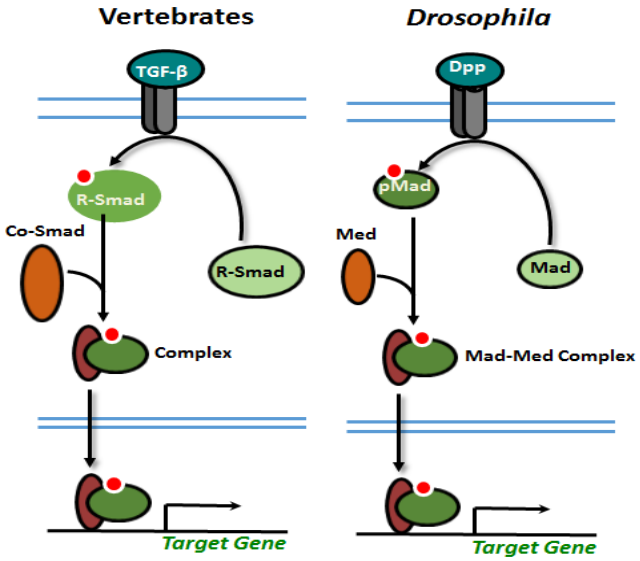

Figure 1.6. TGF-β signaling pathway in vertebrates and *Drosophila* is conserved. TGF-β ligands binding to their receptor activate R-Smad by phosphorylation. Phosphorylated R-Smad form heteromeric complex with Co-Smad and goes in to the nucleus to evoke the expression of target genes. TGF-β signaling work in the similar fashion in *Drosophila* with Dpp as TGF-β and Mad-Medea as R-Smad-Co-Smad homologs.

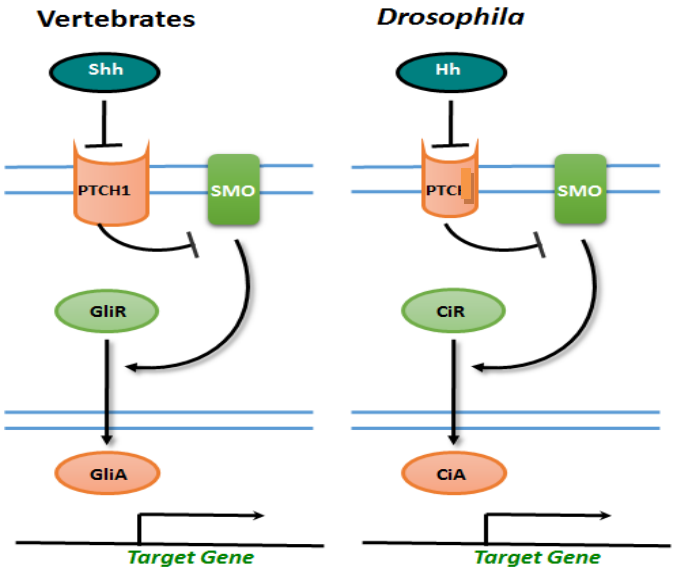

Figure 1.7 Hedgehog signaling pathway in vertebrates and *Drosophila* is conserved. Shh binding to receptor PATCH1 facilitate localization of SMO to the membrane and as a result lead to the activation of its signal transducer Gli. Hh signaling work in the similar fashion in *Drosophila* with PTC and Ci as PATCH1 and Gli homolog respectively.

receptors type-I and type-II that further phosphorylate specific Smad proteins and localization of heteromeric Smad complexes in to the nucleus (Fig.1.6) (Raftery and Sutherland, 1999). Mad belongs to receptors regulated class of proteins Smads or R-Smads while Medea resembles to the Co-Smad class of proteins as their signaling dependent upon R-Smad (Baker and Harland, 1996; Newfeld et al., 1997; Zhang et al., 1996).

The Hedgehog (Hh) signaling pathway was first identified in *Drosophila* by genetic studies and then found to be conserved in vertebrates (Fig. 1.7) (Huangfu and Anderson, 2006; Ingham and McMahon, 2001). Hh binds to its receptor Patched (PTCH) to activate transmembrane protein Smoothened (Smo) (Chen and Struhl, 1996) protein that in-turn activate its signal transducer Cubitus interruptus (Ci). Activated Ci (Ci^{Ac}) goes to the nucleus to activate Hh target genes (Fig. 1.7). In the absence of Hh ligand, Ci undergoes proteolytic degradation to generate a shorter repressive form (Ci^{R}) that transcriptionally represses Hh target genes (Gallet, 2011). Studies in vertebrates have clearly established that the Hh signaling scaffold identified in *Drosophila* is conserved with their counterparts receptor Patch1, transmembrane protein Smo and signal transducer Gli that work in the similar fashion (Fig.1.7) (Ingham and McMahon, 2001).

1.7. Pleotropic response evoked by a morphogen in different tissue types:

Though the canonical signaling pathway of a particular morphogen is highly conserved (Ingham and McMahon, 2001; Weiss et al., 2010) but still it can evoke diverse responses in different cell types. As a result, the same morphogen activity can activate diverse genes expression pattern in cell types of different tissue types. For instance, while Dpp leads to the transcription of *optomotor blind*, *spalt* and *brinker* to pattern the wing imaginal disc (Shen and Dahmann, 2005), along with *eyeless* it regulates the transcription of retinal determination genes *eya*, *dac and so* to regulate eye development in *Drosophila* (Firth and Baker, 2009; Pappu et al., 2005).

Actually, the response of a cell towards a morphogen signaling not only depends upon its concentration of signal received and duration of signal but also influenced by the presence and absence of other transcription factors and cofactors as well as by the status of regulatory region of the target genes. Therefore, the pleotropic response evoked in different cell types is primarily determined by the following factors:

1. Repertoire of transcription factors present in the receiving cells.

2. Epigenetic landscape of the regulatory region of the target genes in the receiving cells.
3. Interaction between different morphogen signalings.

1.7.1. Repertoire of transcription factors available in a particular cell type:

Several studies have clearly explained that along with morphogen mediated extrinsic control, specific cell intrinsic factors play equally important determintal role in regulating specific gene expression for a particular fate (Fig. 1.8) (Grieder et al., 1997; Lee and Frasch, 2005). For instance, in *Drosophila* embryos Dpp signaling alone cannot activate Labial expression in endoderm cells but requires the synergestic binding of Labial and Extradenticle to the labial enhancer (Grieder et al., 1997). Similarly, during embryonic development in Xenopus, Wnt signaling leads to dorsalizing response during early phase while in later phase leads to patterning of lateral and ventral mesoderm without any change in its signal transduction pathway (Hamilton et al., 2001). This differential response of Wnt signaling is determined by the interactions of Wnt pathway nuclear components with tissue specific nuclear factor XTcF3. So, the interaction of ß-catenin with XTcF3 factor determines Wnt signaling mediated dorsal or ventral response. Thereby, intrinsic mechanisms defined by the gamut of cell specific transcription factors are also important to make a cell competent enough for its response to external morphogen signaling.

1.7.2. Epigenetic Landscape:

Along with tissue specific intrinsic factors, epigenetic events also control responsiveness of a cell to a morphogen signaling (Cho et al., 2014; Hirabayashi et al., 2009). Epigenetic memory stabilizes gene expression through a number of cell generations to maintain expression of lineage specific genes (Ringrose and Paro, 2004; Schuettengruber and Cavalli, 2009). Therefore, morphogen signaling can only evoke the expression of its target genes that remain in epigenetically open state in that particular cell type (Fig. 1.9). Thereby, differential epigenetic state determines differential morphogen response in a different cell types and renders certain genes refractory to morphogen signaling. For example, Wnt3a induce osteogenic differentiation only in cells with intrinsic osteogenic potential not in fibroblast or in adipose tissues. Because in non osteogenic tissues Wnt3a remains unable to activate *Bmp2* and osteogenic factor *Alp* due to repression on their promoters posed by epigenetic modifications (Cho et al., 2014). Furthermore, it has been

demonstrated that treatment of pre-adipocytes or fibroblasts with demethylating and histone deacetylase inhibitors makes *Bmp2* and *Alp* responsive to Wnt3a signaling and leads to their transdifferentiation into osteoblasts.

1.7.3. Interactions between different morphogens.

A cell fate is specified by integration of multiple signaling cascades that can act parallel, synergistically or antagonistically with each other. Therefore, integration of differentially activated different morphogens signaling lead to the activation of different set of genes in different cells types (Halfon et al., 2000; Perrimon et al., 2012; Senarath-Yapa et al., 2013; Xia et al., 2010). For instance, differential activation of TGF-β, Wnt, FGF and BMP signaling in neural crest-derived frontal bone and mesoderm-derived parietal bone decide their potential to go into osteogenesis or apoptosis (Fig. 1.10) (Senarath-Yapa et al., 2013). Similarly, during oogenesis in *Drosophila*, Hh signaling brings about degradation of the Tkv, receptor for Dpp signaling that helps in maintaining a steep gradient between germline stem cells and its progeny and thereby activate different genes in the daughter cells (Xia et al., 2010). Therefore, integration of various morphogen signalings work in a tissue specific manner to evoke differential gene response. Thus, combinatorial flexibility at the promoter level is the reason for vast array of responses by small number of signaling pathways during development.

1.8. Cell fate alteration:

Cell fate alteration is a phenomenon of cellular plasticity, where cells deviate from their normal developmental program and get converted into another cell type. It is associated with various natural developmental process as well as various pathophysiological conditions. Cell fate alteration is called transdetermination when a stem or progenitor cell committed for a specific cell fate converted in to another closely related cell type (Maves and Schubiger, 1999; McClure and Schubiger, 2007). Transdetermination is totally different from transdifferentiation, where a already differentiated cells directly switches their fate to another differentiated cell type of different lineage without undergoing intermediate state of de-differentiation (Bjornson et al., 1999; Wagers and Weissman, 2004).

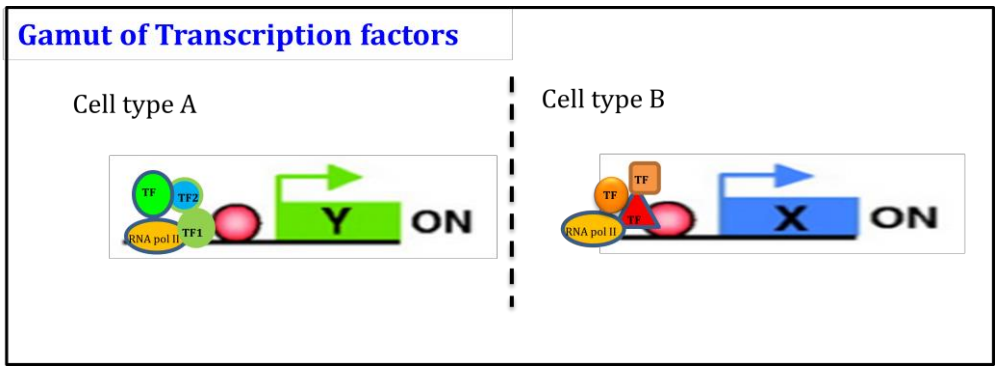

Figure 1.8. Repertoire of transcription factors. transcription factors available in a one cell type are different from another cell type. Interaction of cell specific transcription factors with morphogen signaling determine expression of different genes in different cell type.

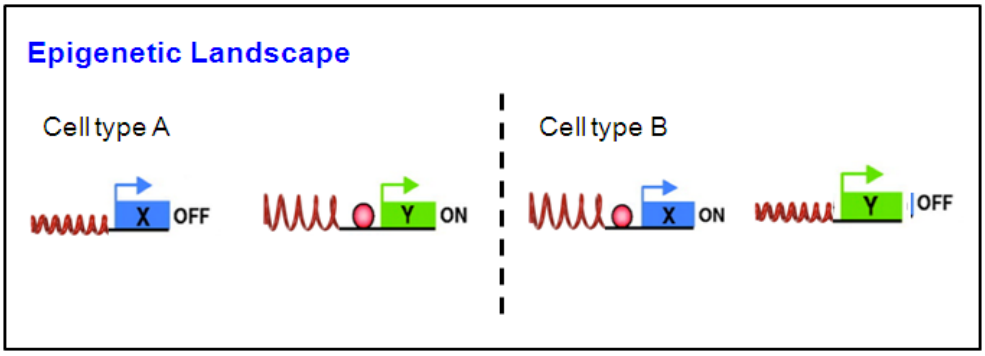

Figure 1.9. Morphogen response in a cell is determined by epigenetic landscspe. Similar morphogen signaling can only activate the expression of gene Y present in open state in cell type A while unable to evoke the expression of similar gene in cell type B where it is in repressed state.

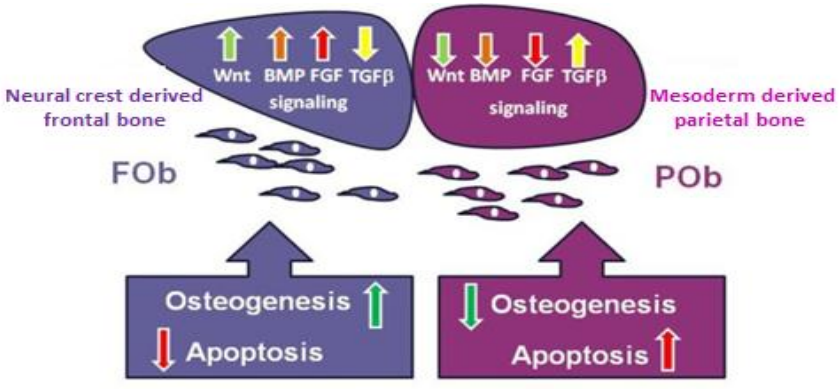

Figure 1.10. Interaction between morphogen signaling. Integration of differentially activated similar morphogen signalings lead to different responses in two different tissue types for example osteogenesis in neural crest-derived frontal bone while apoptosis in mesoderm-derived parietal bone (Senarath-Yapa et al., 2013).

1.8.1. Cell fate alterations during development:

Although very rare, several studies have evidenced the occurrence of cell fate alteration specifically associated with certain conditions like regeneration and wound healing (Echeverri and Tanaka, 2002; Jopling et al., 2011; Suetsugu-Maki et al., 2012). During wound healing and regeneration, epithelial cells surrounding the wound dedifferentiate and proliferate to regenerate the lost structures (Davis et al., 2012; Mann et al., 2007). In segmented decapods, like crab and lobster, it has been shown that while eyestalk or claws normally have the potential to regenerate their lost damaged structure, in some instances, they regenerate in to different structures like antenna. *Drosophila* also demonstrates such kind of plasticity associated with the cells of their imaginal discs. Large bodies of studies have established that in rare instances, cells of the regenerating fragmented imaginal discs can undergo alteration in cell fate specification thereby giving rise to structures not associated with their destined fate (Hadorn, 1968; Schubiger, 1971).

Change in cell fate specification is also seen in marbelled- ballon frog (Mohanty-Hejmadi et al., 1992). It has been observed that ectopic legs can develop in the place of amputated tail of tadpole larvae and this is primarly dependent upon the concentration of available retinoic acid that function as a morphogen molecule. Similarly, iris cells of newt and salamander upon injury can dedifferentiate, proliferate and redifferentiate to form either iris cells or lense cells (Eguchi et al., 1974; Shen et al., 2004). Interestingly, these two cell types (iris cells and lense cells) are strikingly different in their origin, while the iris develops from epithelium and lens develops from neuronal tissue.

Instances of cell fate switching during normal development are also evidenced in mouse. Two interesting examples of such events are: (i) The switch from smooth to skeletal muscles during oesophageal development in mice embryo (Patapoutian et al., 1995) and (ii) transdifferentiation of glomerular epithelium into macrophagic cells during kidney development (Bariety et al., 2003).

Apart from the normal developmental events, change in cell fate specification has also been implicated with some pathophysiological conditions. A well-known example of such an event is in human is Barrett's esophagus. During this process of epithelial transformation, the esophageal cells transdifferentiate in to small intestinal cells (Yu et al., 2005). This situation can lead to neoplasia and then gastro-adenocarcinoma with no

diagnosis. Despite the medical relevance of this and other metaplastic conditions, little is known about the underlying mechanisms.

1.8.2. Cell fate alteration in cultures:

Along with *in vivo* instances there are some *in vitro* examples, where even differentiated cells transdifferentiated in to another cell type. David Tosh observed one of such kind of interesting example in rat pancreatic exocrine cells. These cells got transformed in to hepatocytes under the influence of synthetic harmonedexamethasone that had been given to increase endocrine cells secretion (Tosh et al., 2002). Recently, cell fate alteration has attained a significant applicable platform because of their application in regenerative medicines, where by using a set of transcription factors, fate of differentiated cells can be directly converted in to other cell types. MyoD was discovered by Harold Weintraub in 1989 that can directly convert different kinds of cell lines in to muscle fibers called myotubes (Weintraub et al., 1991). Later on a number of such kinds of transcriptional regulators were discovered like Pdx-1, Ngn etc. that can bring direct reprogramming of hepatic cells to pancreatic cells (Yechoor et al., 2009).

1.9. Transdetermination in *Drosophila* imaginal discs:

Drosophila imaginal discs serve as a wonderful system to study developmental plasticity associated with cell fate alteration. Although, the cells in the imaginal discs are highly determined, but their fate can be easily altered during regeneration associated with disc fragmentation and by ectopic expression of selector or homeotic genes.

1.9.1. Cell fate determination in *Drosophila* imaginal discs.

Previous studies that involves molecular-genetic and transplantation assays have clearly established that the cells of imaginal discs are highly determined for their specific fate (Worley et al., 2012). E. Hadorn along with his colleagues started with some sets of classical transplantation experiments to have an understanding about the determination level of the imaginal discsat different developmental stages of *Drosophila*. They took fragmented imaginal disc cells and transplanted them into the adult fly abdomen as well as in larvae of different developmental stages (Gehring, 1966; Hadorn, 1968). They observed that fragmented disc cells transplanted into younger larvae were only able to regenerate their respective structures. On the other hand, the disc cells remained proliferating in the

abdomen but were able to differentiate into their assigned specific structures when transplanted back into developing larvae. *In vivo* cultures of bisected wing and leg discs fragments resulted into the regeneration of the missing part of the large disc fragment where as smaller disc fragments regenerated mirror image of itself (Hadorn, 1968; Hadorn et al., 1970; Ursprung and Hadorn, 1962).

Further to investigate the determination level of imaginal discs, they isolated the cultured discs cells from the adult fly host and again re-cultured them into new adult fly host. By this method, they were able to maintain disc culture for around 300 transfer of generation for 12 years and found that disc cells remain undifferentiated and proliferating without any limit (Gehring, 1966; Hadorn, 1968). Interestingly, these subculture disc cells differentiated in to their destined fate upon transplantation in to larvae even after a number of transfers of generations.

1.9.2. Transdetermination in regenerating imaginal discs of *Drosophila*:

In very rare instances, however after a number of generations, these cultured discs cells switched their fate and differentiated into adult structures derived from other imaginal discs. These imaginal discs implants generated both autotypic (specified inherent fate) and allotypic (other imaginal discs fate) structures (Hadorn, 1968). Interestingly, allotypic structures were present in the proliferating regenerating parts of the implanted discs tissues. Hadorn termed this phenomenon as transdetermination. Therefore, they concluded that in *Drosophila melanogaster*, larval imaginal discs that are anlagen for specific adult cuticular structure change their fate by transdetermination under specific conditions (damage and regeneration) through various unknown molecular mechanisms and factors. From their analyses on prolonged discs subcultures, they revealed that ceratin types of transdetermination events were more frequent than others and also only a few transformations were reversible. They also concluded that transdetermination events occur in a specific sequence (McClure and Schubiger, 2007). As shown in (Fig. 1.11) genital disc cells can switch to leg or antenna but the transformation in opposite direction was never observed. In contrast, the switch from leg to antennae and antennae to leg occur with similar frequency. Interestingly, few imaginal discs cells have more potential to undergo transdetermination than other cells upon fragmentation known as weak point.

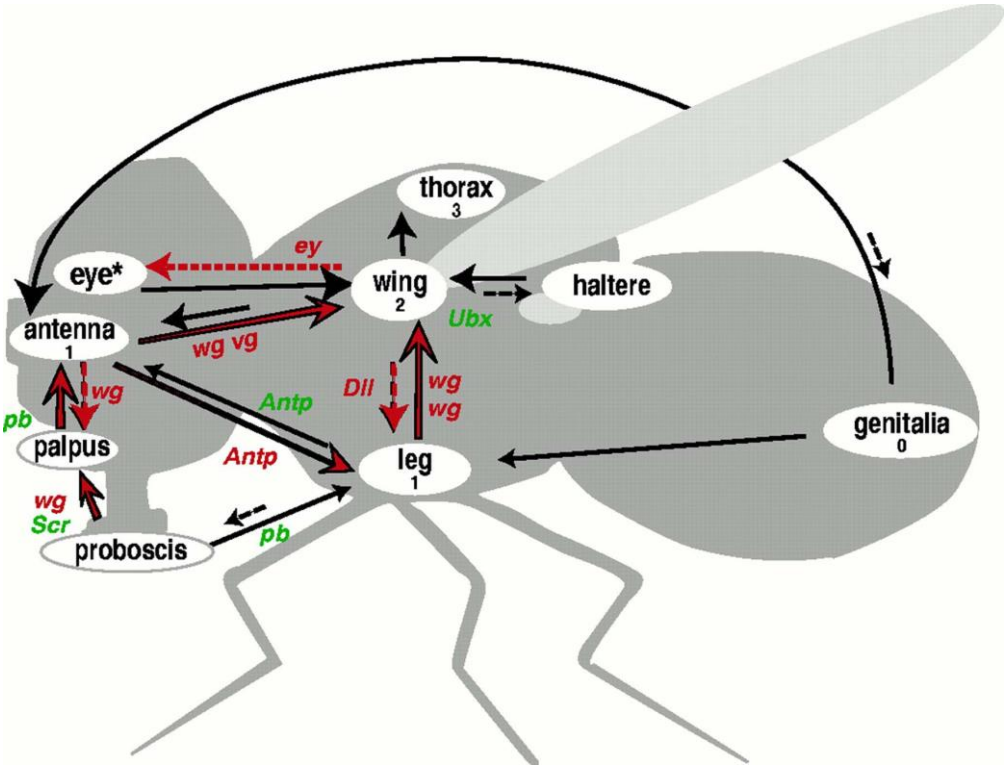

Figure 1.11. Transdetermination events in Drosophila imaginal discs. Transformations occur upon fragmentation and regeneration are shown in black. Length of arrow between two structures determine frequency of transdetermination. Dotted line depicts infrequent transformations. Homeotic alterations correspond to observed transdetrmination events are shown by green letters. Red line shows the transformations that can be carried out by genetic manipulations or upon fragmentation and regeneration (Wei et al., 2000).

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Later on molecular analyses revealed that these weak points correspond to high morphogens activity (Maves and Schubiger, 1995).

1.9. 3. Transdetermination by ectopic gene expression in *Drosophila*

Interestingly, careful observation of transdetermination events mentioned above revealed that many of these events bear a resemblance to homeotic mutations. For instance, transdetermination from antennae to leg resembles *Antennapedia* mutation and haltere to wing resembles *Ultrabithorax* (Ubx) mutation (Morata and Lawrence, 1977). Moreover, ectopic expression of *Antennapedia* can bring about leg to antennae transformation (Schneuwly et al., 1987) while ectopic expression of Ubx induces haltere structure at the place of wing (Lindsley et al., 1972).

Apart from homeotic genes, in several instances, ectopic expression of various selector genes such as *eyeless*, *wingless* and *vestigial* can lead to the formation of heterologus structures in other tissues (Halder et al., 1995; Kim et al., 1996; Maves and Schubiger, 1998, 1999, 2003). It has been evidenced that ectopic expression of *wingless* in leg disc can lead to leg to wing transdetermination in a fashion that resembles the transdetermination process associated with fragmented leg discs (Maves and Schubiger, 1995). Wg is member of Wnt family of signaling molecules that play essential role in the proper patterning of imaginal discs in *Drosophila*. Similarly, ectopic expression of another selector gene *vestigial* (*vg*) triggers ectopic wing generation on leg, antennae and eye imaginal discs. The gene *vg* encodes a nuclear protein majorly involved in regulating growth and differentiation of wing tissue during normal as well as ectopic wing development (Kim et al., 1996; Williams et al., 1991). Another interesting example of transdetermination is the formation of ectopic eyes, where in ectopic eyes can be generated on different body parts other than their normal position by targeted expression of *eyeless* gene in *Drosophila* (Halder et al., 1995). The *Drosophila* gene *eyeless* (*ey*) is homologous to the mouse *Small eye* (*Pax-6*) gene and to the *Aniridia* gene in humans (Halder et al., 1995). By targeted expression of *ey* complementary DNA in various imaginal disc primordia of *Drosophila*, ectopic eye structures were induced on the wings, the legs and on the antennae. The ectopic eyes appeared morphologically normal and consisted of groups of fully differentiated ommatidia with a complete set of photoreceptor cells. Apart from *eyeless*, targeted expression of other early eye determining genes, such as *dachshund (dac)* and *eyes absent (eya)* also resulted in the generation of ectopic eyes in various tissues

including antennae, thorax and legs (Bonini et al., 1997; Salzer and Kumar, 2010; Shen and Mardon, 1997)

1.10. Morphogens and cell fate transformations:

Association of morphogen activity is not only restricted to normal development and differentiation but has also been implicated to cell fate alteration as observed during wound healing and regeneration (Davis et al., 2012; Mann et al., 2007). In general, it has been observed that change in cell fate specification during these processes happen predominantly in the zones of high morphogen activity suggest the involvement of specific role of morphogen to regulate the fate alteration. For instance, it has been demonstrated that cells undergoing leg to wing conversion in surgically fragmented leg discs of *Drosophila* express high levels of Wingless (Wg) (Maves and Schubiger, 1999) that acts synergistically with higher levels of Dpp for proper transdetermination (Ing et al., 2013; Maves and Schubiger, 1998). Dpp is observed to be a mandatory requisite for leg to wing transdetermination because only the wingless expressing cells that were having higher expression of Dpp were able to change their fate in to wing (Ing et al., 2013; Kango-Singh et al., 2003; Maves and Schubiger, 1998). Dpp and Hh are also known to play important role during ectopic photoreceptor differentiation (Kango-Singh et al., 2003).

Ectopic expression of morphogen can also lead to alteration of cell fate specification in *Drosophila*. It has been documented that ectopic expression of wingless (Wnt homolog in *Drosophila*) in to leg imaginal disc cells activates the expression of wing marker Vestigial (Vg) and convert them into wing cells (Maves and Schubiger, 1995). Involvement of morphogen in cell fate alteration is not only restricted to the flies but has also been documented in higher vertebrates.

In a series of elegant experiments, Okubo and Hogan had exhibited that hyperactive Wnt signaling has the capacity to converts early lung progenitor cells of mice into secretory intestinal cells (Okubo and Hogan, 2004). The identity of the transformed cell types was confirmed by checking the expression of intestinal specific genes *Atoh1* that got triggered by ectopic Wnt signaling (Okubo and Hogan, 2004). Likewise, retionic acid facilitates conversion of regenerating tail into limbs in case of marbled-balloon frog (Mohanty-Hejmadi et al., 1992). Increase in the duration and concentration of retinoic acid treatment increased the number of limbs regenerated.

Although the above evidences highlight the requirement of morphogen in cell fate alteration, but our understanding of the mechanistic basis is very rudimentary. It is generally perceived that similar to their role during normal development, high levels of morphogen activity is required to turn on their target genes overriding the normal developmental program.Very little has been explored to determine their role in such other processes apart from their role in activating the normal canonical signaling during normal cell fate specification.

1.11. Objectives of present work:

The fundamental question that prompted me to initiate this project was to know whether morphogen play any role, other than their normal function, in activating their target genes during change in cell fate specification. As shown in schematic representation in Fig. 1.12, let us assume that there are two determined cell types A and B. The genes *a, c, f, g* and *m, q, r, s* are all potential targets of a specific morphogen signaling pathway. However, due to presence of specific transcription factors and different epigenetic landscape of the genome, the same morphogen signaling yields differential responses in these two determined cell types. While it turns on genes *a, c, f, g* in cell type A, in cell type B genes *m, q, r, s* get activated. In this situation, if we change the fate of cell type A to that cell type B then, it poses a unique challenge to morphogen activity. During this altered scenario while the expression of genes *a, c,* f and *g* needs to be shut down, the genes *m, q, r* and *s* need to be activated. Activation of new set of target genes *m, q, r* and *s* demands the withdrawl of epigenetic repression that keeps them silent. Moreover, the manner in which a particular morphogen interacting with another morphogen signaling in cell type A might need to be rewired as the fate changes to cell type B. I was rather intrigued to study the complex role of a morphogen in this process.

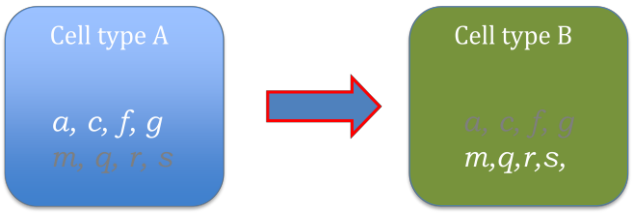

Fig. 1.12 Model showing change in the pattern of gene expression during cell fate alteration. Conversion of cell type A to cell type B requires repression of already expressing genes *a, c, f, g* and activation of repressed genes *m, q, r, s*.

The specific questions that I asked for my study are:

1. Whether any morphogen signaling play any role in withdrawing the epigenetic repression on its target genes during cell fate alteration?
2. If so, then how is this processes coordinated with the known role played by the morphogen in transcriptional activation of the target genes?
3. How the interactions between different morphogen signaling get rewired during the process of cell fate alteration?

Chapter 2.

Materials and Methods

2.1. Fly culture

The flies were reared on standard corn meal food containing maize powder and dextrose as carbohydrates source, yeast as protein source, propionic acid and methyl paraben as preservative and antifungal agent respectively and agar for solidification. All fly stocks were grown at 25°C (if not otherwise mentioned) in standard bottles/vials.

2.2. Fly Stocks and Genotypes:

The different stocks that were used for the study are as follow.

1. ***w^{1118}***: These flies carry white eye mutation on first chromosome and were used as experimental control.

2.2.1. GAL4 driver lines:

1. ***w^{1118}; wg^{Sp-1}/Cyo; P{Gal4-dpp.blk1}40C.6/TM6B,Tb^{1}*** (Treisman and Rubin, 1995): This transgenic line has *Gal4* insertion downstream to *decapentaplegic* (*dpp*) enhancer. The cloned promoter drives Gal4 expression along the anterior-posterior axis of wing disc. This transgenic insertion is on chromosome 3. This fly line is not homozygous viable thereby balanced by Tubby balancer. This stock was obtained from Bloomington Stock Centre (1553).
2. ***w^{1118}; P{Ser-Gal4.GF}1 P{Ser-Gal4.GF}2*** (Fleming et al., 1997; Hukriede et al., 1997): This transgenic line expresses Gal4 under control of *Serrate* (*Ser*) promoter. The *serrate* promoter drives Gal4 expression primarily in dorsal compartment of wing imaginal disc. This fly line is homozygous viable. This transgenic insertion is on second chromosome. This stock was obtained from Bloomington Stock Centre (6791).

2.2.2. UAS responder lines:

1. ***y^1w^{1118}; P{UAS-ey.H}UE11*** (Halder et al., 1995): This transgenic line expresses full length *eyeless* (*ey*) cDNA under UAS promoter. Ey play an important role in cell fate determination during eye development. Targeted expression of *ey* into various imaginal discs primordia leads to the generation of ectopic eyes (Halder et al., 1995). This stock is homozygous viable. This transgenic construct is on the second chromosome. The stock was obtained from Bloomington Stock Centre (6294).
2. ***w^{1118}, P{UAS-bsk.DN}2*** (Adachi-Yamada et al., 1999): These transgenic flies have an insertion on the first chromosome which expresses a dominant negative form of *Drosophila* JNK, basket (bsk), under UAS control. This stock is homozygous viable. The stock was obtained from Bloomington Stock Centre (6409).

3. ***w^{1118}; UAS-Dfos^{bZIP}*** (Rallis et al., 2010): These transgenic flies express a dominant negative form of *Kayak,* transcriptional activator for *Drosophila* JNK. *Kayak* fragment (*Drosophila Fos*) fused with Bzip sequence to create dominant negative form under the control of UAS. This transgenic construct is present on the third chromosome. This stock is homozygous viable. This stock was obtained from Bloomington Stock centre (7215).

4. ***w^{1118}; P{w[+mc]=UAS-dpp.S}42B.4*** (Tracey et al., 2000): This transgenic line expresses full length *Decapentaplegic* (*Dpp*) cDNA under UAS promoter. This line is homozygous viable. This insertion is on third chromosome. This stock was obtained from Bloomington Stock centre (1486).

5. ***w^{1118}; UAS-vg/Cyo*** (Kim et al., 1996): This transgenic line express full length *vestigial (vg)* cDNA under the control of *UAS*. This transgenic insertion is on the second chromosome. This stock was provided by Prof. L.S. Shashidhara, IISER Pune.

6. ***w^{1118}; P{w[+mc]=UAS-Mmp1.f1}3*** (Page-McCaw et al., 2003): This transgenic line expresses the "f1" isoform cDNA of Mmp1 under UAS promoter. This transgenic insertion is on the third chromosome. This stock was a kind gift from Prof. Andrea Page-McCaw, Vanderbilt University School of Medicines.

7. ***w^{1118}; P{w[+mc]=UAS-Timp.P}3*** (Page-McCaw et al., 2003): This transgenic line expresses Tissue inhibitor of metalloproteinases (Timp) under UAS promoter.Timp is an endogenous inhibitor that inhibits both Mmp1 and Mmp2 activity by occupying their active site. This transgenic insertion is on the third chromosome. This stock was a kind gift from Prof. Andrea Page-McCaw, Vanderbilt University School of Medicine.

8. ***P{UAS-AUG-DsRed}*** (Kasuva and Iverson.,2000): This transgenic line expresses red fluorescence protein under UAS promoter. This insertion is on the third chromosome. This stock was obtained from Bloomington Stock centre (6281).

2.2.3. Mutant lines:

1. ***w^{1118}, hep^{r75}/FM7c*** (Glise et al., 1995): This is loss of function allele of *hemipterous* (Glavicet al., 1999) generated by P-element excision by hybrid dysgenesis in *hemipterous* gene locus. This is balanced by FM7 on first chromosome. This stock was obtained from Bloomington Stock centre (6761).

2. ***w^{1118}; Mad12P {neoFRT} 40A/CyO*** (Sekelsky et al., 1995): This is loss of function allele of *Mother against Dpp* (*Mad*) having point mutation in *Mad* locus on second chromosome. This mutation is homozygous lethal. This stock was obtained from Bloomington Stock centre (58785).

3. ***tkv^{7}, cn1, bw1, sp1/CyO*** (Nellen et al., 1994; Penton et al., 1994): Loss of function allele of *Thickvein* (*tkv*). This fly line has point mutation in *tkv* that lead to amino acid replacement (E528K, E474K) in C terminus of the kinase domain. This mutation is homozygous embryonic lethal balanced by *Cyo* on second chromosome. This stock was obtained from Bloomington Stock centre (3242).

4. ***y^{1}, w^{1118}, $Tak1^{(2)}$*** (Vidal et al., 2001): This fly line has point mutation in the kinase domain of the TGF-beta activated kinase1 (Tak1). These flies have defects in immune response and therefore more susceptible to *E. coli* infection. This stock was obtained from Bloomington Stock centre (26272).

5. ***w^{1118}, $Tak1^{(179)}$*** (Delaney and Mlodzik, 2006): This is loss of function allele of TGF-beta activated kinase1 (Tak1) generated by G31D mutation in ATP binding

motif in the Kinase subdomain and result in kinase nonfunctional protein. This stock was obtained from Bloomington Stock centre (26275).

6. ***w^{1118}; $FoXo^{del94}$/TM6B,Tb*** (Slack et al., 2011): *FoXo* mutant generated as a result of imprecise excision of *foxo[BG01018]* region leads to deletion of 20kb fragment comprising promoter region as well as several coding exons. This mutation is balanced by Tubby on third chromosome. These mutants have defects in developmental rate. This stock was obtained from Bloomington Stock centre (42220).

7. ***PSc^{h27}/CyO*** (Wu and Howe, 1995): Loss of function allele of *Posterior Sex combs (Psc)*. This mutation is homozygous lethal and balanced by *CyO* on second chromosome. This stock was obtained from Bloomington Stock centre (5547).

8. ***$ry^{506}hh^{AC}$ /TM3,Sb^{1}*** (Lee et al., 1992): Loss of function allele of *hedgehog*. hh^{AC} mutant generated as a result of deletion at the start of open reading frame. This mutation is homozygous lethal and balanced by *TM3,Sb* on third chromosome. This stock was obtained from Bloomington Stock centre (1749).

9. ***$Mmp1^{Q273}$/CyO*** (Page-McCaw et al., 2003): This mutant is hypomorphic allele of Mmp1 having mutation in the hinge region. The homozygous mutant flies have defects in head eversion. This stock was a kind gift from Prof. Andrea Page-McCaw, Vanderbilt University School of Medicine.

2.2.4. UAS RNAi lines:

The different UAS-dsRNA fly lines used in this study were obtained from Bloomington Stock Centre, Indiana, USA. These lines were generated under Transgenic RNAi Project (TRiP) of Harvard University, USA (Ni et al., 2008). These transgenic RNAi fly lines were generated by using Valium (Vermillion-AttB-Loxp-Intron-UAS-MCS) vector based on phiC31 site specific integration.

1. ***$y^{1}sc\ v^{1}$; UAS-Tak1 (dsRNA):*** (Ni et al., 2011): These transgenic flies express double stranded RNA to knock down the m-RNA of TGF-beta activated kinase1 (TAK1) under the control of UAS. This transgenic construct is homozygous viable. Quantitative analysis by Q-PCR showed 46% knockdown of TAK1 transcript at 29^{o}C with tubulin as reference gene (Sopko et al., 2014). This stock was obtained from Bloomington Stock centre (33404).

2. ***y^1 v^1; UAS-tkv (dsRNA)*** (Ni et al., 2011): expresses double stranded RNA to knock down the m-RNA of *thickvein* (*tkv*) under the control of UAS. This line is homozygous lethal and balanced by Curly (*Cyo*) on second chromosome. Quantitative analysis by Q-PCR showed 79% knockdown of *tkv* transcript at 29^0c with tubulin as reference gene (Sopko et al., 2014). This stock was obtained from Bloomington Stock centre (40937).

3. ***y^1 v^1; UAS-mad (dsRNA)*** (Ni et al., 2008): expresses double stranded RNA to knock down the m-RNA of *Mother against Dpp* (*Mad*), transcriptional activator of Dpp signaling under the control of UAS. This transgenic construct is present on third chromosome. This stock was obtained from Bloomington Stock centre (31315).

4. ***y^1 v^1; UAS-Med (dsRNA)*** (Ni et al., 2009): This transgenic fly line expresses double stranded RNA for RNAi of *Medea* (*Med*) under the control of UAS. Med is a transcriptional activator of Dpp signaling that interact with Mad. This line is homozygous viable. This stock was obtained from Bloomington Stock centre (31928).

5. ***y^1 v^1; UAS-kay (dsRNA)*** (Ni et al., 2011): This transgenic fly line expresses double stranded RNA to knock down the m-RNA of *Kayak* (*Kay*) under the control of UAS. Kay is one of the transcriptional activator of *Drosophila* JNK signaling.This line is homozygous viable. This construct resulted in to embryonic lethality when drivenby Act5c Gal4 (Zeng et al., 2015). This stock was obtained from Bloomington Stock centre (33379).

6. ***y^1 $v^{1;}$ UAS-Pc (dsRNA)/TM3sb*** (Ni et al., 2011): This transgenic flyline expresses double stranded RNA for RNAi of *Poly comb* (*Pc*) under the control of UAS. This transgenic construct is present on third chromosome. Embryonic lethal when driven by Act5c Gal4 (Zeng et al., 2015). This stock was obtained from Bloomington Stock centre (33622).

7. ***y^1 $v^{1;}$ UAS-ph-p (dsRNA)*** (Ni et al., 2011): This transgenic flyline expresses double stranded RNA for RNAi of *polyhomeotic proximal* (*ph-p*) under the control of UAS. This transgenic construct is present on third chromosome.This stock was obtained from Bloomington Stock centre (31777).

8. ***y^1 sc^1 v^1; UAS-FoXo (dsRNA)*** (Ni et al., 2011): This transgenic fly line expresses double stranded RNA for RNAi of *Foxo* under the control of UAS. Foxo is a transcriptional activator of *Drosophila* JNK signaling. This transgenic construct is present on third chromosome. This stock was obtained from Bloomington Stock centre (32427).

9. ***UAS-Mmp1 (dsRNA) (B)*** (Uhlirova and Bohmann, 2006): This transgenic fly line expresses antisense RNA for Mmp1 gene in *Drosophila*. These transgenic flies were generated by using cDNA fragments amplified from mmp1 gene. These fragments were cloned as inverted repeats in to the pWIZ vector and transformed in to flies by standard P-elements mediated germ-line transformation. This transgenic construct present on second chromosome. This stock was gifted by Prof. D.Bohman, University of Rochester Medical Centre, Rochester, NY,USA

10. ***UAS-Mmp1 (dsRNA) (KK)***: This transgenic fly line also expresses antisense RNA that specifically knockdown the Mmp1 transcript in *Drosophila*. This stock was obtained from Veinna Drosophila Resource Centre (101505)

2.2.5 LacZ Insertion Lines

1. ***w^{1118}; puc-LacZ/TM3, Ser*** (Martin-Blanco et al., 1998): This transgenic fly line has an insertion on third chromosome, which expresses β-galactosidase under enhancer region of *puckered* gene. It expresses lacZ in the proximal most region of notum in wing and leg imaginal discs. This stock was kind gift from the laboratory of Prof. Utpal Banerjee.
2. ***Dpp-lacZ/CyO*** (Blackman et al., 1991): This transgenic line contains a 12Kb DNA from the dpp 3' cis-regulatory region fused with the lacz coding region which mimics the pattern expression of dpp genes in the imaginal discs. This fly line was a kind gift from Prof. J.K. Roy, Banaras Hindu University.

3. ***y^1w^{1118}; Thor-LacZ*** (Spradling et al., 1999): This is an enhancer trap line that express lacz in the pattern of Thor. Thor generally used as a reporter for FoXo activity. This line is homozygous viable. This stock was obtained from Bloomington Stock centre (9558).

4. ***FLW-1*** (Cavalli and Paro, 1998): This is a reporter lacz line to check the activity of Polycombs group of proteins. These transgenic flies contain a lacZ reporter gene under the control of Fab7 PRE9 along with Gal4 binding site in between them. Fab7 PRE9 colocalizes with Pc and GAGA factor binding site. So, binding of PcG to PRE9 restricts Gal4 binding and as a result lacz expression (Cavalli and Paro, 1998). This construct is present on first chromosome and is homozygous viable. This stock was gifted by Dr. Renato Paro, ETH, Zurich.

5. ***LW-1*** (Cavalli and Paro, 1998): This is also a reporter lacz line used as a control. These transgenic flies contain a lacz reporter gene under the control of Gal4 binding site. There is no PRE9. This construct is present on third chromosome. This line is homozygous viable. This stock was provided by Dr. Renato Paro, ETH, Zurich

6. ***Mmp1-lacZ*** (Uhlirova and Bohmann, 2006): This transgenic fly line expresses lacZ reporter for Mmp1 expression. These transgenic flies were generated by cloning 4.78-kb mmp1 genomic fragment with three putative AP-1 binding sites into the lacZ coding transformation vector pPelican without a minimal promoter. Therefore, these flies act as a reporter for JNK mediated Mmp1 expression. This transgenic construct is present on second chromosome. This stock was gifted by Prof. D.Bohman, University of Rochester Medical Centre, Rochester, NY,USA.

2.3. Detailed genotypes of mutant fly lines analyzed for this study

Genotype of the original fly stocks	**Genotypes of mutant fly lines generated for this study**
To generate ectopic eyes in the developing imaginal discs of *Drosophila*	
w^{1118};wg^{Sp-1}/Cyo;P{Gal4dpp.blk1}40C.6/TM 6B,Tb^{1}	w^{1118}; UAS-ey/+; Dpp-Gal4/+
w^{1118}; P{Ser-Gal4.GF}1 P{ Ser-Gal4.GF}2	w^{1118}; UAS-ey/ Ser-Gal4; +/+

To study the pattern of Dpp expression	
w^{1118}; P{UAS-AUG-DsRed}	a) *w^{1118}; UAS-ey/UAS-DsRed; Dpp-Gal4/+* b) *$Tak1^{(2)}$/ y; UAS-ey/UAS-DsRed; Dpp-Gal4/+* c) *hep^{r75}/+; UAS-ey/UAS-DsRed; Dpp-Gal4/+*
w^{1118};Dpp-lacZ/CyO	a) *w^{1118}; UAS-ey,Dpp-lacZ; Dpp-Gal4/+* b) *w^{1118}; UAS-ey,Dpp-lacZ/Ser-Gal4*
To study the role of canonical Dpp signaling during ectopic eye formation	
w^{1118}; tkv^7 cn^1 bw^1 sp^1/CyO	a) *w^{1118}; tkv^7/UAS-ey; Dpp-Gal4/+*
w^{1118};Mad^{12}P{neoFRT}40A/CyOA ct GFP	a) *w^{1118}; Mad^{12} P{neoFRT}40A/UAS-ey; Dpp-Gal4/+*
y^1 v^1; UAS-tkv (dsRNA)	a) *y^1; UAS-ey/+; Dpp-Gal4/ UAS-tkv (dsRNA)* b) *y^1; UAS-ey/Ser-Gal4; UAS-tkv (dsRNA)/+*
y^1 v^1; UAS-Mad (dsRNA)	a) *y^1; UAS-ey/+; Dpp-Gal4/ UAS-Mad (dsRNA)* b) *y^1; UAS-ey/Ser-Gal4; UAS-Mad (dsRNA)/+*
y^1 v^1; UAS-Med (dsRNA)	a) *y^1; UAS-ey/+; Dpp-Gal4/ UAS-Med (dsRNA)* b) *y^1; UAS-ey/Ser-Gal4; UAS-Med (dsRNA)/+*
To check the involvement of Tak1 during ectopic eye formation	
y^1 w^{1118}, $Tak1^{(2)}$	a) *y^1 w^{1118};$Tak1^{(2)}$/ y; UAS-ey/+; Dpp-Gal4/+* b) *y^1 w^{1118},$Tak1^{(2)}$/ y; UAS-ey/+; DppGal4/UAS-Dpp* c) *y^1 w^{1118},$Tak1^{(2)}$ / y; UAS-ey/Ser-Gal4; +/*
y^1 sc v^1; UAS-Tak1 (dsRNA)	a) *y^1; UAS-ey/+; Dpp-Gal4/ UAS-Tak1 (dsRNA)* b) *y^1; UAS-ey/Ser-Gal4; UAS-Tak1 (dsRNA)*
To investigate JNK activation during ectopic photoreceptor differentiation	
w^{1118}, P{UAS-bsk.DN}2	a) *UAS-bsk.DN/y; UAS-ey/+; Dpp-Gal4/+* b) *UAS-bsk.DN/y; UAS-ey/Ser-Gal4*
w^{1118},$hepr^{75}$/FM7c	a) *$hepr^{75}$/+; UAS-ey/+; Dpp-Gal4/+* b) *$hepr^{75}$/+; UAS-ey/+; Dpp-Gal4/UAS-Dpp* c) *$hepr^{75}$/+; UAS-ey/Ser-Gal4; +/+*
w^{1118}; UAS-$Dfos^{bZIP}$	a) *w^{1118};UAS-ey/+; Dpp-Gal4/UAS-$Dfos^{bZIP}$* b) *w^{1118};UAS-ey/Ser-Gal4; UAS-$Dfos^{bZIP}$*

y^1 $v^{1;}$ UAS-kay (dsRNA)	a) *y^1 v^1;UAS-ey/+; Dpp-Gal4/UAS-kay (dsRNA)* b) *y^1 v^1;UAS-ey/Ser-Gal4; UAS-kay (dsRNA)*
w^{1118}; FoXo^{del94}/TM6B,Tb	a) *y^1 v^1;UAS-ey/+; Dpp-Gal4/ FoXo^{del94}* b) *y^1 v^1;UAS-ey/Ser-Gal4; FoXo^{del94}/+*
y^1 sc^1 $v^{1;}$ UAS-FoXo (dsRNA)	a) *y^1;UAS-ey/+; Dpp-Gal4/UAS-FoXo(dsRNA)* b) *y^1;UAS-ey/Ser-Gal4; UAS-FoXo(dsRNA)/+*
w^{1118}; puc-LacZ/TM3,Ser	a) *w^{1118}; UAS-ey/+; Dpp-Gal4/ Puc-lacZ* b) *w^{1118}; UAS-ey/Ser-Gal4; Puc-lacZ/+* c) *w^{1118}; tkv^7/UAS-ey; Dpp-Gal4/ Puc-lacZ* d) *w^{1118};Mad^{12}P{neoFRT}40A/UASey;Dpp-Gal4/Puc-lacZ* e) *y^1; UAS-ey/Ser-Gal4; UAS-Med (dsRNA)/Puc-lacZ* f) *y^1; UAS-ey/Ser-Gal4; UAS-Mad (dsRNA)/Puc-lacZ* g) *$Tak1^{(2)}$/ y; UAS-ey/+; Dpp-Gal4/Puc-lacZ* h) *$Tak1^{(2)}$/y; UAS-ey/Ser-Gal; Puc-lacZ* i) *UAS-bsk.DN/y; UAS-ey/+; Dpp-Gal4/ Puc-lacZ* j) *UAS-bsk.DN/y; UAS-ey/Ser-Gal4; Puc-lacZ/+*
y^1 w^{1118}; Thor-LacZ	a) *y^1 w^{1118}; UAS-ey/+; Dpp-Gal4/Thor-LacZ* b) *y^1 w^{1118}; UAS-ey/Ser-Gal4; Thor-LacZ /+*
To check Dpp mediated PcGdownregulation during ectopic eye formation	
FLW-1	a) *FLW-1/y; Dpp-Gal4/+* b) *FLW-1/y; UAS-ey/+; Dpp-Gal4/+* c) *FLW-1/y; UAS-ey/+; Dpp-Gal4/UAS-tkv (dsRNA)* d) *FLW-1/y; UAS-ey/+; Dpp-Gal4/UAS-Tak1(dsRNA)* e) *FLW-1/y; UAS-ey/+; Dpp-Gal4/UAS-Dfos^{bZIp}* f) *FLW-1/y; UAS-ey/+; Dpp-Gal4/UAS-kay (dsRNA)* g) *FLW-1/y; UAS-ey/+; Dpp-Gal4/UAS-Mad (dsRNA)* h) *FLW-1/y; Dpp-Gal4/UAS-Dpp*
LW-1	*LW-1/ Dpp-Gal4*
y^1 $v^{1;}$ UAS-ph-p (dsRNA)	*y^1 w^{1118}; UAS-ey/+; Dpp-Gal4/ UAS-ph-p (dsRNA)*
y^1 $v^{1;}$ UAS-Pc (dsRNA)/ TM3sb	*y^1 w^{1118}; UAS-ey/+; Dpp-Gal4/ UAS-Pc (dsRNA)*
Psc^1/CyOAct-GFP	*w; Psc^1/UAS-ey; Dpp-Gal4/+*
PSc[h27]/CyO	a) *w^{1118}; PSc[h27]/UAS-ey; Dpp-Gal4/+*

	b) *Tak1$^{(2)}$/ y; PSc[h27]/UAS-ey; Dpp-Gal4/+*
Dac^{1}/CyOAct-GFP	*w^{1118}; Dac^{1}/UAS-ey; Dpp-Gal4/+*
w^{1118}; UAS-vg/Cyo	a) *w^{1118}; UAS-vg/+; Dpp-Gal4/+* b) *w^{1118}; UAS-vg/UAS-GFP+; Dpp-Gal4/+* c) *Tak1$^{(2)}$/ y; UAS-vg/+; Dpp-Gal4/+* d) *UAS-bsk.DN/ y; UAS-vg/+; Dpp-Gal4/+* e) *y^{1};UAS-vg/+; Dpp-Gal4/UAS-kay (dsRNA)* f) *w^{1118};UAS-vg/+; Dpp-Gal4/UAS-Dfos^{bZIP}*
To study the role of Mmp1 during ectopic eye formation	
Mmp1^{Q273}/CyO	*w^{1118}; Mmp1^{Q273}/UAS-ey; Dpp-Gal4/+*
UAS- Mmp1(dsRNA) (B)	*w^{1118}; UAS- Mmp1(dsRNA) (B) /UAS-ey; Dpp-Gal4/+*
UAS-Mmp1(dsRNA) (V)	*w^{1118}; UAS- Mmp1(dsRNA) (V) /UAS-ey; Dpp-Gal4/+*
w^{1118}; P{[+mc]=UAS-Timp.P}3	*w^{1118}; UAS-ey/+; Dpp-Gal4/ UAS-Timp*
w^{1118}; P{[+mc]=UAS-Mmp1.f1}3	a) *w^{1118}; UAS-ey/+; Dpp-Gal4/ UAS-Mmp1* b) *w^{1118}; Mmp1^{Q273}/UAS-ey; Dpp-Gal4/ UAS-Mmp1*
Mmp1-lacZ	*w^{1118}; Mmp1-lacZ /UAS-ey; Dpp-Gal4/+*
ry^{506}hh^{AC} /TM3,Sb^{1}	a) *w^{1118}; UAS-ey/+; Dpp-Gal4/ hh^{AC}* b) *w^{1118}; Mmp1^{Q273}/UAS-ey; Dpp-Gal4/hh^{AC}*

2.4. Immunostaining of larval imaginal discs

Larvae of late third instar or desired developmental time period were selected and dissected on ice in 1X PBS (10mM NaH_2PO4, 2mM KH_2HPO4, 137mM NaCl, and 2.7mM KCl). The dissected tissues were fixed in 4% formaldehyde prepared in 1X PBS for 45 minutes followed by two quick washes in 1X PBS. Tissues were then permeabilized by washing with 0.3% PBT (0.3% Triton-X100 in 1X PBS) for 1 hr (4 washings, 15 minutes each) at room temperature on shaker. Blocking was done in 10% NGS (prepared in 0.3% PBT) for 45 minutes-1hour at room temperature on shaker. Tissues were then incubated in primary antibody with appropriate dilution at 4^{o}C for 18-24 hours. After incubation in primary antibody tissues were washed with 0.3% PBT

again for 1 hour (4 washings, 15 minutes each). Tissues were again blocked with 10% NGS for 20-30 minutes in room temperature. Tissues were incubated in Secondary antibody (specific to primary antibody used) prepared in 10% blocking solution (10% NGS in 0.3% PBT) and were kept at 4^{o}C for 18-24 hours or at room temperature for 2 hours. Secondary antibody incubation was followed by washings with 0.3% PBT. After washing with 1X PBS tissues were stained with chromatin binding dyes by incubating them in Dapi or in TO-PRO-3 solution in 1X PBS at at room temperature for 1 hour. Then tissues were subsequently washed with 1X PBS and mounted in Vectashield, Vector Laboratories (ZA0825). Tissues were examined under fluorescence and confocal microscope.

2.5. Immunological Detection of Proteins (Antibodies):

2.5.1 Primary Antibodies:

The different antibodies used for this study are as follow:

- **Anti-Eyeless** (Anti eyeless, Developmental Studies Hybridoma Bank, Iowa): This is monoclonal antibody raised in mouse that recognizes 48 kDalinker region of Eyeless protein of *Drosophila*. The working dilution is 1:100.
- **Anti-Eyes Absent** (eya10H6, Developmental Studies Hybridoma Bank, Iowa): This is monoclonal antibody raised in mouse against 456-469 amino acids of Eyes absent protein of *Drosophila*. The working dilution is 1:2.

- **Anti-Dachshund** (mABdac2-3, Developmental Studies Hybridoma Bank, Iowa): This antibody is a bacterially expressed protein corresponding to the amino acids 378-599 of *Drosophila* Dac. It mimics the expression pattern of *dac* as observed by *in situ* hybridization and expression of enhancer trap insertion in *dac* (Mardon et al., 1994). This is used at a dilution of 1:20.

- **Anti-ELAV** (Embryonic Lethal Abnormal Vision) (Rat-Elav-7E8A10, Developmental Studies Hybridoma Bank, Iowa): This rat monoclonal antibody recognizes *Drosophila* protein Elav that marks most differentiated neurons in peripheral and central nervous system. The working dilution for immunostaining is 1:100.

- **Anti Wingless** (4D4, Developmental Studies Hybridoma Bank, Iowa): This mouse monoclonal antibody is specific for *Drosophila* Wingless (Wg) protein. The working dilution is 1:4.

- **Anti Engrailed/Invected** (4D9, Developmental Studies Hybridoma Bank, Iowa): This mouse monoclonal antibody recognizes both Engrailed and Invected proteins. This marks patterning of segmentation from cellular blastoderm stage onward, peripheral nervous system neuron as well as central nervous system neurons in *Drosophila*. The working dilution is 1:2.

- **Anti p-Mad** (Gancz et al., 2011): This rabbit antibody recognizes the phosphorylated form of Mother against Dpp (Mad) protein to give a read out for Dpp activity. The working dilution is 1:1000. This antibody was a kind gift from Dr. E. Laufer, Columbia University, NY, USA.

- **Anti β- galactosidase:** (Promega, Z3781): Monoclonal purified antibody raised in mouse against β-galactosidase protein of E.coli. The working dilution is 1:100.

- **Anti β- galactosidase** (Molecular Probe, USA, A11132): Highly purified antibody raised in rabbit against E.coli β-galactosidase to detect the level of β-galactosidase reporter lacz expression in tissue. The working dilution is 1:2000.

- **Anti Mmp1:** (14A3D2, 3A6B4, 3B8D12, 5H7B11, Developmental Studies Hybridoma Bank, Iowa): These are four different antibodies raised against different domains of Mmp1. 14A3D2 recognizes hemopexin domain while 3A6B4, 3B8D12, 5H7B11 recognize catalytic domain of Mmp1. These four antibodies were used in mixture in 1:1:1:1 dilution.

2.5.2. Secondary antibodies for immunostaining

The different secondary antibodies, used to detect the primary antibodies, were as follows.

- **Cy^{TM3}-Conjugated affinipure donkey anti rabbit IgG (H+L)** (Jacksons Immuno Research Laboratories, USA # 711-165-152). This affinity purified secondary antibody conjugated with cyanine Cy^{TM3} dye (absorption maxima/ emission

maxima is 550 nm/ 570 nm) was used at a dilution of 1:600 to detect primary antibodies raised in rabbit.

- **(FITC)-Conjugated affinipure goat anti rabbit IgG (H+L)** (Jacksons Immuno Research Laboratories, USA # 711-096-152):This affinity purified secondary antibody conjugated with Fluorescein FITC dye (absorption maxima/ emission maxima is 492 nm/ 520 nm) was used at a dilution of 1:600 to detect primary antibodies raised in rabbit.

- **Cy^{TM3}-Conjugated affinipure goat anti mouse IgG (H+L)** (Jacksons Immuno Research Laboratories, USA # 115-166-062): This affinity purified secondary antibody conjugated with cyanine Cy^{TM3} dye (absorption maxima/ emission maxima is 550 nm/ 570 nm) was used at a dilution of 1:600 to detect primary antibodies raised in mouse.

- **(FITC)-Conjugated affinipure goat anti mouse IgG (H+L)** (Jacksons Immuno Research Laboratories, USA # 115-095-166): This affinity purified secondary antibody conjugated with Fluorescein FITC dye (absorption maxima/ emission maxima is 492 nm/ 520 nm) was used at a dilution of 1:600 to detect primary antibodies raised in mouse.

- **Cy^{TM5} 649-Conjugated affinipure goat anti rat IgG (H+L)** (Jacksons Immuno Research Laboratories, USA # 712-495-153): This affinity purified secondary antibody conjugated with cyanine 649 dye (absorption maxima/ emission maxima is 650 nm/ 670 nm) was used at a dilution of 1:600 to detect primary antibodies raised in rat.

- **Cy^{TM3}-Conjugated affinipure donkey anti rat IgG (H+L)** (Jacksons Immuno Research Laboratories, USA # 712-165-153): This affinity purified secondary antibody conjugated with cyanine Cy^{TM3} dye (absorption maxima/ emission maxima is 550 nm/ 570 nm) was used at a dilution of 1:600 to detect primary antibodies raised in rat .

- **(FITC)-Conjugated affinipure donkey anti rat IgG (H+L)** (Jacksons Immuno Research Laboratories, USA # 712-165-153): This affinity purified secondary antibody conjugated with Fluorescein FITC dye (absorption maxima/ emission

maxima is 492 nm/ 520 nm) was used at a dilution of 1:600 to detect primary antibodies raised in rat.

2.5.3. Stains:

1. **Phalloidin- Texas Red** (Molecular Probe, India # T7471): It is a phallotoxin isolated from poisonous fungus *Amanita phalloides*, which specifically binds to polymeric filamentous actin and prevents its depolymerization. This F-actin probe is conjugated with red fluorescent Texas red dye (excitation maxima: 591nm, emission maxima: 608). The working dilution was 1:500. Tissues after secondary antibody treatment were washed thrice for 5 minutes each with 1X PBS. Then, tissues were incubated in phalloidin Red (1:500) in 1X PBS for 1hour at room temperature. After incubation, tissues were washed with 1X PBS and observed after mounting in vectasheild.

2. **DAPI** (4',6-diamidino-2-phenylindole dihydrochloride, Molecular Probe, India # ZB1130). This is a blue fluorescent dye that binds to A-T rich region in dsDNA. It is generally used to stain nuclei in live as well as fixed tissues. Its absorption maxima/emission maxima are 351nm/461nm. The working dilution was 1µg/ml. Tissues after secondary antibody treatment were washed thrice for 5 minutes each with 1X PBS. Then, tissues were incubated in DAPI solution (1µg/ml) in 1X PBS for 30 minutes at room temperature. After incubation, tissues were washed with 1X PBS and observed after mounting in vectashield.

3. **TO-PRO-3 iodide** (Molecular Probe, India # T3605): This is a highly sensitive carbocyanine nucleic acid stain with far red fluorescence. It is used as counterstain for nucleus and dead cell indicator. Its absorption maxima/emission maxima are 642nm/661nm. The working dilution was 1:500.

2.6. Cryosectioning of imaginal discs:

Tissues were immunostained following the standard immunostaining protocol as described before in section 2.4. After immunostaining, tissues were re-fixed with 4% formaldehyde for 30 minutes at room temperature. After the formaldehyde fixation, tissues were given a quick wash with 1X PBS and then incubated in 30% sucrose solution (prepared in 1X PBS) for overnight at 4^{o}C.

Tissue blocks were prepared by embedding imaginal discs in the Tissue-Plus O.C.T (Optimal Cutting Temperature) solution (Fisher Healthcare # 4585). The imaginal discs were properly aligned in the solution and immediately transferred inside the Leica Cryotome (CM3050) instrument maintained at -25^{0}C temperature. After the block (containing the tissues) was frozen completely, sections of 25µm were taken on glass slides either manually or by controlling the automatic panel. These slides having tissues were washed carefully with 1X PBS and incubated in phalloidin solution (1:500 in PBS) for 10 minutes at room temperature and quickly, carefully washed with 1X PBS to remove excess phalloidin and mounted in the mounting medium, Vectashield and imaged under confocal microscope (LSM 780, Carl Zeiss).

2.7. Imaging and analysis

2.7.1. Confocal microscopy

Immunoflourescence images were captured in Laser Scanning Confocal Microscope (LSM 780, Carl Zeiss). Optical sectioning was done using line mode in confocal microscope. 3D movies were prepared by doing bridge mounting of immunostained samples and then images were processed in Imaris-X64 software to make movies and 3D models.

2.7.2. Scaning Electron Microscopy:

For recording the external morphology of ectopic eyes on wings and legs and ectopic wing on eyes, flies of desired genotypes were etherized and desired structures were oriented properly and imaged using scanning electronic microscope (Jeol JCM 5000).

2.7.3. Quantification of fluorescence intensity:

Intensities of the levels of Dachshund and Eyes Absent expression were quantified in terms of gray value of Dachshund and Eyes Absent expressing area by using ImageJ. Area of Dachshund and Eyes Absent expressing domains were selected by using selection tool in ImageJ. Fluorescence intensity and the area of the selected expression domains were calculated by using measure function in ImageJ. To be more precise, each experiment was repeated three times along with control and imaged at the similar parameter settings. pMad activity was quantified in terms of pixel intensities within

fixed area by using plot profile function of ImageJ of wing discs, imaged at the same settings. Average of the pixel intensities of five imaginal discs were taken to calculate final pMad intensity profile.

2.7.4. Quantification of the domain of expression:

We used ImarisX64 for calculating the area of puc-lacZ as well as Flw-1 reporter lacZ expressing cells andnormalized it with respect to total area of disc to account for variability in discs size. The calculated areas in experimental samples were compared to their corresponding controls to determine ratio. The results shown here are outcome of three independent experiments.

Ommatidial number was also calculated by using Imaris X64.

2.7.5. Statistical analysis:

Data are expressed as mean±standard deviation (SD) of values from three independent experiments. Statistical analysis was performed using two-tailed Student's t-test. Significance was accepted with P values of <0.01; <0.001 and <0.0001, mentioned as *, **, *** respectively was considered as statistically significant.

2.8. RNA isolation from whole larvae

Appropriate number of (10 larvae for control) synchronized hatched larvae were collected and washed thoroughly with PBS to remove food particles. Larvae were homogenized in TRiZOL (Invitrogen # 15596018) and extracted with 200µl of chloroform (Sigma # C2432). Further purification was performed by using RNeasy Mini Kit (Quiagen # 74104) as instructed. To remove residual DNA samples were incubated in RNase free 2U DNase (Qiagen-79254) in RDD buffer for 25 minutes at 37^0C. RNA pellet was dissolved in nuclease free DEPC treated water (Sigma # 95284). Quantitation of RNA was performed by using nanodrop-2000 spectrophotometer (Thermo-Scientific).

2.9. Isolation of total RNA from imaginal discs.

100-150 imaginal disc were dissected on ice in 1X PBS and homogenized in 1 ml TRIzol reagent (Ambion # 15596018) using homogenizer. After proper

homogenization sample was kept at room temperature for 10 minutes for complete dissociation. 200µl of chloroform was added and incubated at room temperature for 10-15 minutes. The resulting mixture was then centrifuged at 4°C for 15 minutes at 12000 RPM. Three layers were separated out, uppermost aqueous layer containing RNA, middle layer containing proteins and lowermost organic layer containing DNA. The upper layer was carefully taken out in to a separate RNase-free tube so that middle layer remained unaffected. The sample was then incubated with 0.6 volumes of isopropanol for 10 minutes at room temperature to precipitate out the RNA and then centrifuge at 13,000 rpm for 15minutes at 4 °C to pellet out the RNA. RNA pellet was washed with 70% ethanol and dissolved in DEPC water. Finally, the sample was incubated at 37^0c with RNase free 2U DNase (Qiagen-79254) in RDD buffer for 25 minutes to remove residual DNA. Quantitation of RNA was performed by using nanodrop spectrophotometer.

2.10. Synthesis of c-DNA

c-DNA was synthesized using Verso c-DNA synthesis kit (Molecular Probe # AB1453A) following the manufacturer's recommended protocol. 500 ng of RNA was used for c-DNA synthesis. RNA was incubated with cDNA reaction mix at 42˚C for 30 min and then kept on 95˚C for 2 min. c-DNA mix was prepared using 5X RT buffer, 500µM dNTP mix, OligodT and random Hexamer primer mix, RT Enhancer to remove contaminating DNA and Verso enzyme mix which acted as reverse transcriptase for c-DNA manufacture. c-DNA mix was added to the RNA and the reaction was mixed properly by pipetting and short spin. This mixture was then incubated at 42˚C for first 30 min. Reaction was terminated by incubating at 95˚C for last 2 min c-DNA samples were stored at -20˚C.

2.10.1. Preparation of Reaction Mixture:

Constituents	**Final Concentration**	**Volume For 20*µ*l reaction**
5 X RT Buffer	1X	4µl
dNTPs Mix (500µM)	50 µM	2µl
RT Enhancer	1.5 mM	1µl
RNA Primer	1X	2µl

Verso Enzyme Mix	1 unit	1µl
RNA (500ng/µl)	500 ng	1-5µl
Nuclease-free water	---	9-13 µl
TOTAL		**20*µ*l**

2.11. RT PCR

The 20 µl reaction mixture was prepared by adding reagents in the following sequence: PCR water; 10X Taq Buffer; dNTPs mix; $MgCl_2$; Forward Primer; Reverse Primer; and Taq Polymerase.

2.11.1. Preparation of PCR Reaction Mixture:

Constituents	**Final Concentration**	**Volume for 20*µ*L reaction**
10 XTaq Buffer	1X	2.0µl
dNTPs Mix (10mM)	0.2mM	0.4µl
$MgCl_2$	1.5 mM	0.8µl
Forward Primer (10µM)	1µM	0.4µl
Reverse Primer (10µM)	1µM	0.4µl
Taq Polymerase (3 unit/ µl)	0.6 units	0.2µl
DNA (500ng/µl)	500ng	1 µl
PCR water		14.8 µl
Total		**20**µl

The amplification reaction consisted of 40 cycles with initial denaturation at 94˚C for 5 minutes, followed by 39 cycles of denaturation at 95˚C for 30 seconds, annealing at 59˚C (Variable for different set of primers) for 45 seconds and extension at 72˚C for 30 seconds. Reaction was terminated after final extension for 10 minutes at 72˚C. 10µl of PCR products were loaded on 1% agarose gel along with 50bp DNA ladder as the marker to check the amplification.

2.12. Real Time PCR

Transcript level of different genes were compared by quantitative RT- PCR on RNA isolated from 50-60 late third instar larval imaginal discs of each genotype by using standard TRIzol method. Real time qRT-PCR was performed using SYBR green mix Biorad on Biorad CFX96 instrument, following the instructions provided in the

manual. After setting up the reactions with SYBR Green, plate form was made on instrument and reaction was set up at 95^0c for 15 sec and 59^0c for 15 sec and 72^0c for 15 sec for 40 reactions. Melting curve was performed from 95^0c to 72^0c for 5 min, to analyse Tm of the amplicon. Expression analysis was performed on instrument using ΔΔCt method.

Expression level was normalized to Ribosomal protein (Rp49) level and then compared with respect tocontrol. Primers were designed from NCBI. We specifically designed primers from exon-exon junction to avoid any misinterpretation from DNA contamination. Specificity of primers were checked with primer blast.

2.12.1. Primer used:

Gene	**Primer Sequence**	
Tak1	Forward	5′ CTCGGGCCAACTGGACAATA 3′
	Reverse	3’ ATCAAAGCCATTCGCCTCCA 5’
dachshund (*dac*)	Forward	5’ CTCCAGGAATTGCTCTCCCA 3’
	Reverse	3’ TCGTCCTCGACGCCGATTAT 5’
eyes absent (*eya*)	Forward	5’ ATCGTGGAAATGTTGGCACC 3’
	Reverse	3’ GGAGGTTACCAGCACGTTGA 5’
sine oculis (*so*)	Forward	5’ GTGTTTGCGAGGTTCTCCAGC 3’
	Reverse	3’ AATGCGCTTTCAACCACAGG 5’
polyhomeotic proximal (*php*)	Forward	5’ CAGACCCAGCAAAACCAGATT 3’
	Reverse	3’ TCCATTGTGGGGCATGACAG 5’
poly comb (*pc*)	Forward	5’GAGTAAGGGGAAGTTGGGGC 3’
	Reverse	3’ TCCACGACGCCCTTCTTAAC 5’
Thickveins (*tkv*)	Forward	5’TTCTCATGTGCAAGGTAGCC3’
	Reverse	3’GCGTCGCTTGTAGGTGAAAC5
Kayak (*kay*)	Forward	5’GACCGATACTTCAAGTGCCC3’
	Reverse	3’ATCAAAGCCATTCGCCTCCA5’

Ribosomal Protein49 (Rp49)	Forward	5'CTAAGCTGTCGCACAAATGGC3'
	Reverse	3'TTCTGCATGAGCAGGACCTC5'
Matrix metalloproteinases1 (*Mmp1*)	Forward	5' GCGTGTGAAGAACCTCACCT 3'
	Reverse	3' TCTCCACGAACTTGATCTCG 5'

Chapter 3.

The morphogen, Decapentaplegic (Dpp), employs a two-tier mechanism to activate its target retinal determining genes during ectopic eye formation in *Drosophila*.

3.1. Introduction

The gene *eyeless* (*ey*) in *Drosophila* codes for a Pax6 family of transcription factor that plays an essential role in the development of eyes (Gehring and Ikeo, 1999). It is homologous to the *Aniridia* gene in human and *small eye* gene in mouse (Quiring et al., 1994). Loss of function mutation in *eyeless* gene leads to reduction and complete elimination of eye structure (Quiring et al., 1994) while its ectopic expression in various *Drosophila* imaginal discs leads to the formation of ectopic eyes on wings, legs and on antennae (Halder et al., 1995; Quiring et al., 1994; Salzer and Kumar, 2010). Molecular and genetic studies involved during *Drosophila* eye development have established that *eyeless* can lead to the activation of all the genes that are involved in eye development (Halder et al., 1998). Therefore, ectopic eye induction in larval wing imaginal discs of *Drosophila* by ectopic expression of *eyeless* has been an extensively used model to understand various questions associated with normal eye development as well as different cell biological processes associated with cell fate alteration.

Apart from *eyeless*, targeted expression of other early eye determining genes, such as *dachshund (dac)* and *eyes absent (eya)* also resulted in the generation of ectopic eyes in various tissues including antennae, thorax and legs (Bonini et al., 1997; Salzer and Kumar, 2010; Shen and Mardon, 1997). Interestingly, ectopic expression of *dac* and *eya* also induced ectopic *eyeless* expression in a way similar to their activation by ectopic *eyeless* expression. Loss of function mutation of any of these early eye determining genes led to the loss of eye formation (Bonini et al., 1993; Cheyette et al.,

1994; Mardon et al., 1994). In fact, previous studies have revealed that normal eye development in *Drosophila* is regulated by the co-ordinated activities of the following genes: *eyeless* (*ey*), *sine oculis* (*So*), *eyes absent* (*eya*) and *dachshund* (*dac*) that constitute a gene regulatory network generally termed as the Retinal Determining (RD) gene regulatory network (Fig.3.1) (Desplan, 1997; Heberlein and Treisman, 2000; Silver and Rebay, 2005). Mutant analysis as well as expression pattern studies have clearly established that *ey* acts upstream to all these RD genes and transcriptionally activates the expression of *So*, *eya* (Halder et al., 1998; Halder et al., 1995; Pignoni et al., 1997; Quiring et al., 1994).

Furthermore, yeast two hybrid and *in vitro* studies have shown that even So and Eya physically interact with each other to form a transcription factor complex and synergistically activate *dac* expression (Chen et al., 1999; Pignoni et al., 1997). Similarly, Eya also act as transcriptional co-activator in a complex with Dac to regulate RD genes expression (Chen et al., 1997). Therefore, as shown in Fig.3.1, RD genes regulate each other's expression at the level of transcription as well as by various protein-protein interactions and thereby form a very complex regulatory network that help to establish the cell fate during eye development.

Importantly, it has also been established that Eyeless acts in co-ordination with Dpp to activate the expression of *dac*, *eya* and *so* during normal as well as ectopic eye development (Fig.3.2) (Chen et al., 1999; Kango-Singh et al., 2003; Pappu et al., 2005). Failure of ommatidial differentiation upon attenuation of Dpp signaling confirms its role during normal eye development. Further studies revealed that, Dpp loss of function mutants eye discs as well as Mad^{1-2} loss of function clones failed to express *eya*, *So* and *dac* (Chen et al., 1999; Curtiss and Mlodzik, 2000) while ectopic expression of Dpp in the anterior part of eye disc led to ectopic Eya, So and Dac expression (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). Consistent with these results, even during ectopic eye formation, ommatidial differentiation was always found to be restricted to the cells already having Dpp expression. Moreover, co-expression of *ey* with *dpp* always resulted in enhanced ectopic eye differentiation (Kango-Singh et al., 2003). Functional genomics studies also revealed that Eya and So along with phosphorylated Mad bind to the cis-regulatory elements in *dac* genomic region to regulate 3'EE enhancer of *dac* gene (Pappu et al., 2005).

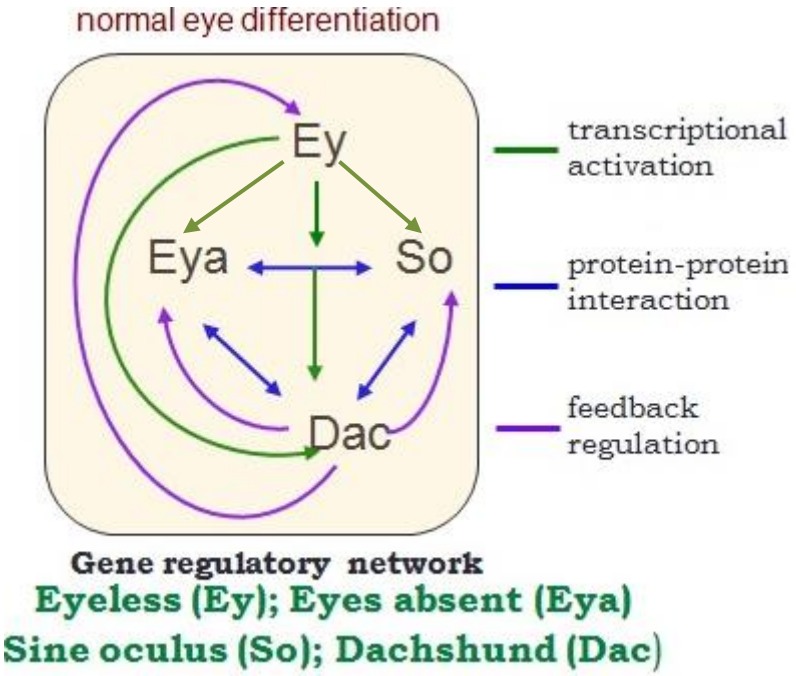

Figure3.1. Gene regulatory network involved in retinal cell fate specification.

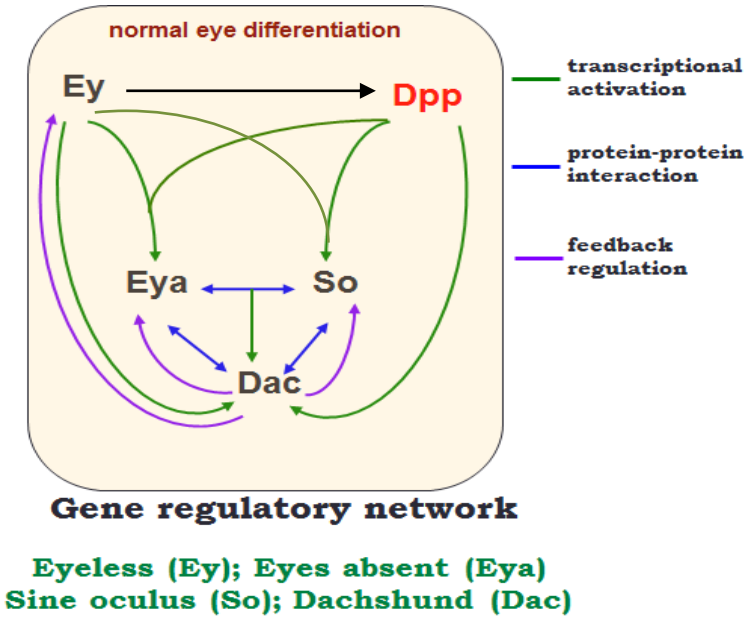

Figure 3.2. Model depicting synergistic involvement of Dpp along with Ey to regulate the expression of RD genes.

Although, the role of Dpp signaling in triggering RD genes network have been elucidated in eye disc, Dpp signaling is equally important to turn on completely different subset of genes including *optomotor-blind*, *spalt* and *brinker* in developing wing disc which are essential for wing development. Interestingly, other than *dac*, no other members of RD genes network are turned on in the wing discs and therefore do not play any role in wing development. *dac* also expresses in a very small domain in the anterior compartment and till now, its role in wing development is not understood. Therefore, Dpp evokes the expression of a set of target genes in the developing wing disc that is entirely different from that what it does in the developing eye disc. As explained earlier in Chapter-1 (section-1.7.1 and 1.7.2), this pleotropic response of Dpp signaling in developing eye and wing discs of *Drosophila* is an outcome of availability of different gamut of transcription factors as well as different epigenetic modifications present in different cell types.

However, during ectopic eye formation in developing wing disc, Dpp has to activate the genes of RD network that otherwise remain repressed in wing disc. Therefore, I wanted to understand the regulation of RD genes expression by Dpp in non-retinal tissue during wing to eye transformation. Specifically, I was interested to know whether apart from transcriptional activation of its target genes, Dpp plays any role in derepression of these target genes during the process of cell fate alteration. Although, understanding the mechanism underlying this process and the role of Dpp, if any, in co-coordinating this seems to be intriguing and this area is still unexplored.

3.2. Results

3.2.1. Generation of ectopic eyes in the larval wing imaginal discs of *Drosophila melanogaster.*

Ectopic eyes were generated in the developing larval wing imaginal discs by targeted expression of *eyeless* employing the bipartite *UAS-Gal4* system (Brand and Perrimon, 1993). *eyeless* expression was induced to generate ectopic eyes as *eyeless* is one of the most potent inducers for eye development in *Drosophila* (Halder et al., 1995).

Two independent *Gal4* driver lines *Dpp-Gal4* and *Ser-Gal4* were used that express in different compartments of wing discs (Fig.3.3A). As shown by reporter RFP expression, Dpp is expressed along the anterior/posterior (A/P) boundary of wing disc (Fig.3.3B) and accordingly, ectopic Eyeless expression was observed along the entire Dpp expressing domain in the AP-axis upon driving *eyeless* with *Dpp-Gal4* (Fig.3.3C). Expression of Ser, however, shows dynamic pattern at different stages of wing disc development. In the early third instar larval stage, Ser expresses in the entire dorsal compartment (Fig.3.3D). While during the middle and late third instar stages, Serrate expression gets restricted along the DV boundary under the action of Notch and Wingless signaling (Yan et al., 2004) (Fig.3.3E). Since Serrate is expressed in the entire dorsal compartment of the developing wing disc in early stages, ectopic Eyeless expression was also observed in the entire dorsal compartment of the mid/late third instar wing imaginal discs of *UAS-ey*/*Ser-Gal4* larvae (Fig.3.3F). Therefore, in both the cases ectopic *eyeless* expression mimicked the expression pattern of driving Gal4 lines. Analysis of adult flies revealed the generation of ectopic eyes at the places of wings in *UAS-ey*/+*; Dpp-Gal4*/+ (100%) as well as *UAS-ey*/*Ser-Gal4* (95%) adult flies (Fig.3.3G, H).

3.2.2. Ectopic photoreceptor differentiation in the wing disc is restricted to the domain of Dpp expression

To study the differentiation of ectopic photoreceptors in the developing larval wing discs, third instar larval wing discs were immune-stained with antibody against ELAV.

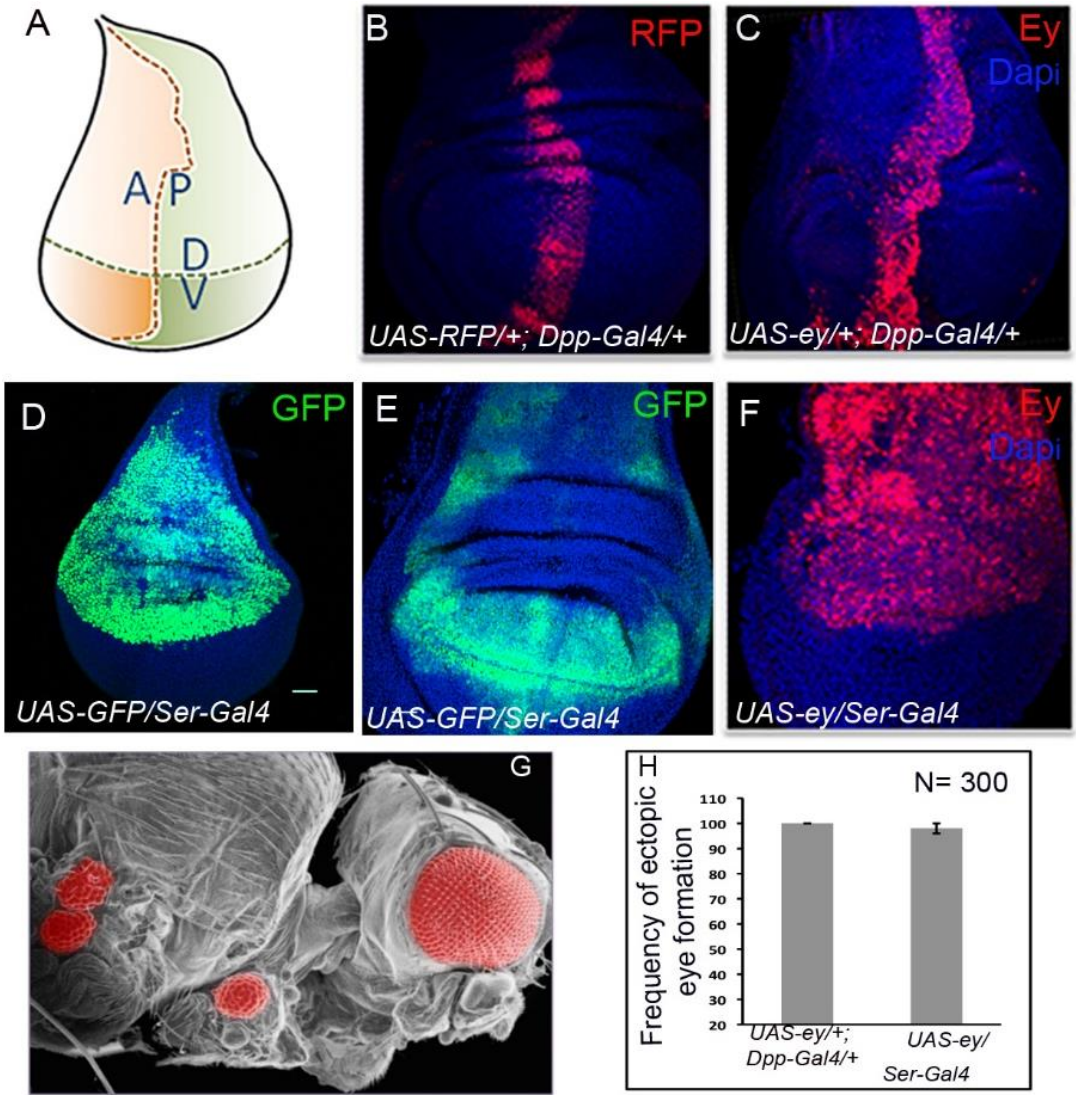

Figure 3.3. Generation of Ectopic eyes in the developing larval wing imaginal discs of *Drosophila melanogaster*. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A)** Schematic representation of third instar larval wing imaginal disc showing the A/P and the D/V boundaries, marked in red and green respectively. **(B)** Reporter RFP expression in the Dpp domain along the A/P boundary in third instar larval wing discs. **(C)** Ectopic expression of Eyeless in *UAS-ey/+; Dpp-Gal4/+* third instar larval wing discs. **(D, E)** Expression of reporter GFP in the wing discs of *UAS-GFP/Ser-Gal4* larvae. GFP expression was observed in the entire dorsal compartment in the early third instar larval wing discs **(D)** while it gets restricted to D-V boundary in late third instar larval wing discs **(E). (F)** Ectopic expression of Eyeless in the entire dorsal compartment in wing disc of *UAS-ey/Ser-Gal4* third instar larvae. **(G)** Scanning Electron Micrograph (SEM) image of adult fly showing ectopic eyes at the place of wings. **(H)** Quantitative estimation of the frequency of ectopic eye formation at the places of wings in *UAS-ey/+; Dpp-Gal4/+* and *UAS-ey/Ser-Gal4* adult flies.

ELAV (Embryonic Lethal Abnormal Vision) is a pan neuronal marker (Robinow and White, 1991). It is a mRNA binding protein that normally expresses in the differentiating photoreceptors of eye discs as well as in all other neurons but does not normally expresses in the wing discs (Yao et al., 1993). Immunostaining of *UAS-ey*/+*; Dpp-Gal4*/+ third instar larval wing discs with ELAV antibody revealed the generation of ectopic photoreceptors along the AP-axis, the domain in which Dpp is known to express (Fig.3.4A). However, in case of wing discs of *UAS-ey*/*Ser-Gal4* larvae, despite *eyeless* being expressed in the entire dorsal compartment, ectopic photoreceptors differentiated only in a specific region of the posterior compartment (Fig.3.4B). Quantitation of the number of ectopic ommatidia in the wing discs of late third instar larvae revealed that the average number of ectopic ommatidia per imaginal disc was around (60 ± 6) in case of *UAS-ey*/+*; Dpp-Gal4*/+ larvae while it was (45 ± 8) in wing discs of *UAS-ey*/*Ser-Gal4* larvae (Fig.3.4C).

To understand this spatial restriction of ectopic ommatidial differentiation in wing discs, the expression of Retinal determining (RD) proteins were checked for Eyes absent (Eya) and Dachshund (Dac) in *UAS-ey*/+*; Dpp-Gal4*/+ and *UAS-ey*/*Ser-Gal4* larval wing discs. Immonostaining with antibodies against Eyes absent and Dachshund revealed their ectopic expression along the A-P boundary in wing discs of *UAS-ey*/+*; Dpp-Gal4*/+ larvae (Fig.3.4D, F). In contrast, in wing discs of *UAS-ey*/*Ser-Gal4* larvae, ectopic Eya and Dac expression were observed in a subset of cells located in the posterior-dorsal compartment (Fig.3.4E, G). Interestingly, this domain of ectopic Eya and Dac expression overlapped with the area that exhibited ectopic photoreceptors differentiation in these discs (Fig.3.4A, B). Furthermore, the expression level of another RD gene *sine oculis* (*So*) was also checked at mRNA level by RT-PCR in wing imaginal discs undergoing ectopic photoreceptor differentiation. As compared to wild type wing discs, robust increase in the level of *sine oculis* expression was observed during ectopic photoreceptor differentiation in the wing discs of both *UAS-ey*/+*; Dpp-Gal4*/+ and *UAS-ey*/*Ser-Gal4* larvae (Fig.3.4J). Together, these results demonstrated that there was ectopic expression of the three RD genes *eya*, *so* and *dac* upon ectopic expression of *eyeless*. More importantly this RD genes expression got restricted to a specific domain of Eyeless expressing cells in the wing disc. Despite being direct transcriptional targets of Eyeless, since the expression of Dac, Eya were restricted to a subset of Eyeless expressing cells in *UAS-ey*/*Ser-Gal4* larval wing

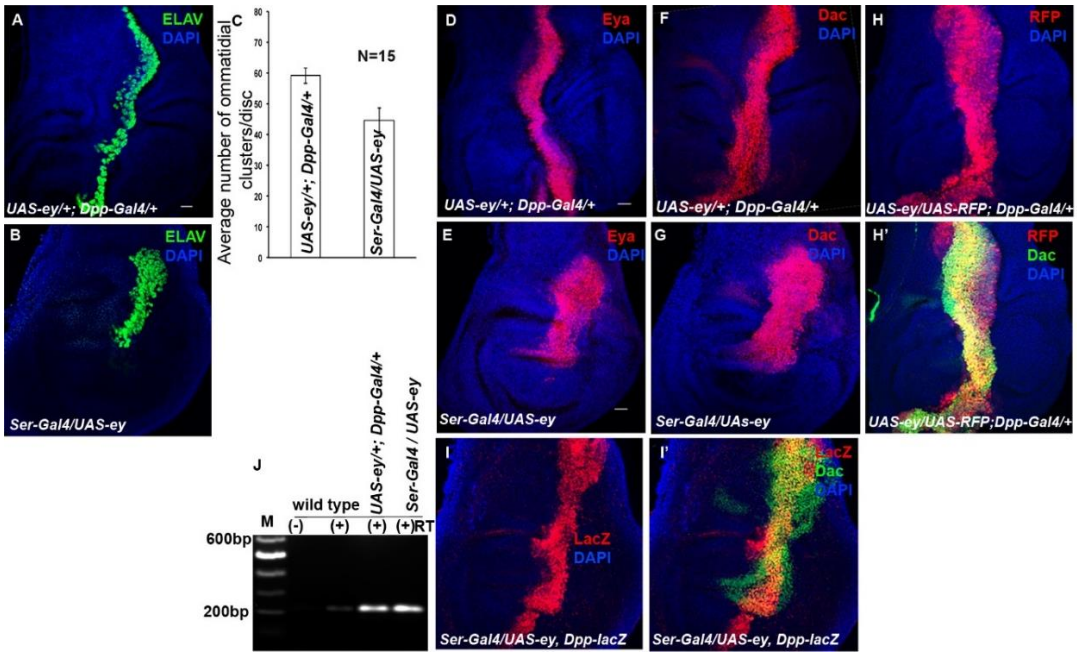

Figure 3.4. Ectopic photoreceptor differentiation in the wing disc is restricted to the domain of Dpp expression. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A, B)** Ectopic photoreceptors marked with ELAV in the wing discs of **(A)** *UAS-ey/+; Dpp-Gal4/+* late third instar larvae and **(B)** *UAS-ey/Ser-Gal4* larvae. **(C)** Graph showing average number of ectopic photoreceptors in the wing discs of *UAS-ey/+; Dpp-Gal4/+* and *UAS-ey/Ser-Gal4* late third instar larvae. **(D, E)** Ectopic Eya expression in the wing discs of **(D)** *UAS-ey/+; Dpp-Gal4/+* and **(E)** *UAS-ey/Ser-Gal4*late third instar larvae. **(F, G)** Ectopic Dac expression in the **(F)** *UAS-ey/+; Dpp-Gal4/+* and **(G)** *UAS-ey/Ser-Gal4*late third instar larval wing discs. **(H - I')** Ectopic *eyeless* expression leads to higher level of Dpp expression in **(H)** *UAS-ey/+; Dpp-Gal4/+* and **(I)** *UAS-ey/Ser-Gal4 larval* wing discs. Ectopic Dac expression exactly overlap with extended Dpp expression domain in the wing discs of **(H')** *UAS-ey/+; Dpp-Gal4/+* and **(I')** *UAS-ey/Ser-Gal4* larvae. **(J)** RT PCR results revealed elevated level of *sine oculis* (*so)* transcript in *UAS-ey/+; Dpp-Gal4/+* and *UAS-ey/Ser-Gal4* third instar larval wing imaginal discs as compared to w^{1118} larval wing imaginal discs.

discs, this indicated the involvement of some other factors to collaborate with Ey for evoking RD genes expression. Previous studies have also clearly established that Eyeless needs Dpp to transcriptionally activate *so*, *dac* and *eya* (Chen et al., 1999; Firth and Baker, 2009; Pappu et al., 2005). Therefore, in an attempt to understand whether Dpp plays any role in restricting the domain of Eya and Dac expression, the domains of ectopic Eya and Dac expression were mapped with respect to Dpp expression along A/P boundary. Dpp expression was analyzed either by reporter RFP expression or by Dpp-lacZ expression. In wild type wing imaginal discs, Dpp expression was observed in a narrow strip of cells along the anterior posterior boundary (Fig.3.3B). In contrast, ectopic expression of *eyeless* by *Dpp-Gal4* led to overexpression of Dpp in the endogenous Dpp domain as revealed by reporter RFP expression (Fig.3.4H). Similarly, over expression of Dpp was also observed in wing discs of *UAS-ey*/*Ser-Gal4* larvae specifically in the region undergoing ectopic ommatidial differentiation as shown by Dpp-lacZ expression (Fig.3.4I). Interestingly, when expression of Dac was analyzed with respect to Dpp expression, there was complete overlap of Dac expression domain with the elevated Dpp expression in case of *UAS-ey*/+*; Dpp-Gal4*/+ larval wing imaginal discs (Fig.3.4H′). Even in case of *UAS-ey*/*Ser-Gal4* larval wing discs the domain of Dac expression completely overlapped with Dpp domain in the posterior-dorsal compartment (Fig.3.4I′).

These results clearly demonstrated that conversion of wing disc cells to photoreceptors was always restricted to the domain of elevated Dpp expression normally observed in these discs. These results corroborate with the previous results that established the synergistic role of Mad dependent canonical Dpp signaling along with Ey in regulating RD genes expression during normal eye development

3.2.3. Attenuating Canonical Dpp signaling pathway inhibits ectopic photoreceptor differentiation

Signaling by Dpp is triggered upon its binding to its receptor consisting of thickveins (Tkv) and Punt (Put). Activated tkv, in turn, phosphorylates Mad (Mothers against dpp) (fly homolog of R-SMAD in vertebrates) that forms a heterodimer with Medea (fly homolog of C-SMAD in vertebrates) (Raftery and Sutherland, 1999; Sekelsky et al., 1995) and translocate into the nucleus to activate transcription of Dpp target genes.

Dpp signaling was blocked by co-expressing *UAS-tkv (dsRNA)* to knock down the expression of its receptor *tkv* specifically in these transdetermining cells and checked for the expression of RD genes in wing discs undergoing ectopic photoreceptor differentiation. Knocking down of *tkv* resulted in complete elimination of ectopic Dac expression both in *UAS-ey/+; Dpp-Gal4/+* as well as *UAS-ey/Ser-Gal4* larval wing discs (Fig.3.5A, B). Staining with anti ELAV antibody revealed that there was no differentiation of photoreceptors (Fig.3.5A, B). Furthermore, the knockdown efficiency of *UAS-tkv (dsRNA)* construct used in this study was checked by ubiquitously driving *UAS-tkv (dsRNA)* with *daughterless-Gal4* (*da-Gal4*) at 29ºC and analyzing the transcript level of *tkv* in embryos. qRT-PCR analysis revealed 84% drop in *tkv* transcript level upon knocking down the expression of *tkv* when compared to its wild type expression level (Fig.3.5J).

Drastic reduction was also observed in the level of expression of RD genes upon knocking down the expression of its signal transducer molecule, Mad, specifically in the wing disc cells undergoing photoreceptor differentiation. Knocking down of Mad in these transdermining cells led to drastic reduction in Dac and Eya expression that finally led to complete inhibition of neuronal differentiation (Fig.3.5C-F). To check the efficiency of *UAS-Mad (dsRNA)*, expression of pMad was checked in the wing imaginal discs of *UAS-ey/+; Dpp-Gal4/UAS-Mad (dsRNA)* larvae. As evident from (Fig.3.5H), a significant reduction was detected in the expression of pMad. Intensity profiling along the axis drawn from anterior to posterior compartment showed a drastic drop in pMad level in Dpp region (Fig.3.5I) when compared to corresponding control (Fig.3.10K). Upon quantitation of differentiating photoreceptors, 95% reduction in the number of ectopic ommatidia was found by inhibiting canonical Dpp signaling either upon knocking down the expression of *tkv* or *Mad* as well as by inducing ectopic photoreceptor differentiation in tkv^{7} or Mad^{12} (loss of function allele) heterozygous mutants wing discs (Fig.3.5G).

In sum, these results clearly established the role of canonical Dpp signaling during ectopic neuronal differentiation in the developing wing discs and corroborate with previous findings where Eyeless along with Dpp is known to activate the transcription of RD genes i.e. *eyes absent*, *sine oculis* and *dachshund* (Chen et al., 1999; Firth and Baker, 2009; Pappu et al., 2005) in normal eye development. Therefore, in a manner similar to normal eye development, during ectopic eye formation also, Dpp induces the expression of its target retinal determining genes by its canonical signaling pathway that includes Mad.

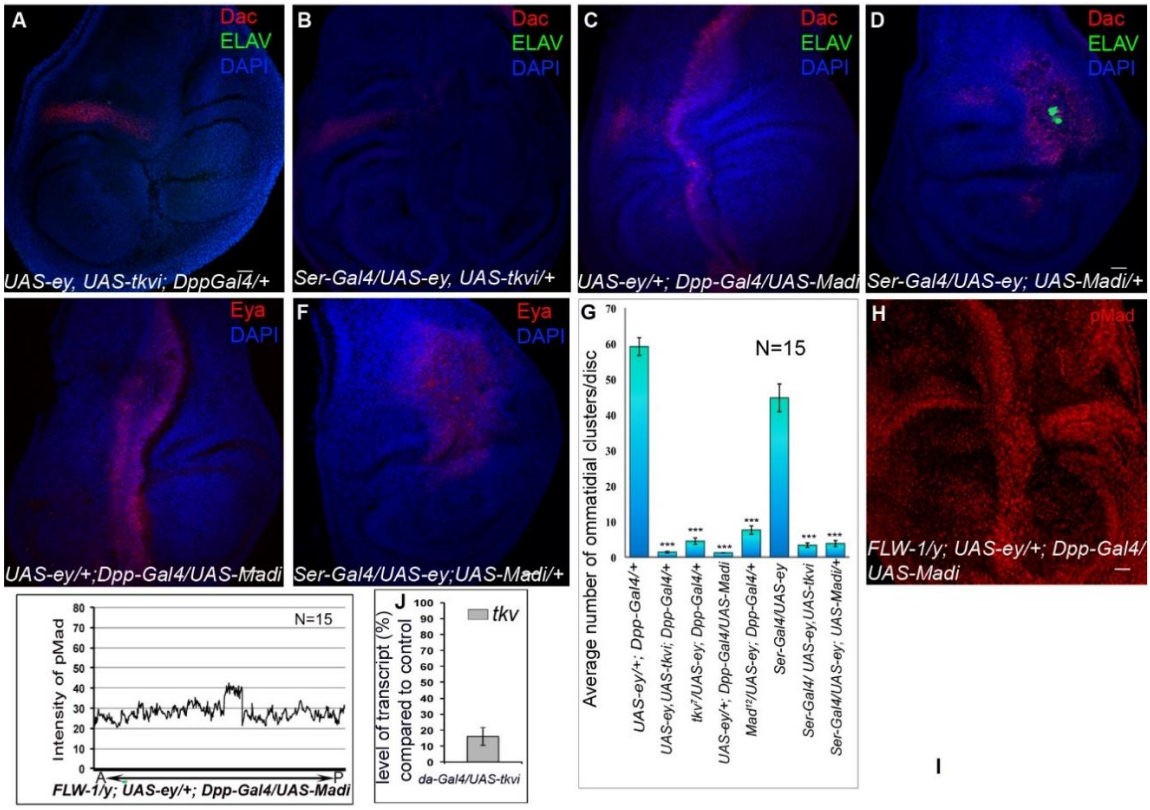

Figure 3.5. Knocking down of Canonical Dpp signaling pathway inhibits ectopic photoreceptor differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A, B)** Impairing Dpp signaling by knocking down the expression of its receptor *tkv* leads to the complete loss of Elav expression and drastically reduced ectopic Dac expression in both **(A)** *UAS-ey/+; Dpp-Gal4/+* and **(B)** *UAS-ey/Ser-Gal4* late third instar wing discs demonstrates complete loss of ectopic eye induction. **(C - F)** Similar loss in ELAV expression and drastic reduction in Dac expression **(C, D)** as well as in Eya expression **(E, F)** was observed by knocking down the activity of transcriptional activator Mad for Dpp canonical signaling in the larval imaginal discs of genotypes mentioned. **(G)** Graph showing average number of ectopic ommatidia in the late third instar larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001). **(H)** Reduced level of pMad expression by knocking down the expression of *Mad* in the Dpp domain.**(I)** Intensity profile of pMad expression in the larval wing imaginal discs of genotype mentioned. **(J)** Graph depicting drastic drop in the level of *tkv* transcript upon driving *UAS-tkv (dsRNA)* with a ubiquitous *da-Gal4* when compared to its level in wild type larvae.

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However, it is important to note that ectopic eye formation is a totally different and more complex process than the normal eye development. During normal eye development, Ey along with Dpp has to activate the RD genes in a cell otherwise determined to form photoreceptors. In contrast, during ectopic eye induction they need to activate eye specific genes in wing cells that are not normally destined to form photoreceptors. Since epigenetic modifications play very significant role in modulating genes expression as the fate of cell gets determined, it is expected that the epigenetic landscape of a wing disc cell is totally different from eye imaginal disc cell. Therefore, to activate the expression of target RD genes in a wing cell by Dpp is more challenging as it involves withdrawal of epigenetic repression. Therefore, I was interested to determine whether Dpp is playing any role beyond its expected known function to activate RD genes in non-neuronal wing cells during ectopic eye formation.

3.2.4. Attenuating the expression of dTak1 affects ectopic photoreceptor differentiation by regulating the level of retinal determining genes:

Studies that include mammalian cell lines and *in vivo* model organisms ranging from fly to mouse have demonstrated that apart from the canonical pathway, members of the TGF-ß family of proteins can modulate various cellular responses by activation of the TGF-ß Activated Kinase 1(TAK1) (Delaney and Mlodzik, 2006; Mihaly et al., 2001; Silverman et al., 2003). TAK1 is a member of MAPKK Kinase family and a key component of various signaling pathways. It activates and phosphorylates MAP2K family members that include MEK1/2, MKK3/6, MKK4/7, and MEK5. As shown in Fig.3.6, in vertebrates, TAK1 forms a multiple protein complex with TAB1, TAB2 or TAB3 and TRAF6 that are functionally conserved and leads to auto phosphorylation and activation of TAK1 (Dai et al., 2012; Delaney and Mlodzik, 2006; Sakurai, 2012). Activated Tak1, in turn, plays very important role in regulating various biological processes including embryonic development, apoptosis, and tumour suppression in prostate and liver tissues apart from its major role in innate and adaptive immunity (Delaney and Mlodzik, 2006; Sakurai, 2012). To investigate the role of *Drosophila* Tak1 (dTak1) in ectopic photoreceptor differentiation, the expression of *dTak1* was knocked down by co expressing *UAS-dTak1 (dsRNA)* specifically in the cells where *eyeless* was ectopically expressed. Interestingly, knocking down of *dTak1* in these cells led to almost complete elimination of ectopic ommatidial differentiation in wing discs (Fig.3.7A, B, G). Knockdown efficiency of *UAS-dTak1 (dsRNA)* was analyzed by driving it ubiquitously with *daughterless-Gal4* (*da-Gal4*) at 29ºC and analyzing the transcript level of *dTak1* in embryos. qRT-PCR analysis

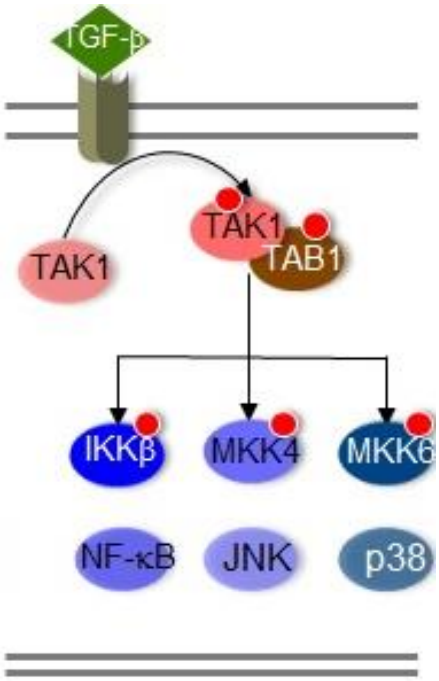

Figure 3.6. Figure showing different signaling pathways triggered by TAK1. TAK1 upon phosphorylation can lead to the phosphorylation of different downstream targets including IKKß, MKK4 and MKK6 thereby can activate various different signal transduction pathways mediated by NF-κB, JNK and p38.

revealed 50.1% drop in *dTak1* transcript level upon knocking down the expression of *dTak1* when compared to its expression level in wild type embryos (Fig.3.8G). Importantly, reduction in ectopic photoreceptors differentiation to similar extent was also observed when ectopic photoreceptor differentiation was induced by either *Dpp-Gal4* or *Ser-Gal4* in the wing discs of $dTak1^2$ (loss of function allele) mutant larvae (Fig.3.7C, D, G). Around 75-80% reduction in number of photoreceptor was observed in $dTak1^2$*/y; UAS-ey/+; Dpp-Gal4/+* as well as in $dTak1^2$*/y; UAS-ey/+; Dpp-Gal4/+* larval wing discs.

To know whether the drop in ectopic photoreceptor differentiation was a result of any change in RD genes expression, the expression of RD proteins Eya and Dac was checked in *dTak1* knockdown as well as in *dTak1* mutant background. Although, no appreciable change in their domains of expression was observed, there was drop in their level of expression. The drop in ectopic photoreceptor differentiation, however, was not due to drastic reduction in Dac and Eya expression as observed upon attenuating the canonical Dpp signaling. Rather, reduced level of Dac expression was found upon attenuating the activity of dTak1 in $dTak1^2$ mutant background (Fig.3.7C, D). Similar reduction in the expression level of ectopic Dac and Eya was also observed by knocking down the expression of *dTak1* (Fig.3.7E, F and 3.7H, I). To have quantitative estimate, fluorescence intensity of Dac and Eya was quantified in their expression domains and calculated the fold change in their intensity compared to their respective controls. Quantitative estimation revealed 30-40% (n=15) drop in Dac and Eya protein level in *UAS-ey/+; UAS-dTak1 (dsRNA)/Dpp-Gal4* as well as in $dTak1^2$*/y; UAS-ey/+; Dpp-Gal4/+* larval wing discs (Fig 3.8A). Almost similar 25-35% (n=15) drop in Dac expression level was observed in *UAS-ey/+; UAS-dTak1 (dsRNA)/Ser-Gal4* and $dTak1^2$*/y; UAS-ey/Ser-Gal4* larval wing discs (Fig.3.8B). Similarly, Around 22% (n=15) drop in the level of Eya expression was observed in these wing discs (Fig.3.8B). However, even though, there was significant drop in the level of expression of RD proteins, change in the domain of their expression was not appreciable as revealed upon quantitatation of the area of expression (Fig.3.8C, D).

Furthermore, to determine whether the regulation of RD genes by dTak1 was at the level of transcription, transcript level of *dac*, *eya* and *so* was analyzed by qRT-PCR analysis. Interestingly, drastic drop i.e. 70-80% in their transcript levels was found in case of *UAS-ey/+; UAS-dTak1(dsRNA)/Dpp-Gal4* larval imaginal discs as compared to *UAS-ey/+; Dpp-Gal4/+* larval wing discs (Fig.3.8E).

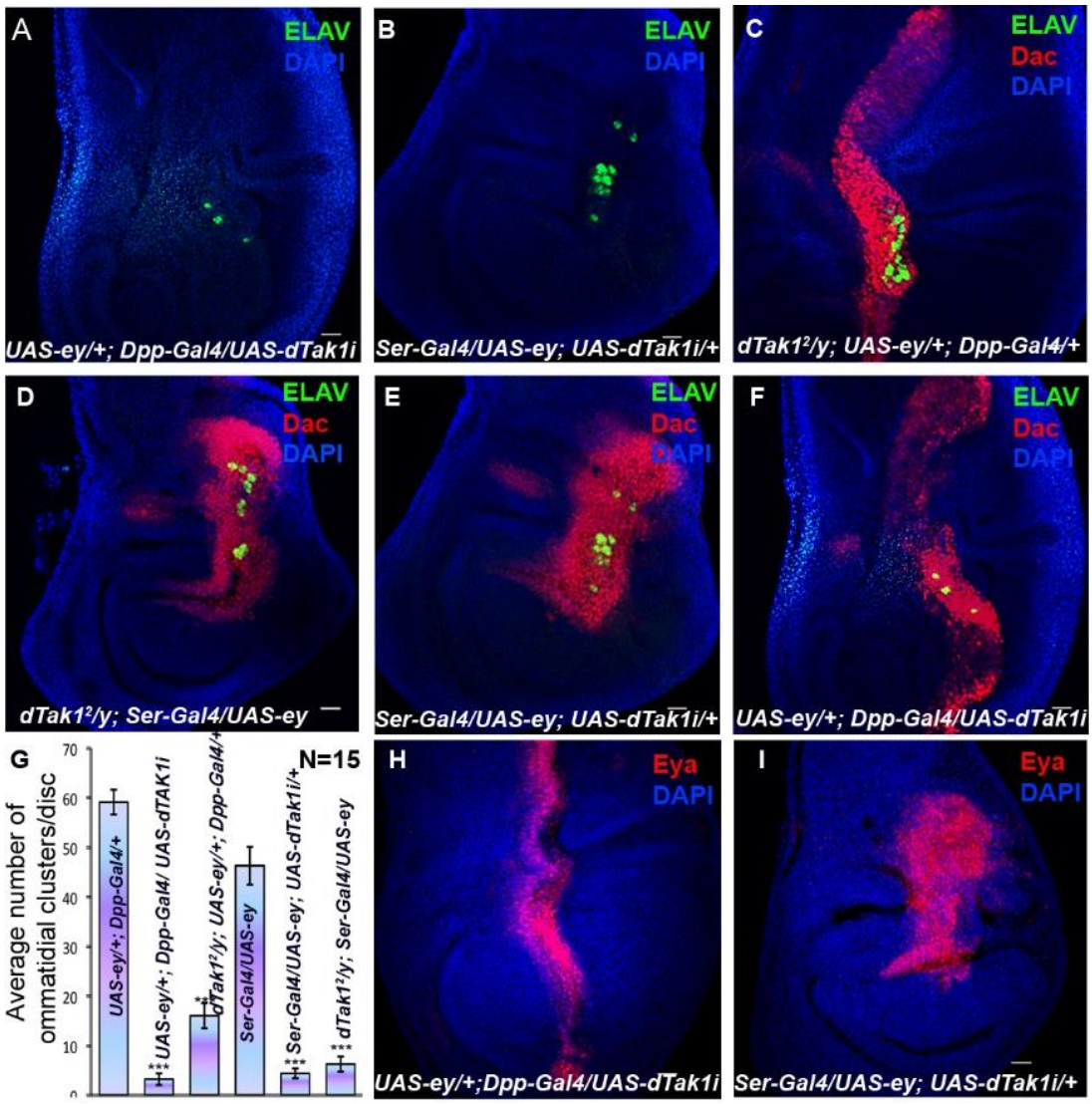

Figure 3.7. Knocking down the expression of dTak1 affects ectopic photoreceptor differentiation by regulating the level of Retinal Determining (RD) genes expression. (A, B) Knocking down the expression of *dTak1* significantly affects the ectopic neuronal differentiation as revealed by ELAV immunostaining in the wing discs of **(A)** *UAS-ey/+; Dpp-Gal4/UAS-dTak1 (dsRNA)* and **(B)** *UAS-ey/Ser-Gal4; UAS-dTak1 (dsRNA)/+* third instar larvae. **(D, E)** Reduced level of Dac expression and consequent decrease in ectopic photoreceptor differentiation was observed upon attenuation of *dTak1* activity in the wing discs of **(D)** *dTak1^2/y; UAS-ey/+; Dpp-Gal4/+* and **(E)** *dTak1^2/y; UAS-ey/Ser-Gal4* larvae. **(E, F)** Knocking down the expression of *dTak1* significantly affects the ectopic neuronal differentiation as shown by ELAV expression as well as results in reduced Dac expression in the wing discs of **(F)** *UAS-ey/+; Dpp-Gal4/UAS-dTak1 (dsRNA)* and **(E)** *UAS-ey/Ser-Gal4; UAS-dTak1 (dsRNA)/+* larvae. **(G)** Quantitative estimate of average number of ectopic ommatidia in the late third instar larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001). **(H, I)** Knocking down the expression of *dTak1* significantly affects ectopic Eya expression in wing imaginal discs of **(H)** *UAS-ey/+; Dpp-Gal4/UAS-dTak1 (dsRNA)* and **(I)** *UAS-ey/Ser-Gal4; UAS-dTak1 (dsRNA)/+* larvae.

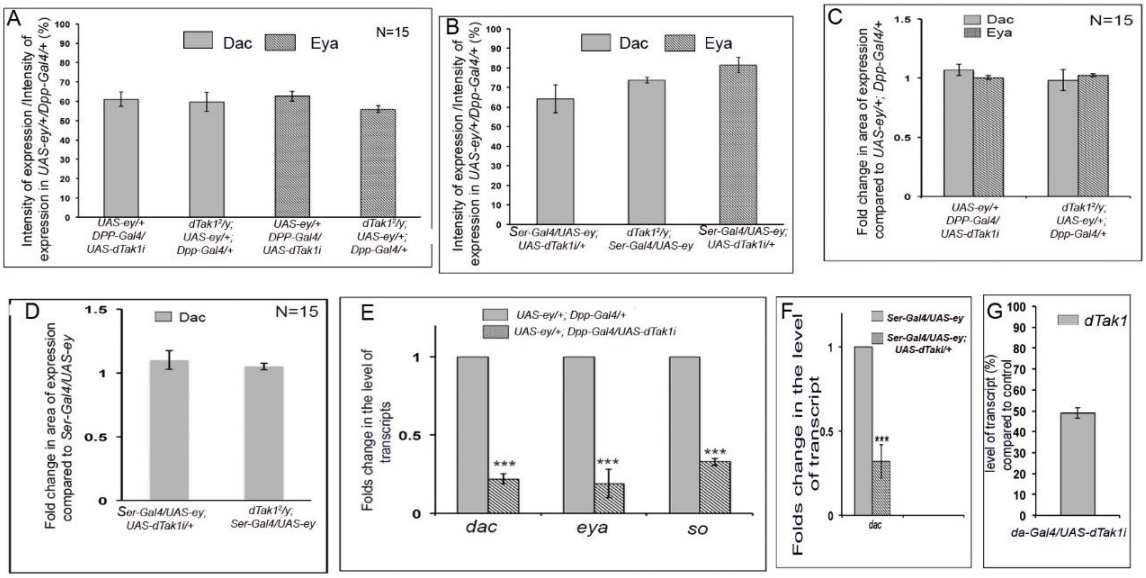

Figure 3.8. Knocking down the expression of dTak1 affects the level of expression of retinal determining genes. (A, B) Quantitative analysis of fluorescence intensity in ectopic Dac and Eya expression domains in wing discs of genotypes mentioned when compared to their controls. **(C, D)** No significant change observed in the area of Dac and Eya expression domain in wing discs of genotypes mentioned when compared to their controls. **(E, F)** Real time PCR results exhibiting drastic drop in the level of *dac*, *eya* and *so* transcripts in larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001)**. (G)** Graph depicting drastic drop in the level of *dTak1* transcript upon driving *UAS-Tak1 (dsRNA)* with a ubiquitous *da-Gal4* driver when compared to its level in wild type larvae

Similarly, Around 70% drop in the transcript level of *dac* in case of *UAS-ey/ Ser-Gal4; UAS-dTak1 (dsRNA)/+* larval imaginal discs as compared to that of *UAS-ey/Ser-Gal4* larval wing discs (Fig.3.8F).

From all these results it is evident that attenuation of dTak1 leads to significant drop in the expression level of RD genes both at the transcript level as well as in protein level that finally results in reduced ectopic ommatidial differentiation. So, these results suggest that a threshold level of expression of RD genes is critical for ectopic eye formation. Since by attenuating the activity of dTak1, that level was not achieved, as a result ectopic photoreceptors failed to differentiate. Moreover, these results clearly show that dTak1 is playing a specific and critical role to regulate the expression of RD genes during ectopic photoreceptor differentiation

3.2.5. Impairing dTak1 activity does not alter ommatidial differentiation during normal eye development

It is quite possible that during normal ommatidial differentiation in developing eye discs dTak1 plays a yet to be known role in regulating RD genes expression. Therefore, in order to ascertain whether dTak1 plays any role during normal eye development, Dac and Eya expression were analyzed in the eye imaginal discs of $dTak1^{2}$ (loss of function allele) as well as $dTak1^{179}$ (loss of function allele) mutant larvae. No alteration was observed in the level as well as pattern of expression of Dac and Eya in these mutant eye discs (Fig.3.9B, C, and E) when compared with wild type eye imaginal discs (Fig.3.9A, D). Moreover, upon immunostaining with antibody against ELAV, normal photoreceptors differentiation was observed in the eye discs of $dTak1^{2}$ and $dTak1^{179}$ larvae (Fig 3.9G, H) similar to that observed in wild type eye imaginal discs (Fig.3.9F)

Together, these results indicate that during normal eye development dTak1 is not involved in regulating the expression of Dac, Eya and as a consequence photoreceptor differentiation in developing eye imaginal discs. This in turn confirms that the role-played by dTak1 in modulating Dac and Eya expression during ectopic eye induction is specific to that process.

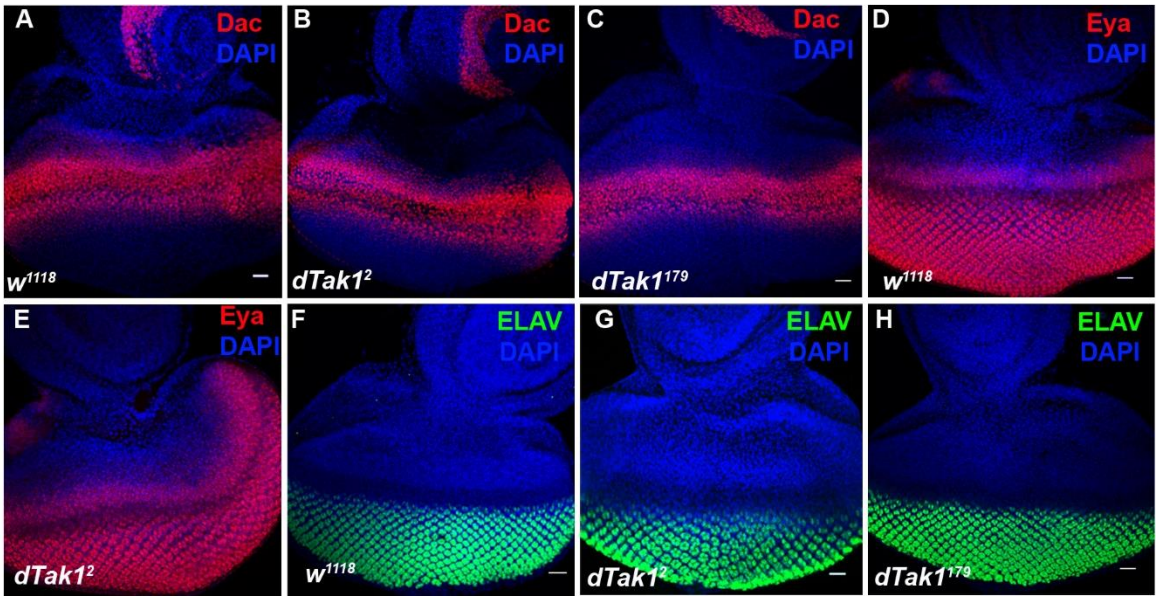

Figure 3.9. Tak1 has no effect on ommatidial differentiation during normal eye development. For all eye discs genotypes are as mentioned. Scale= 20µ **(A-C)** No change in Dac expression is observed in the eye imaginal disc of larvae mutant for *dTak1* in **(B)** *dTak1*^{2}*and* **(C)** *dTak1*^{179} when compared to that observed in **(A)** w^{1118} eye imaginal disc. **(D, E)** No change in Eya expression is observed in the eye imaginal disc of larvae mutant for **(E)** d*Tak1*^{2}when compared to **(D)** w^{1118} eye imaginal disc. **(F-H)** No effect on photoreceptor differentiation as revealed from ELAV expression is observed in the eye imaginal disc of larvae mutant for **(G)** *dTak1*^{2} and **(H)** *dTak1*^{179} when compared to **(F)** w^{1118} eye imaginal disc.

3.2.6. Activation of dTak1 by Dpp is independent of the canonical Dpp signaling pathway

To further investigate the role of dTak1, the level of *dTak1* expression was analyzed in wild type wing disc and compared it with change in expression if any in wing discs upon ectopic expression of *eyeless*. But, results of both RT-PCR (Fig. 3.10A) as well as qRT-PCR (Fig.3.10B) analyses did not reveal any appreciable change in the expression level of *dTak1* in *UAS-ey/+; Dpp-Gal4/+* and *UAS-ey/Ser-Gal4* wing discs as compared to wild type wing imaginal discs.

Next, genetic epistasis experiment was performed to check whether dTak1 acts in a Dpp dependent manner. For this analysis, Dpp was overexpressed in the discs undergoing ectopic photoreceptor differentiation by driving *UAS-Dpp* with *Dpp-Gal4*. This resulted in robust increase in the number of ectopic photoreceptor differentiation (average ommatidial number = 349, n=12) in wing discs of *UAS-ey/+; Dpp-Gal4/UAS-Dpp* larvae (Fig.3.10C, E) as compared to that observed in (average ommatidial number = 64, n=12) *UAS-ey/+; Dpp-Gal4/+* third instar larval wing imaginal discs (Fig.3.4A, C). Interestingly, photoreceptor differentiation got drastically reduced upon attenuation of dTak1 activity in wing discs of $dTak1^{2}/y$; *UAS-ey/+; Dpp-Gal4/UAS-Dpp* larvae (average ommatidial number = 40, n=12) (Fig.3.10D, E). These results clearly show that dTak1 is acting downstream of Dpp.

Furthermore, to understand whether dTak1 has any feedback mechanism to regulate Dpp expression, expression of Dpp was monitored upon attenuating the activity of dTak1 in the wing discs undergoing ectopic eye differentiation. For this purpose, the line *UAS-ey/UAS-RFP; Dpp-Gal4/+* was employed where reporter RFP expresses in the Dpp domain. For quatitative analysis, the fluorescence intensity of RFP expression as well as the area in which reporter RFP expresses were measured and compared to that in control wing discs. No change in Dpp expression was observed both in terms of intensity as well as in its area of expression as revealed by reporter RFP expression in *UAS-ey/+; UAS-dTak1 (dsRNA)/Dpp-Gal4* third instar larval wing imaginal discs (Fig.3.10G-I) when compared to control *UAS-ey/+; Dpp-Gal4/+* larval wing discs (Fig.3.10F). Together these results clearly establish that dTak1 acts downstream of Dpp during ectopic eye formation.

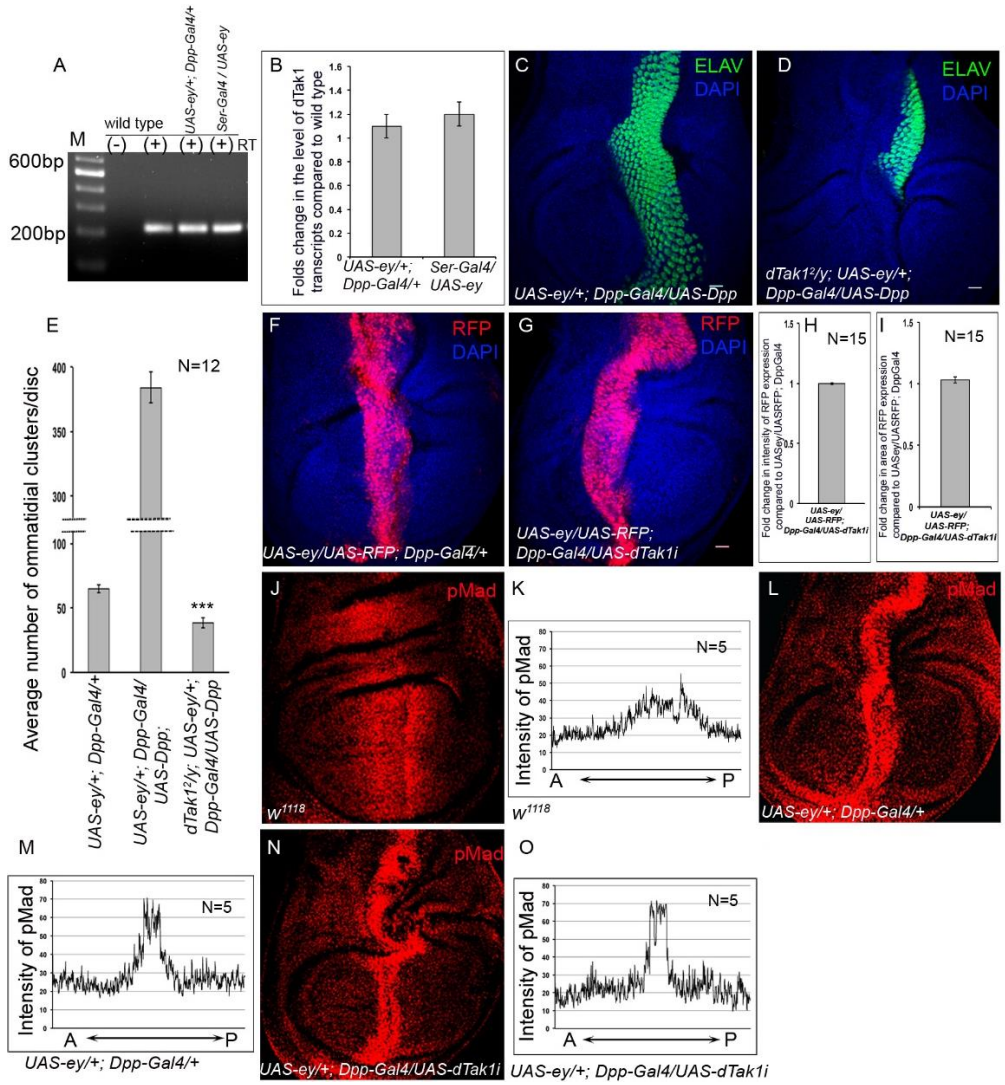

Figure 3.10. dTak1 acts downstream of Dpp, independent of the canonical pathway, to regulate ectopic eye differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A)** RT PCR analysis shows no change in the level of *dTak1* transcript in wing imaginal discs of *UAS-ey/+; Dpp-Gal4/+* and *UAS-ey/Ser-Gal4* third instar larvae as compared to the wing imaginal discs of w^{1118} third instar larvae. **(B)** Real time qPCR analysis also does not reveal significant changes in the transcript expression of *dTak1* in the wing imaginal of *UAS-ey/+; Dpp-Gal4/+* and *UAS-ey/Ser-Gal4* third instar larvae as compared to w^{1118} third instar larval wing discs.**(C, D)** Robust increase in ectopic ommatidial differentiation as revealed by ELAV expression by co-expression of *UAS-Dpp* along with *UAS-ey* in the wing discs of *UAS-ey/+; Dpp-Gal4/UAS-Dpp* third instar larvae **(C)** that gets significantly reduced upon impairing dTak1 activity in this background in the wing discs of $dTak1^2/y$*; UAS-ey/+; Dpp-Gal4/UAS-Dpp* third instar larvae **(D). (E)** Quantitative estimate of average number of ectopic ommatidia in late third instar larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001). **(F, G)** No appreciable changes in the reporter RFP expression for Dpp upon **(G)** generating ectopic eyes in the wing discs where the expression of *dTak1* was knocked down when compared to its expression in the wing discs of **(F)** *UAS-ey/+; Dpp-Gal4/+* late third instar larvae. **(H, I)** Quantification of changes in the expression of Reporter RFP fluorescence intensity **(H)** and area of expression **(I)** by knocking down the expression of *dTak1* in wing discs of *UAS-ey/+; Dpp-Gal4/UAS-dTak1(dsRNA)* late third instar larvae as compared to its expression in *UAS-ey/+; Dpp-Gal4/+* late third instar larval wing discs. **(J, L, N)** Expression of pMad in larval imaginal discs of genotypes mentioned. **(K, M, O)** Averege intensity profile of pMad expression in larval imaginal discs of genotypes mentioned.

Next, to determine whether the observed drop in the expression of RD genes expression in dTak1 knockdown background was due to any alteration in the canonical Dpp signaling cascade, the expression of phosphorylated Mad (pMad), the signal transducer for canonical Dpp signaling was monitored in *dTak1* knockdown background. Interestingly, high level of pMad expression was observed at the place of ectopic photoreceptor differentiation (Fig.3.10L, M) as compared to wild type larval wing discs (Fig.3.10J, K). However, this increase in the level of pMad remained unaltered upon knocking down the expression of *dTak1* in the wing discs during ectopic photoreceptor differentiation (Fig.3.10N, O). Intensity analysis performed along the axis drawn from anterior to posterior compartment also revealed that expression of pMad remains unaltered upon knocking down the expression of *dTak1* (Fig.3.10M, O). Therefore, these results clearly show that dTak1 modulates the levels of RD genes during ectopic eye formation independent of canonical Dpp signaling.

So, collectively these results obtained so far establish two things,

1) Dpp is activating both canonical and a noncanonical signaling cascade during ectopic photoreceptor differentiation to regulate the expression of RD genes.

2) Both canonical as well as noncanonical pathways are indispensable for ectopic photoreceptor differentiation because impairment of any of these pathways leads to complete inhibition or drastic reduction of ectopic photoreceptor differentiation.

3.2.7. Imairing the activity of members of Jun-Kinase (JNK) pathway affects ectopic photoreceptor differentiation.

To identify the downstream target of dTak1 responsible for regulating the RD genes expression, components of many different signal transduction pathways (including NFkB, p38 and JNK) that are known to get activated by dTak1 (Dai et al., 2012) were systematically eleminated and screened for their role in ectopic eye formation. The different genotypes analyzed are shown in table 3.1.

Table 3.1: Genotypes of flies used to screen for downstream target of dTak1.

JNK pathway	
1	*UAS-bsk^{DN}/y; UAS-ey/+; Dpp-Gal4/+*

2	*UAS-bsk^{DN}/y; UAS-ey/Ser-Gal4*
3	*hep^{r75}/+; UAS-ey/+; Dpp-Gal4/+*
4	*hep^{r75}/+; UAS-ey/Ser-Gal4*
P38 pathway	
1	*UAS-ey/+; UAS-p38a (dsRNA)/Dpp-Gal4*
2	*UAS-ey/Ser-Gal4; UAS-p38a (dsRNA)/+*
3	*UAS-ey/+; UAS-p38b(dsRNA)/Dpp-Gal4*
4	*UAS-ey/Se-rGal4; UAS-p38b(dsRNA)/+*
5	*UAS-p38b^{DN}/y; UAS-ey/+; Dpp-Gal4/+*
6	*UASp38b^{DN}/y; UAS-ey/Ser-Gal4*
IMD pathway	
1	*UAS-ey/UAS-IKKb(dsRNA); Dpp-Gal4/+*
2	*UAS-ey/+; UAS-IKKb(dsRNA)/Dpp-Gal4*
3	*UAS-ey/Ser-Gal4; UAS-IKKb(dsRNA)/+*
4	*UAS-ey/DmIKKg^{Key1} (Kenny1); Dpp-Gal4/+*
5	*UAS-ey/+; rel^{E20}/Dpp-Gal4*
6	*UAS-ey/Ser-Gal4; rel^{E20}/+*

In doing so, the involvement of JNK pathway was found in this process. In *Drosophila*, JNK is known as *basket* (Sluss et al., 1996). JNK is MAPK known to get phosphorylated by MAPKK. hep is MAPKK homolog in *Drosophila*, which phosphorylate JNK (Glise et al., 1995). Hep in turn gets phosphorylated by MAPKKKs of which one is dTak1 (Davis, 2000). JNK gets activated in response to various kinds of stress and plays diverse role in development and disease (Adachi-Yamada et al., 1999; Agnes et al., 1999; Sluss et al., 1996; Weston and Davis, 2007).

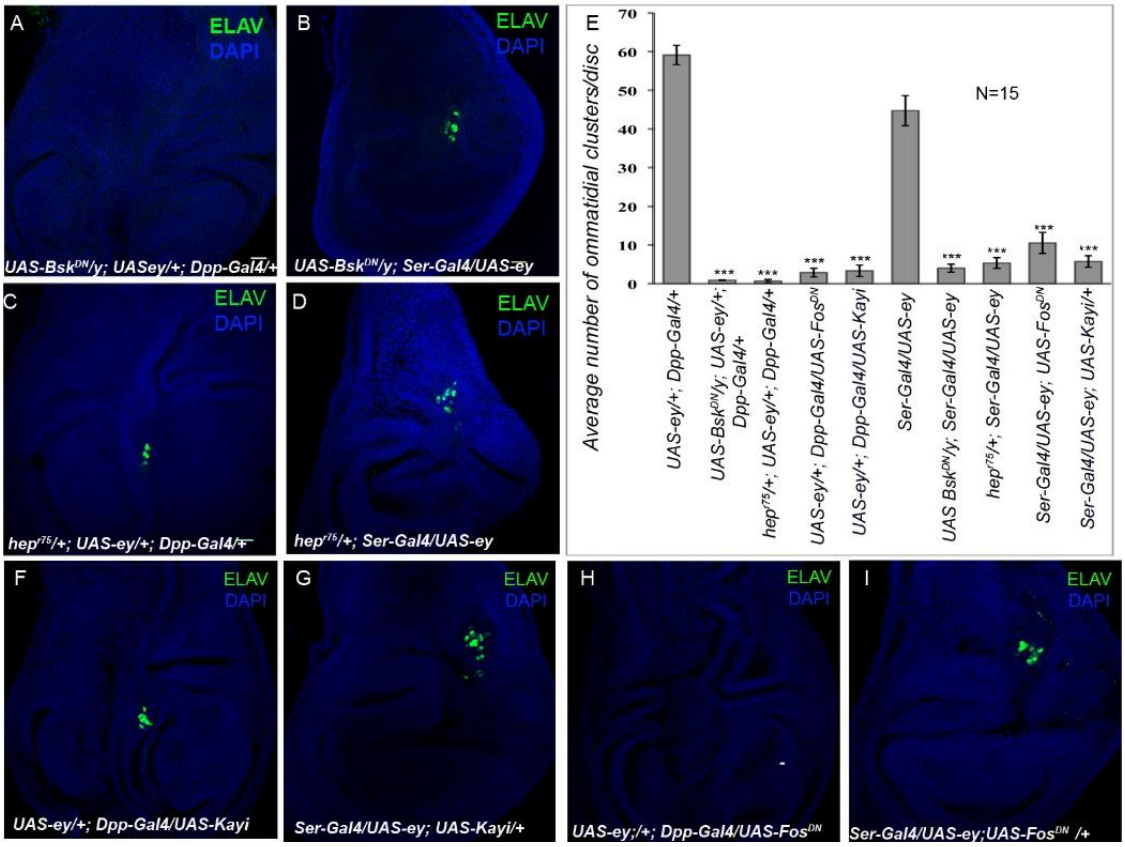

Figure 3.11. Impairing the activity of Jun-N-terminal Kinase (JNK) pathway affects ectopic photoreceptor differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A, B)** Inactivation of JNK signaling by co-expression of dominant negative form of *basket* along with *eyeless* drastically suppressed ectopic differentiation of photoreceptors. **(C, D)** Impairing JNKK activity by bringing ectopic eye formation in the background mutant for hep^{r75} also resulted in to drastic drop in ectopic eye induction. **(E)** Quantitative estimate of average number of ectopic ommatidia in the late third instar larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001). **(F-I)** Attenuating JNK activity by knocking down the expression of *kayak* **(F, G)** and by co-expressing dominant negative form of dFos **(H, I)** also resulted in drastic drop in ectopic eye induction.

Simultaneous over-expression of dominant negative form of *basket* along with *eyeless* by *Dpp-Gal4* and *Ser-Gal4* led to a dramatic (90-95%) reduction in the number of differentiated ectopic photoreceptors (Fig.3.11A, B, and E). Similar 90-95% reduction in ectopic ommatidial differentiation was observed when ectopic eyes were generated in the background mutant for *hemipterous* (*hep*) (Fig. 3.11C-E). Put together, these results clearly indicate the involvement of JNK in this process.

Activated JNK can lead to the transcriptional activation of target genes by the two transcription factors, one is AP-1 (heterodimer of dJun and dFos) (Kockel et al., 2001; Riesgo-Escovar and Hafen, 1997) and other one is FoxO (Wang et al., 2005) (Fig.3.12). dJun after phoshphorylation binds with dFos, encoded by the gene *kayak* (*kay*) in *Drosophila*, to form the transcriptional activator protein 1 (AP-1) (Kockel et al., 2001; Riesgo-Escovar and Hafen, 1997). It was observed that either over-expressing a dominant negative form of dFos or knocking down the expression of *kay* in the cells undergoing ectopic photoreceptor differentiation led to drastic decrease (90%) in the number of ectopic photoreceptors (Fig. 3.11E-I).

The knockdown efficiency of *UAS-kay (dsRNA)* construct used in this study was checked by ubiquitously driving *UAS-kay (dsRNA)* with *da-Gal4* at 29ºC and analyzing the transcript level of *kay* in embryos by qRT-PCR analysis. qRT-PCR analysis revealed 58% drop in *kay* transcript level upon knocking down the expression of *kay* when compared to its wild type expression level (Fig.3.13G).

To establish that activation of JNK pathway is triggered by Dpp signaling, genetic epistasis experiment was performed. As mentioned earlier, overexpression of Dpp in the discs undergoing ectopic photoreceptor differentiation led to robust increase in the number of ectopic photoreceptor number (Fig.3.10C). However, this increase in number got drastically suppressed when ectopic photoreceptors were induced in a genetic background hep loss of function allele hep^{r75} (Fig. 3.13A, D). In conjuction with this observation, no change in the expression of Dpp was observed in terms of its intensity as well as its area of expression as revealed by reporter RFP expression when ectopic eyes were induced in the wing discs mutant for *hep* (Fig. 3.13B, C, E, F). Together these results clearly establish that JNK acts downstream of Dpp during ectopic eye formation and is involved in regulating the number of ectopic photoreceptors.

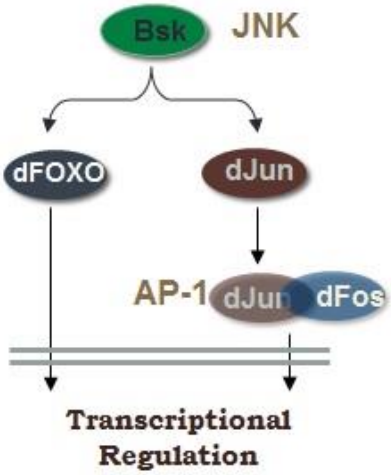

Figure 3.12. Signal transduction pathway of JNK. JNK regulates target gene expression by activating dFOXO or AP-1 transcription factor

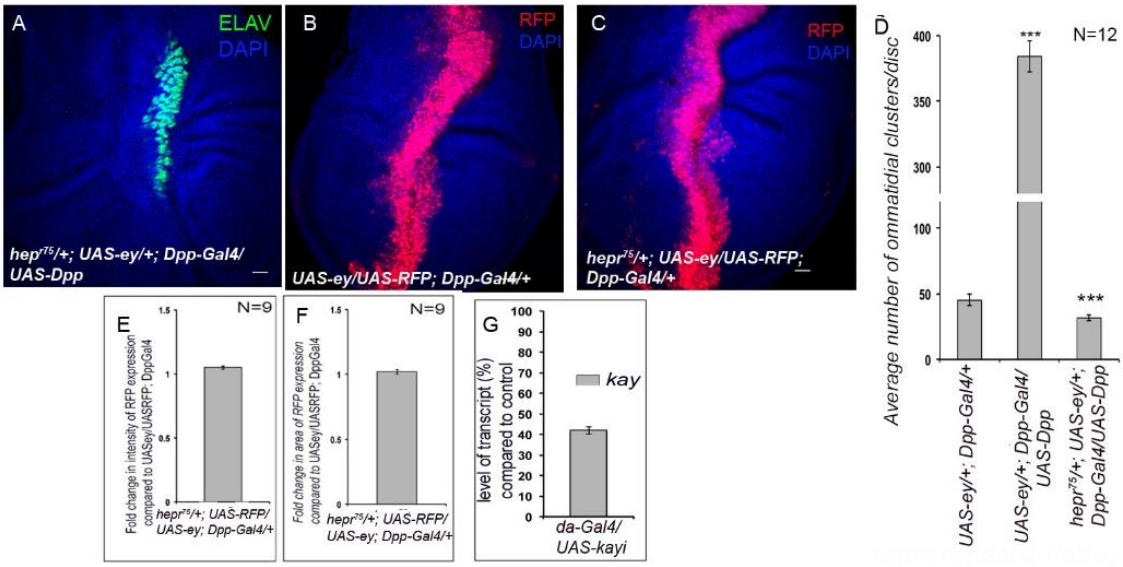

Figure 3.13. Impairing the activity of Jun-N-terminal Kinase (JNK) pathway affects ectopic photoreceptor differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A)** Robust increase in ectopic ommatidial differentiation as seen upon over expression of Dpp got significantly reduced by impairing *hep* activity. **(B, C)** No appreciable change in reporter RFP expression for Dpp was seen upon attenuating the activity of *hep* by generating ectopic eye in the wing discs of **(C)** *hepr*^{75}*/+; UAS-ey/+; Dpp-Gal4/+* larvae when compared to its expression in the wing discs of **(B)** *UAS-ey/+; Dpp-Gal4/+* late third instar larvae. **(D)** Quantitative estimate of average number of ectopic ommatidia in the late third instar larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001). **(E, F)** Quantification of changes in Reporter RFP intensity **(E)** and its area of expression **(F)** in the wing discs of *hepr*^{75}*/+; UAS-ey/+; Dpp-Gal4/+* larvae when compared to their control wing discs of *UAS-ey/+; Dpp-Gal4/+* late third instar larvae. **(G)** Quantitative estimation showing drastic drop in the level of *kayak* transcript in the wing discs upon driving *UAS-kay (dsRNA)* with a ubiquitous *da-Gal4* when compared to its level in the wing discs of wild type larvae.

3.2.8. Attenuation of FoxO activity has no effect on ectopic photoreceptor differentiation

Involvement of FoxO, the other transcriptional factor of the JNK pathway was also checked. First, the activation of FoxO was analyzed by using reporter *thor-lacZ* line. *Thor is a* downstream target of FoxO and it is normally known to express in ring glands, fat bodies but not in wing imaginal discs (Puig et al., 2003; Teleman et al., 2008).

While, normal Thor-lacZ expression in ring glands of *thor-lacZ* larvae was detected (Fig.3.14C), but, wing disc cells undergoing ectopic photoreceptor differentiation as well as wild type wing discs were devoid of any Thor-lacZ expression (Fig. 3.14A, B). In order to have a genetic correlate, ectopic neuronal differentiation was carried in the wing discs where the the expression of *FoxO* was knocked down. There also, ectopic photoreceptor differentiation remained unaffected as revealed by ELAV immunostaining (Fig. 3.14D, E). Similar results were observed when ectopic photoreceptor differentiation was carried out in the wing discs otherwise heterozygous mutant for *FoxO* (Fig. 3.14F, G). Moreover, quantitative analysis also did not reveal any significant alteration in the number of ectopic photoreceptors upon attenuation of FoxO activity (Fig. 3.14H). All these results clearly demonstrate that FoxO is not involved in regulating ectopic photoreceptors differentiation mediated by JNK.

3.2.9. Expression of puc-lacZ during ectopic photoreceptor differentiation is modulated by Dpp signaling through dTak1.

In an attempt to study the activation of JNK during ectopic eye formation**,** the expression of the gene *puckered* (*puc*), which is a direct transcriptional target of the JNK pathway, was analyzed as a reporter for JNK activity in the wing discs undergoing ectopic photoreceptors differentiation (Martin-Blanco et al., 1998). Normally *puc* is known to express in a small set of cells in the dorsal-anterior most notal part while the disc proper cells in the pouch region are devoid of any *puc* expression (Agnes et al., 1999) (Fig. 3.15A). However, immunostaining with anti ß-galactosidase antibody revealed high level of ectopic Puc expression in the wing disc cells undergoing ectopic photoreceptors differentiation. Interestingly, intense pattern of ectopic puc-lacZ expression was observed along the A-P axis in case of *UAS-ey*/+; *Dpp-Gal4*/+ larval wing discs (Fig. 3.15B). Similarly, ectopic puc-lacZ expression was induced in the dorsal posterior cells undergoing

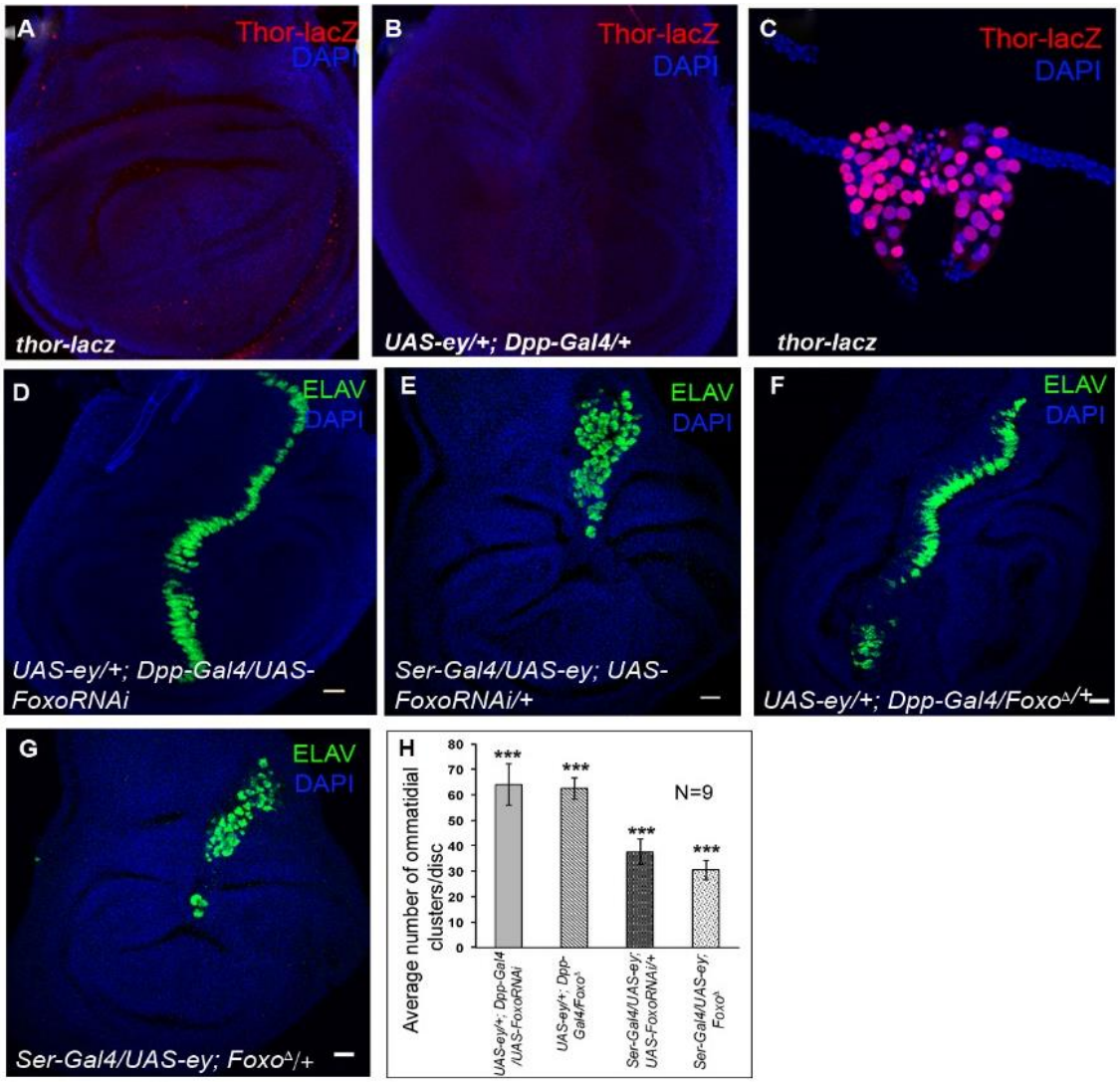

Figure 3.14. FoxO does not affect ectopic photoreceptor differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A-C)** Thor lacZ expression in the wing discs of **(A)** *thor-lacZ* **(B)** *UAS-ey/+; Dpp-Gal4/+* and **(C)** ring gland of *thor-lacz* third instar larvae. **(D, E)** No effect on ectopic neuronal differentiation by knocking down the expression of *Foxo* by co-expressing *UAS-Foxo (dsRNA)* as revealed by ELAV expression. **(F, G)** Similarly, ectopic neuronal differentiation remains unaffected in the wing discs of third instar larvae heterozygous mutant for *Foxo*. **(H)** Quantitative estimate of average number of ectopic ommatidia in the late third instar larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001).

ectopic photoreceptors differentiation in *UAS-ey*/*Ser-Gal4* third instar larval wing discs (Fig. 3.15C).

To determine whether this puc-lacZ expression is induced by the Dpp-dTak1 signaling cascade, Dpp signaling was attenuated by knocking down the expression of one of its receptor *thickveins* (*tkv*) or by inducing ectopic eyes in wing discs heterozygous mutant for *tkv* loss of function allele tkv^7 and checked for puc-lacZ expression by anti ß-galactosidase staining. Interestingly, attenuation of Dpp signaling by impairing the activity of tkv resulted in drastic reduction in the level of puc-lacZ expression in these cells, suggesting that the the activation of JNK was mediated by Dpp (Fig. 3.15D). On quantitative analysis, almost 80% reduction in the domain of puc-lacZ expressing cells was found upon attenuating the activity of *tkv* (Fig. 3.15L). Similar reduction was observed in the level of puc-lacZ expression in these wing cells when they were induced to undergo photoreceptor differentiation in a genetic background otherwise mutant for *dTak1* in $dTak1^2$*/y; UAS-ey/+; Dpp-Gal4/+* and $dTak1^2$*/y; UAS-ey*/*Ser-Gal4* larval wing discs (Fig. 3.15E, F). 70-80% reduction in the domain of puc-lacZ expressing cells was observed upon attenuation of dTak1 activity in the cells undergoing ectopic photoreceptors differentiation (Fig. 3.15L, M). These results clearly show that the activation of JNK is mediated by dTak1 during ectopic photoreceptor differentiation.

Likewise, co-expression of dominant negative form of *bsk* in the cells undergoing photoreceptors differentiation also resulted in drastic drop in puc-lacZ expression in *UAS-*Bsk^{DN}*/y; UAS-ey/+; Dpp-Gal4/puc-lacZ* and *UAS-*Bsk^{DN}*/y; UAS-ey*/*Ser-Gal4; puc-lacZ*/+ larval wing discs (Fig. 3.15J, K). However, the puc-lacZ expression remained unaltered when ectopic eyes were generated in a genetic background heterozygous mutant for *Mad* (Fig. 3.15G, L). Similarly, no appreciable change in the puc-lacZ expression was detected upon knocking down the expression of *Mad* and *Medea* (*Med*) in the cells undergoing ectopic photoreceptors differentiation (Fig. 3.15H, I, M). These results clearly establish that activation of JNK is independent of canonical Dpp signaling.

3.2.10. Activated Jun-N-terminal Kinase (JNK) facilitates ectopic eye formation by regulating the level of RD genes expression.

To investigate the role of JNK in regulating the expression of RD genes during ectopic photoreceptor differentiation, the expression of Dac and Eya was observed in these cells

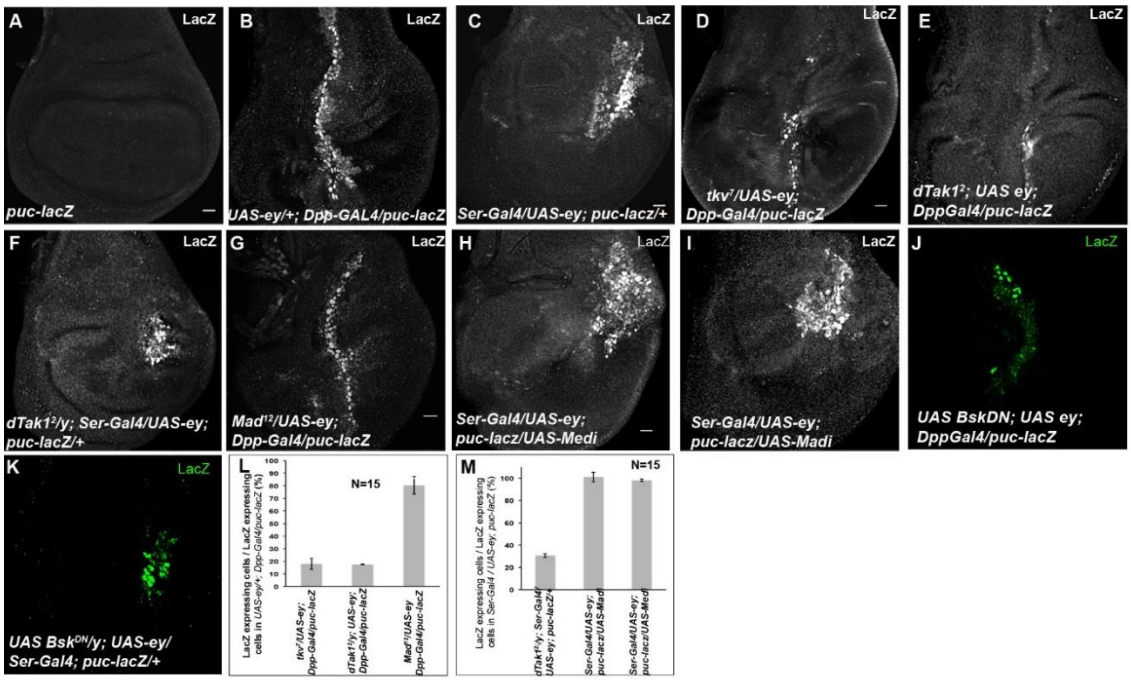

Figure 3.15. Expression of puc-lacZ during ectopic photoreceptor differentiation is modulated by Dpp signaling through dTak1. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. (A) No puc-lacZ expression was observed in the pouch region of wing discs of *puc-lacZ* larvae. (B, C) Ectopic puc-lacZ expression was detected in the wing discs of (B) *UAS-ey/+; Dpp-Gal4/+* and (C) *UAS-ey/Ser-Gal4* third instar larvae. (D-F) Sharp reduction in ectopic puc-lacZ expression was observed after attenuating *tkv* (D) and *dTak1* (E, F) activity in the wing discs undergoing ectopic photoreceptor differentiation. (G-I) However impairing the activity of Mad (G) as well as knocking down the expression of *Mad* and *Med* (H, I) did not affect ectopic puc-lacZ expression. (J, K) Significant drop in ectopic puc-lacZ expression was seen upon inactivating JNK signaling by co-expressing *UAS-bsk^{DN}* along with *UAS-ey*. (L, M) Quantitative analysis of area of puc-lacZ expression domain in wing discs of genotypes mentioned when compared to their respective control wing discs in (L) *UAS-ey/+; Dpp-Gal4/+* and (M) *UAS-ey/Ser-Gal4* larvae.

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upon co-expressing dominant negative form of *basket* as well as in the background otherwise mutant for *hep*. Importantly, 30-50% drop in expression of Dac protein was observed in *UAS-Bsk^{DN}/y; UAS-ey/+; Dpp-Gal4/+* and *UAS-Bsk^{DN}/y; UAS-ey/Ser-Gal4* larval wing discs as revealed upon quantitation of fluorescence intensity in Dac expressing domain (Fig. 3.16E-H). Similar reduction of around 40% in the expression of Dac was found upon attenuating hep activity by inducing ectopic photoreceptor differentiation in the wing discs heterozygous mutant for *hep* (Fig. 3.16A, B, G, H). Likewise, around 30% drop was observed in the level of Eya expression upon attenuation of hep activity during ectopic photoreceptor differentiation (Fig. 3.16C, D, G, and H). Quantitative analyses of the domains of Dac and Eya expression, however, did not reveal any change in their area (Fig. 3.16I, J).

Furthermore, the transcript level of *dac*, *eya* and *so* were analyzed by qRT-PCR analyses. qRT-PCR analyses revealed drastic drop (70-80%) in their transcript levels in case of *hep^{r75}/+; UAS-ey/+; Dpp-Gal4/+* wing discs as compared to of *UAS-ey/+; Dpp-Gal4/+* wing discs (Fig. 3.16K). Similarly, around 70% drop in the transcript level of *dac* was observed in case of *hep^{r75}/+; UAS-ey/Ser-Gal4* larval wing discs as compared to of *UAS-ey/Ser-Gal4* wing discs (Fig. 3.16L). Taken together, the results obtained so far further depict the involvement of JNK signaling in regulating a threshold level of RD genes expression for ectopic eye formation. Since, that level is not getting attained upon attenuating JNK signalling, therefore ectopic phoreceptors failed to differentiate.

3.2.11. Threshold level of RD genes expression is critical for ectopic eye formation.

Inactivation of Dpp mediated dTak1-JNK cascade led to significant drop in the level of RD genes expression and as a result consequent drop in ectopic photoreceptors differentiation. In order to confirm that whether a threshold level of expression of these RD genes is important, the level of Dac expression was manipulated by generating ectopic eyes in the wing discs heterozygous mutant for *dac^{1}*, *Mad^{12}* and *tkv^{7}* (Fig. 3.17A-C). Comparable (40-50%) drop in Dac expression was analyzed (Fig.3.17D) as observed upon attenuating the Dpp-dTak1-JNK cascade and as a consequence of this, there was failure in ectopic photoreceptor differentiation (Fig. 3.17A-C, E). These results confirm the requirement for the expression of RD genes beyond a threshold level for ectopic eye formation.

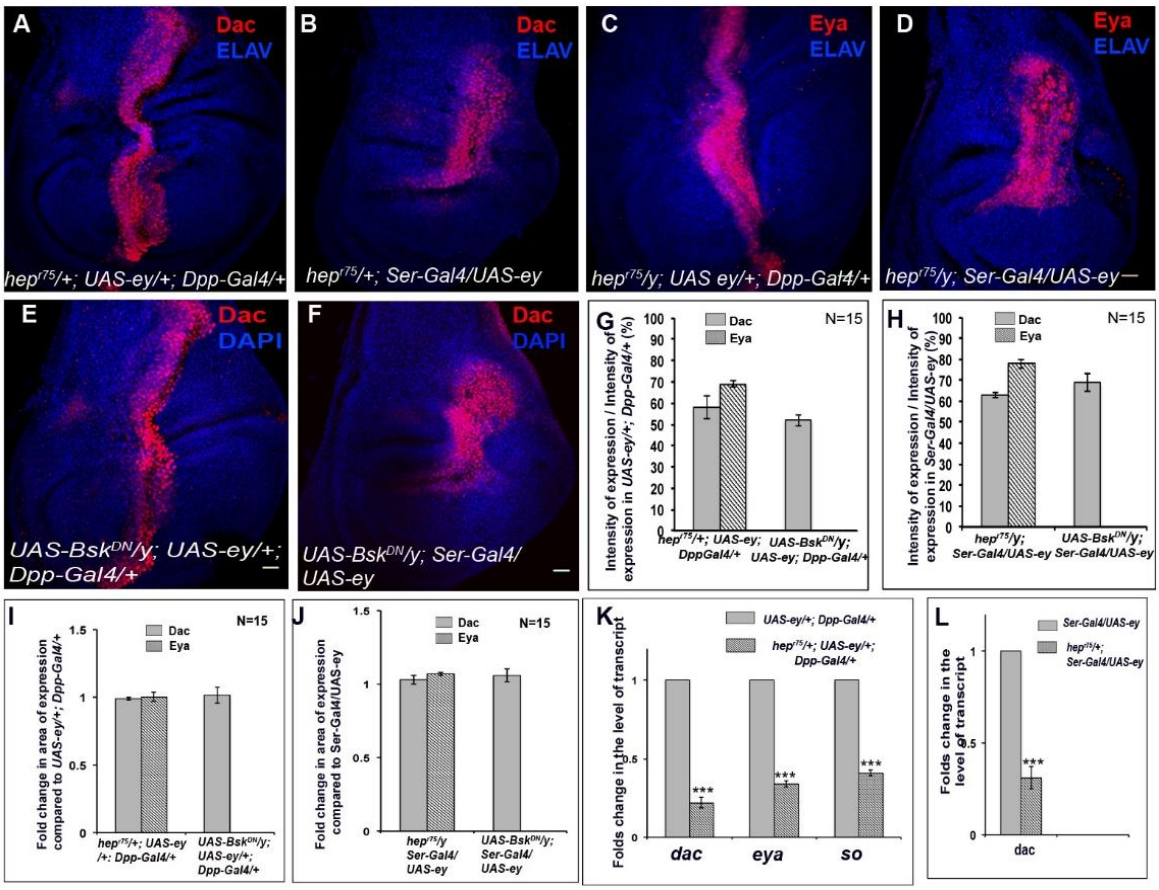

Figure 3.16. Jun-N-terminal Kinase (JNK) modulates the expression of RD genes during ectopic eye formation. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A-D)** Ectopic eyes when induced in wing discs of larvae heterozygous mutant for *hemipterus* also resulted in a drop in Dac **(A,B)** and Eya **(C,D)** expression. **(E, F)** Ectopic Dac expression got reduced upon attenuating JNK signaling by co-expressing *UAS-bsk^{DN} with UAS-ey*. **(G, H)** Quantitative analysis of fluorescence intensity in ectopic Dac and Eya expression domain in wing discs of genotypes mentioned when compared to their respective controls **(G)** *UAS-ey*/+; *Dpp-Gal4*/+ and **(H)** *Ser-Gal4*/*UAS-ey*. **(I, J)** Changes in the area of ectopic Dac and Eya expression domain in the larval wing discs of the genotype mentioned when compared to their respective controls **(I)** *UAS-ey*/+; *Dpp-Gal4*/+ **(J)** *Ser-Gal4*/*UAS-ey*. **(K, L)** Real time analysis showing changes in level of Dac and Eya transcripts in larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001)**.**

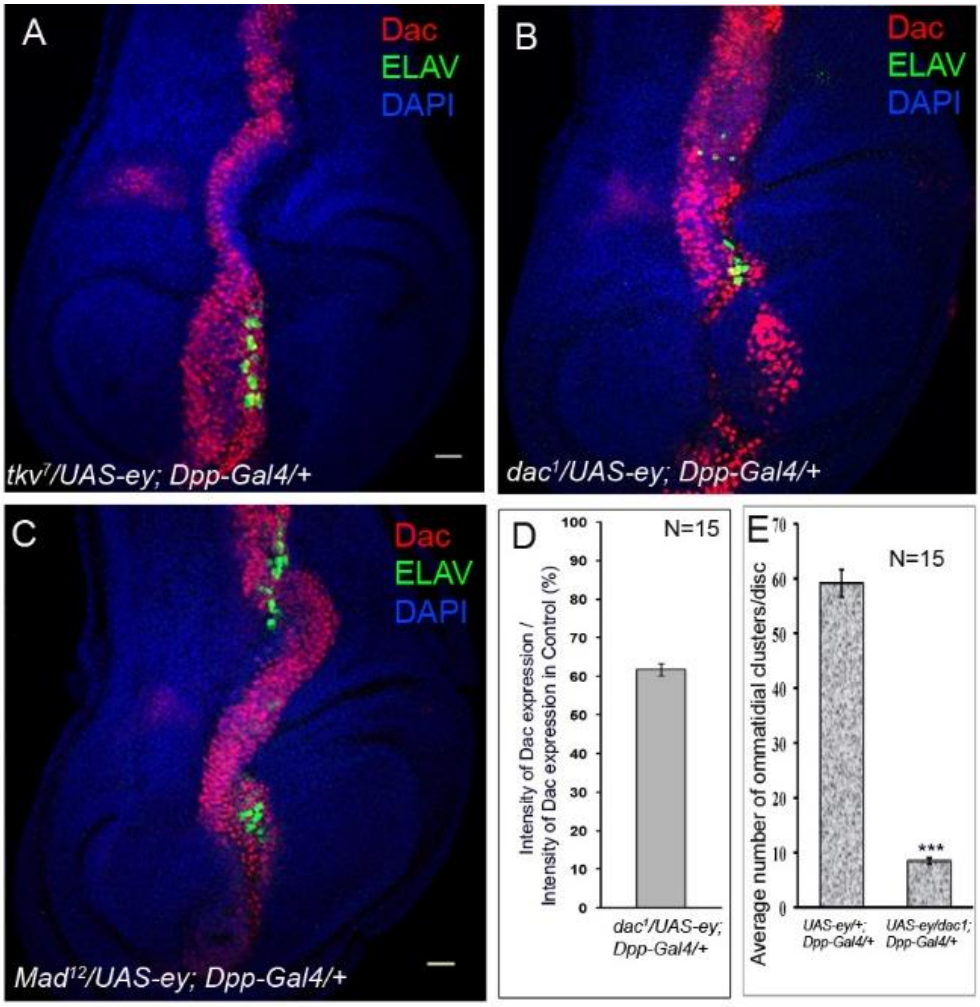

Figure 3.17. Optimal level of RD genes expression is essential for ectopic photoreceptor differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A-C)** Significant drop in ectopic Dac expression and consequent decrease in ectopic photoreceptors differentiation as revealed by Elav expression was observed upon generating ectopic eyes in the wing discs of larvae heterozygous mutant for **(A)** *tkv*^{7} **(B)** *dac*^{1} and **(C)** *Mad*^{12}. **(D, E)** Quantitative estimate of Dac expression **(D)** and ELAVexpression **(E)** in *dac*^{1}mutant larval wing discs undergoing ectopic photoreceptor differentiation as compared to their control wing discs of *UAS-ey*/+*; Dpp-Gal4*/+ larvae (mean ± SD; p value ***<0.0001).

3.2.12. Ectopic photoreceptor differentiation is associated with reduction in Polycomb-group (PcG) genes activity.

During normal development fate of a cell is determined by stable changes in the genome by epigenetic modifications. Therefore, during cell fate alteration, where specified fate of a cell gets altered, it is expected to bring about changes in epigenome to provide plasticity for alteration in cell fate. Polycomb group of proteins are one of the major group of chromatin remodelers that form heteromeric complexes to repress genes expression and thereby render epigenetic modifications to the genome essential for development and differentiation (Oktaba et al., 2008; Otte and Kwaks, 2003; Schuettengruber and Cavalli, 2009).

During normal eye development, RD genes get activated by canonical Dpp signaling in the eye disc but Dpp does not activate RD genes during wing development because they do not normally express in wing imaginal disc except for Dac in a small rudimentary region in the anterior compartment of wing disc (Fig.3.19A, B). Since the RD genes do not get activated during normal development in wing disc so it might be possible that they remain epigenetically repressed there. So, RD genes can be activated in wing disc only after removal of PcG mediated repression on these genes, Various studies involving Chip-qPCR and *in silico* analysis have revealed *dac*, *so*, *eya* as a potent target for PcG proteins mediated repression (Schwartz et al., 2006; Tolhuis et al., 2006; Zeng et al., 2012).

To investigate the involvement of Dpp signaling, if any, in removing any PcG mediated repression on these RD genes during ectopic eye formation, PcG activity was checked by employing a reporter lacZ line. For this purpose two reporter *lacZ* transgenic fly lines *LW-1* and *FLW-1* (Cavalli and Paro, 1998; Lee et al., 2005) were used. *LW-1* construct has Gal4 binding site upstream to reporter *lacZ* (Fig 3.18). Therefore, the binding of Gal4 will lead to lacZ expression. On the other hand, *FLW-1* construct has binding sites for both Gal4 as well as for Polycomb group of proteins (PcG response elements; PRE) upstream to the reporter lacZ (Fig.3.18). Therefore, in a cell where there is normal level of PcG activity, binding of PcG will repress the Gal4 binding and as a result there will be no lacz expression. As a result, FLW-1 will express lacz only when there is low level of PcG activity in a cell. As expected, upon driving the reporter line *LW-1* by *Dpp-Gal4* resulted in a very strong expression of lacZ along the A/P boundary of the wing disc (Fig. 3.19C).

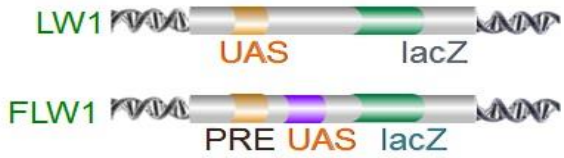

Figure 3.18. Schematic representation of the LW-1 and FLW-1 reporter constructs.

In contrast, very low level of reporter lacZ expression was observed in wing disc upon driving *FLW-1* reporter line with *Dpp-Gal4* (Fig. 3.19D). Intrestingly, lacZ expression got significantly restored after driving *FLW-1* reporter lacZ line in the wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae (Fig. 3.19E). Since increase in lacZ expression can only be possible when there is no PcG mediated repression or there is low level of PcG activity in a cell, this restoration in the expression of the reporter lacZ clearly demonstrates that during ectopic eye formation the activity of PcG proteins gets down regulated. In consistence with these observations, when the expression level of PcG genes, *polyhomeotic proximal* (*ph-p) and polycomb* (*Pc*) transcripts were checked, around 65% drop was found in their transcripts levels in the wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae (Fig. 3.19F). In sum, these results establish that during ectopic eye formation, there is downregulation in the level of PcG activity.

3.2.13. Reporter PcG lac-Z expression overlap with the hot spots region identified in wing, leg and haltere disc:

Hot spots are specific cell populations identified in leg, wing, eye and haltere imaginal discs having more plasticity towards cell fate alteration (Salzer and Kumar, 2010). Interestingly, careful observation of the lacZ expression in *FLW-1/y; Dpp-Gal4/+* larval wing disc correlated with the topological domain that has been identified as hot spots in the developing wing disc (Fig. 3.20A). To determine whether reporter lac-Z expression overlaps with already identified hot spots in other imaginal discs, its expression was analyzed in different imaginal discs of *FLW-1/y; Dpp-Gal4/+* larvae. Interestingly, this correlation was not only limited to wing discs but was also present in haltere and leg discs (Fig. 3.20B, C). Since lacZ expression of this polycomb reporter line actually reports low PcG activity, these results clearly establish that the chromatin is in more dynamic open state in these hot spot areas. In other words, these cells seems to be more plastic in nature and are prone for cell fate alteration.

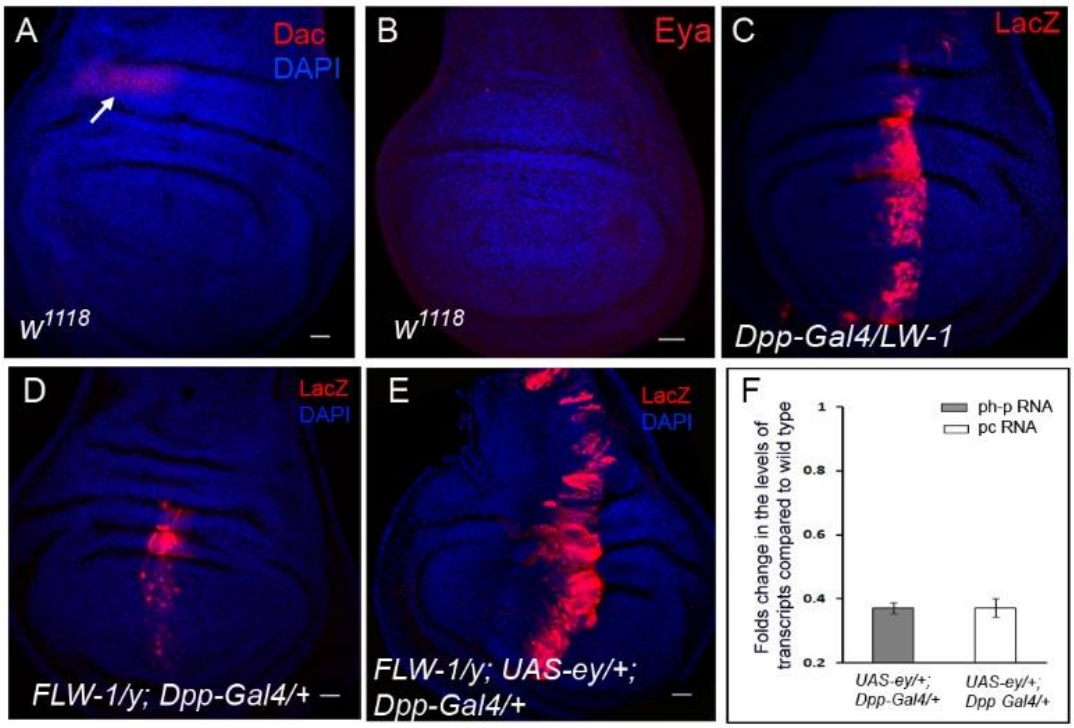

Figure 3.19. Downregulation of Polycomb-group (PcG) genes during ectopic eye differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A)** Dac expression in wild type wing imaginal disc. **(B)** No Eya expression was observed in wild type wing imaginal disc. **(C)** Reporter lacZ expression in the wing discs of *LW-1* larvae. **(D)** Reduction in the level of polycomb reporter lacZ expression was observed in the wing disc of *FLW-1/y*; *Dpp-Gal4*/+ larvae. **(E)** Significant recovery in polycomb lacZ expression was observed in *FLW1/y; UAS-ey/+; Dpp-Gal4*/+ larval wing imaginal disc. **(F)** qRT-PCR analysis shows drop in the level of *php* and *pc* transcripts in larval wing discs upon ectopic expression of *eyeless*.

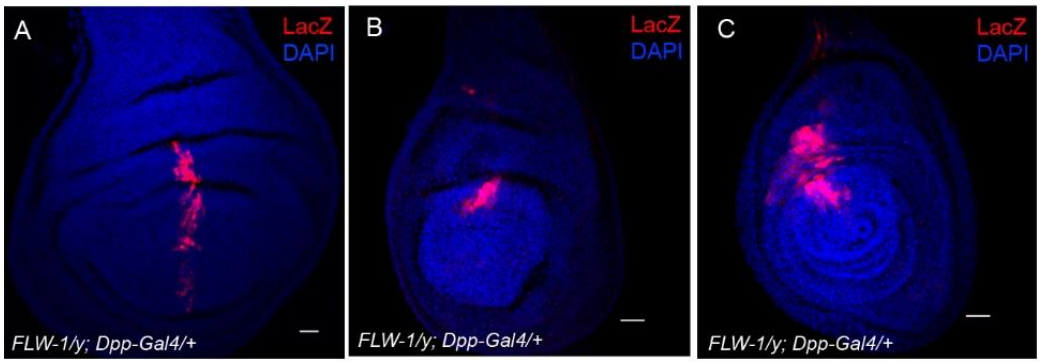

Figure 3.20. Reporter PcG lac-Z expression overlaps with hot spots in wing, leg and haltere discs. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A-C)** Expression of polycomb reporter lacZ in wing disc **(A)** haltere disc **(B)** leg disc **(C)** of genotypes mentioned.

3.2.14. Downregulation of PcG genes is mediated by the Dpp-dTak1-JNK pathway.

To determine the involvement of Dpp-dTak1-JNK signaling pathway in regulating the activity of PcG proteins, this signaling pathway was impaired by various means and checked for PcG reporter lacZ expression. Attenuating Dpp signaling by knocking down the expression of one of its receptor *thickveins* (*tkv*) (≈40% knockdown) resulted in reduction in the level of reporter lacZ expression in these cells (Fig. 3.21A, F). Similarly, around 40% reduction in reporter lac-Z expression was observed in these wing cells when they were induced to undergo photoreceptor differentiation in a genetic background knocked down for *dTak1* expression (Fig. 3.21B, 21F). Importantly, inactivating the JNK signaling pathway either by overexpressing a dominant negative form of *dFos* in the transdetermining tissue or upon knocking down *kayak* by expressing *kayak (dsRNA)* (Fig. 21C, D, F) also led to substantial (30%) decrease in reporter lacZ expression.

However, no alteration in the lacZ expression was observed when ectopic eyes were generated in a genetic background where expression of *Mad* was knocked down in the cells undergoing ectopic photoreceptors differentiation (Fig. 3.21E, F). In consistence with these observations, the drop in expression level of PcG genes, *polyhomeotic proximal* (*ph-p) and polycomb* (*Pc*) transcripts observed in the wing discs of *UAS-ey/+; Dpp Gal4/+* larvae got significantly restored upon attenuating the activity of dFos in *UAS-ey/+; Dpp-Gal4/UAS-Fos*^{DN} larval wing discs (Fig. 3.21G).

In this context, it is important to note that no involvement of dTak1 has been observed in normal photoreceptor differentiation in eye imaginal disc (Fig.3.9E-H). Since, the downregulation of the expression of PcG genes, as observed during ectopic eye differentiation is dependent on dTak1-JNK signaling and as dTak1 does not affect normal photoreceptor differentiation in eye disc, it appears that this phenomenon is specifically associated with ectopic eye differentiation. Furthermore, even over-expression of Dpp in larval wing disc never led to activation of JNK pathway as well as downregulation of reporter lacZ expression for PcG activity (Fig. 3.21I, J). Only during change in cell fate specification Dpp seems to be capable of modulating epigenetic modifications for altered cell fate. Together, these results confirm the involvement of Dpp mediated JNK signaling in down regulating the expression of PcG genes during ectopic photoreceptor differentiation.

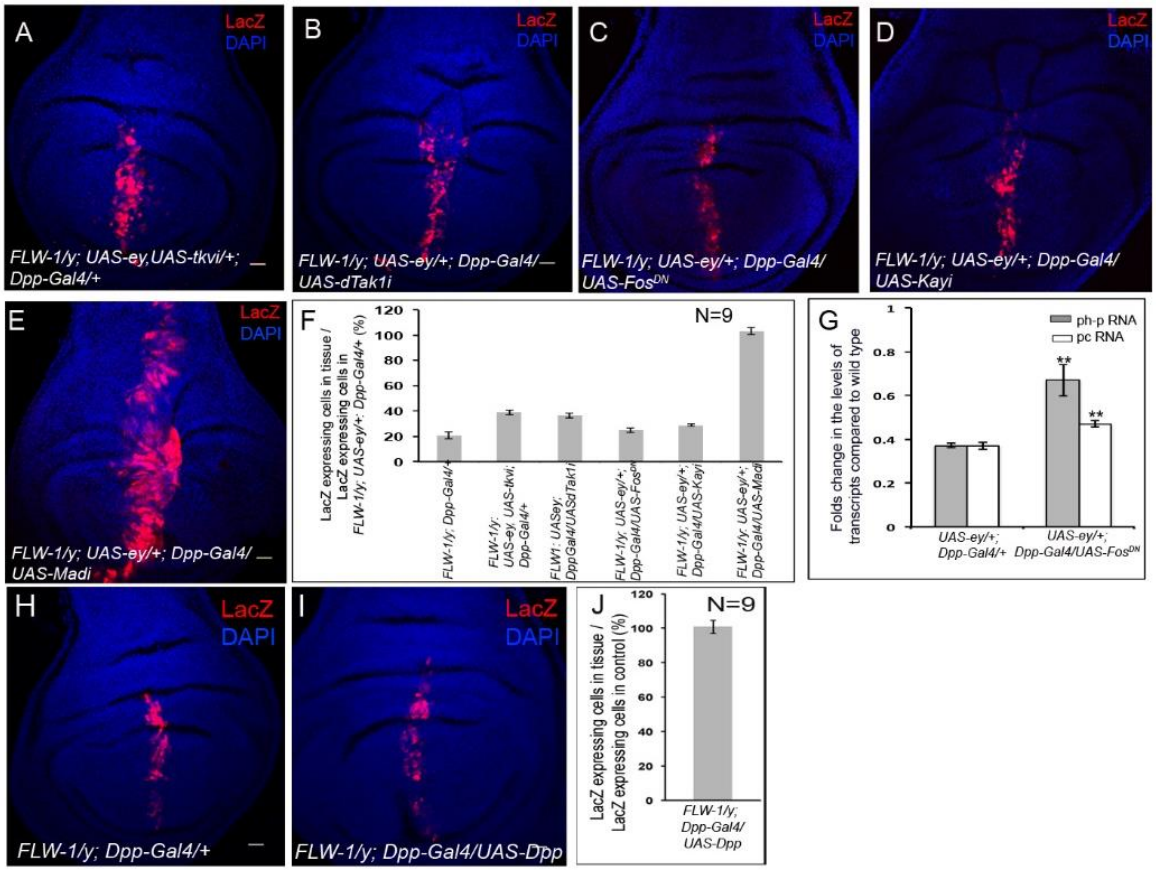

Figure 3.21. Downregulation of PcG is mediated by Dpp-dTak1-JNK pathway. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ**. (A, B)** Reduced Polycomb reporter lacZ expression was observed in the wing discs undergoing ectopic photoreceptors differentiation upon knocking down the activities of *tkv* **(A)** and *dTak1* **(B)**. **(C, D)** Inactivating JNK pathway by either coexpressing*UAS-Fos*^{DN} **(C)** or by knocking down the expression of *dFos* **(D)** led to considerable decrease in PcG reporter lacZ expression in the wing discs undergoing ectopic photoreceptors differentiation. **(E)** However, no change in lacZ expression was observed by knocking down *Mad* expression in the wing discs undergoing ectopic photoreceptors differentiation. **(F)** Quantitative estimate of area of Polycomb reporter lacZ expression domain in larval wing discs of genotype mentioned when compared to their respective controls. **(G)** Real time analysis showing drop in *php* and *pc* transcript levels during ectopic eye formation get recovered upon attenuating the activity of dFos in this background (mean ± SD; p value **<0.001). **(H, I)** No appreciable change in the expression of polycomb reporter lacZ was observed upon overexpression of Dpp **(I)** when compared to respective control in wing discs **(H)** of genotypes mentioned. **(J)** Quantification of changes in area of polycomb reporter lacZ expression in the wing discs of genotype mentioned.

3.2.15. Differentiation of ectopic photoreceptors get enhanced in PcG mutant background.

In order to have a functional correlate of these results, ectopic eyes were generated in wing discs of larvae heterozygous mutant for two different independent *Posterior sex comb* (*Psc*) loss of function alleles Psc^{h27} *and* Psc^{1}. Analysis was resorted to early third instar larval wing discs to avoid gross morphological distortions associated with late third instar wing discs of these genotypes undergoing ectopic photoreceptor differentiation. As depicted by the Fig. 3.22 A, the number of ectopic photoreceptors was very less in the early third instar wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae. Interestingly, the number of ectopic photoreceptors got increased to four to five fold in both Psc^{1}*/UAS-ey; Dpp-Gal4/+* and Psc^{h27}*/UAS-ey; Dpp-Gal4/+* larval wing discs (Fig. 3.22B, C) as compared to control wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae suggesting that lowering down the level of *Psc* expression favors or supports ectopic eye formation. A similar three to four fold enhancement in ommatidial differentiation was observed when the expression of two other PcG genes, *polyhomeotic proximal* (*ph-p*) and *polycomb* (*Pc*) were knocked down in the cells undergoing photoreceptor differentiation by co-expressing their dsRNA (Fig. 3.22D). This increase in the number of photoreceptors in the early third instar discs results from precocious differentiation of photoreceptors.

Knockdown efficiency of *UAS-ph-p (dsRNA)* was checked by ubiquitously driving it with *daughterless-Gal4* at 29ºC and analyzed the transcript level of *ph-p* in embryos by qRT-PCR analysis. 69% drop in the transcript level of *ph-p* was observed upon knocking down *ph-p* and *Pc* when compared to its expression level in wild type embryos (Fig. 3.22E)

These results conclude that the down regulation in the level of PcG proteins activity further facilitate ectopic photoreceptor differentiation. However, this observation is in contrast to photoreceptor differentiation observed in somatic clones of PcG genes in eye imaginal disc. It has been demonstrated that loss of PcG activity inhibits photoreceptor differentiation during normal eye development (Janody et al., 2004).

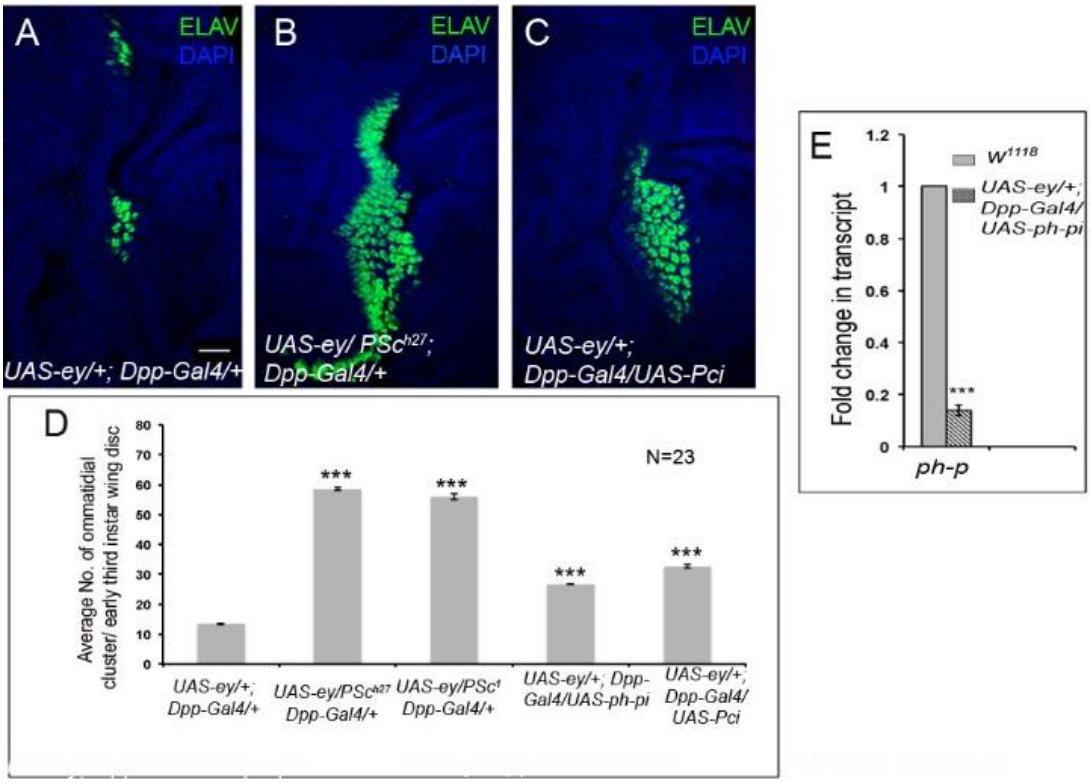

Figure 3.22. Differentiation of ectopic photoreceptors gets enhanced in Polycomb-group (PcG) mutant background. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ**. (A-C)** An increase in the number of differentiating ectopic photoreceptors was observed in wing imaginal discs of early third instar larvae heterozygous mutant for *PSc* **(B)** and in *Pc* knocked down background **(C)** as compared to controlwing discs of **(A)** *UAS-ey*/+; *Dpp-Gal4*/+ early third instar larvae. **(D)** Quantitative estimate of average number of ectopic ommatidia in the early third instar larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001). **(E)** Graph depicting drastic drop in the level of *ph-p* transcripts upon driving *UAS ph-p (dsRNA)* with a ubiquitous *da-Gal4* when compared to their levels in wild type larvae (mean ± SD; p value ***<0.0001).

3.2.16. Downregulation of PcG activity is required for the derepression of *dac*, *so* and *eya*.

To ascertain whether increase in ectopic photoreceptor differentiation was actually caused by increase in RD genes expression upon attenuation of PcG activity, expression of RD genes Dac and Eya were observed in the wing discs where the expression of PcG genes was knocked down during ectopic photoreceptors differentiation. Knocking down the expression of *pc* and *ph-p* in *UAS-ey/+; Dpp-Gal4/UAS-pc (dsRNA)* and *UAS-ey/+; Dpp-Gal4/UAS-php (dsRNA)* larval wing discs resulted in almost two fold increase in the level of Dac and Eya expression (Fig. 3.23A-F). However, even though quantitative analysis of fluorescence intensity revealed significant increase in the level of Dac and Eya expression; no significant change was found in their area of expression (Fig. 3.23G). Furthermore, the transcript level of *dac*, *eya* and *so* was analyzed by qRT-PCR in the wing discs of *UAS-ey/+; Dpp-Gal4/UAS-php (dsRNA)* larvae. In tune with the previous results, 2-2.5-fold increase in their transcript levels was observed in case of *UAS-ey/+; Dpp-Gal4/UAS-php (dsRNA)* wing discs as compared to of *UAS-ey/+; Dpp-Gal4/+* wing discs (Fig. 3.23J).

To further ascertain the involvement of PcG proteins in modulating Dac expression by dTak1 mediated Dpp signaling, I wanted to determine if the decrease in Dac expression as observed in *dTak1* mutants (Fig. 3.23H) could be rescued by down regulating PcG activity. Indeed, generating ectopic eyes in the wing discs of larvae double mutant for *Psc* (Psc^{h27}) and *dTak1* resulted in a dramatic increase in Dac expression (Fig. 3.23I) and consequently, recovery in the number of differentiating photoreceptors was also observed (Fig. 3.23I). Together these results establish that Dpp signaling mediated by dTak1 is instrumental in down regulating the expression of PcG genes and in the process released PcG mediated repression on RD genes.

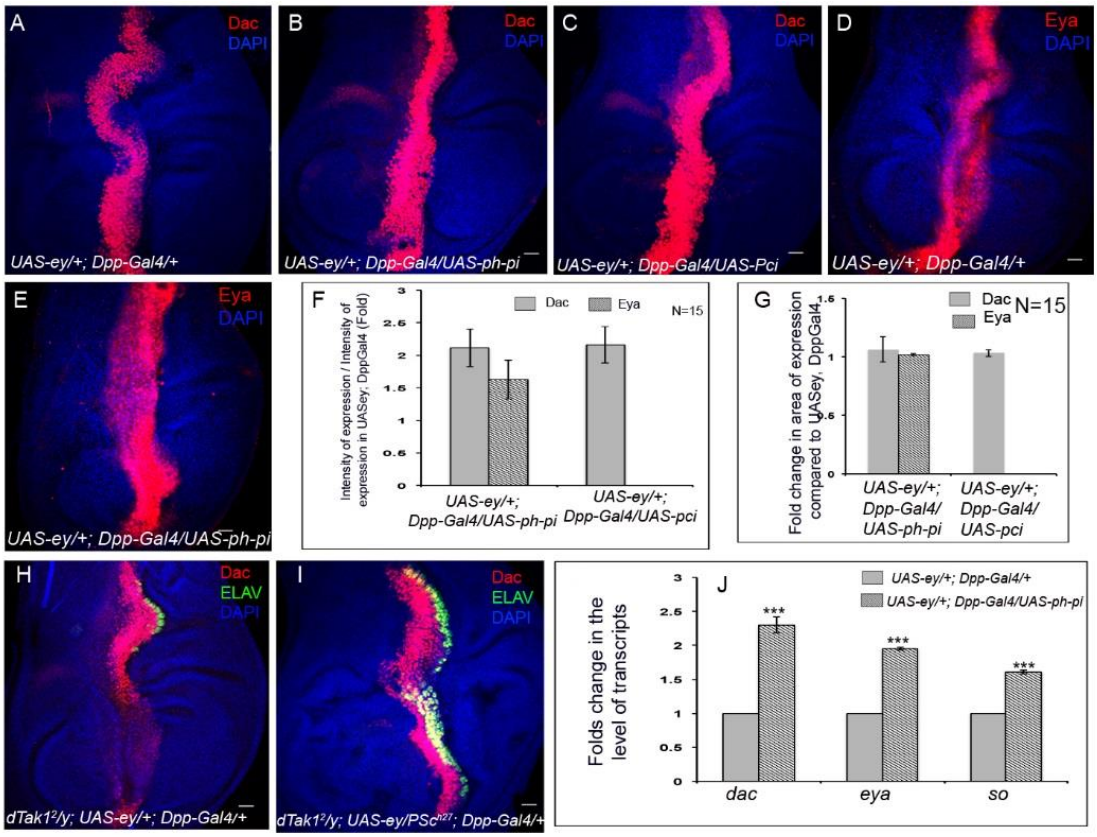

Figure 3.23. Downregulation of PcG activity is required for the derepression of *dac*, *so* and *eya*. For all wing discs anterior is to the left and genotypes are as mentioned. Scale= 20µ**. (A-C)** Increase in the level of ectopic Dac expression was observed upon knocking down the expression of *Pc* **(B)** and *ph-p* **(C)** in the third instar larval wing discs undergoing ectopic eye induction when compared to control wing discs of **(A)***UAS-ey/+; Dpp-Gal4/+* third instar larvae. **(D, E)** Increase in the level of ectopic Eya expression was observed upon knocking down the expression of*ph-p* **(E)** in the third instar larval wing discs undergoing ectopic eye induction when compared to control wing discs of **(D)** *UAS-ey/+; Dpp-Gal4/+* third instar larvae. **(F)** Quantitative analysis of fluorescence intensity in ectopic Dac and Eya expression domain in wing discs of genotypes mentioned when compared to their control wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae. **(G)** No significant changes in the area of ectopic Dac and Eya expression domain was found in the larval wing discs of the genotype mentioned when compared to their control wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae. **(H, I)** The drop in the level of ectopic Dac expression and subsequent reduction in Elav expression observed upon generating ectopic eyes in *dTak1* mutant **(H)** got significantly rescued upon reducing the level of of*PSc* **(I)** in this background. **(J)** Real time analysis showing significant increase in the level of *dac* and *eya* transcripts upon lowering down PcG activity in larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001).

3.3. Discussion:

Dpp, a key morphogen in *Drosophila*, plays a very important role in development and pattern formation. Similar to all other morphogens, Dpp can directly activates the transcription of its target genes via the binding of the transcriptional activator pMad to the promoters of the target genes as well as by regulating the expression of target genes by activation of repressor. For instance, during normal as well as ectopic eye development, Dpp transcriptionally activates the retinal determining genes that involves *eya*, *so* and *dac* (Chen et al., 1999; Pappu et al., 2005). These RD genes along with *ey* regulate each other's expression by multiple feedback loops and even the proteins interact physically to constitute complex regulatory RD gene network responsible for eye development (Fig.3.1; modified from (Pappu et al., 2005; Silver and Rebay, 2005). Although the RD genes are involved in promoting each others expression in the initial stages of induction , the levels of Dac and So when crosses a particular threshold leads to activation of a negative feed back loop that inhibits *eyeless (Atkins et al., 2013)*. In contrast, in wing discs along with direct transcription of its target genes *optomotor-blind* and *spalt*, Dpp also regulates target gene expression indirectly by activating transcriptional repressor *brinker* (Campbell and Tomlinson, 1999). Brinker competes with pMad for their occupancy to Dpp responsive elements in Dpp target genes to bring about repression of *optomotor-blind* and *spalt* (Minami et al., 1999). In this study, an *in vivo* genetic evidence is provided for yet another important regulatory mechanism by which Dpp can regulate the expression of its target RD genes independent of its bonafide canonical signaling specifically during induction of ectopic eyes. It was observed that apart from previously known transcriptional activation of its target RD genes, during ectopic eye induction, Dpp regulates RD genes expression by simultaneously triggering another cascade involving dTak1-mediated activation of JNK. In turn, activated JNK down regulates the expression of PcG genes to alleviate PcG mediated repression on its target RD genes in non-retinal tissues (Fig.3.24 modified from (Pappu et al., 2005; Silver and Rebay, 2005). This result, however deviates from that observed during normal development where it has been documented that loss of PcG activity inhibits photoreceptor differentiation (Janody et al., 2004). Therefore, this study establishes that by derepressing RD genes, the morphogen Dpp also creates a condition permissive for change in cell fate

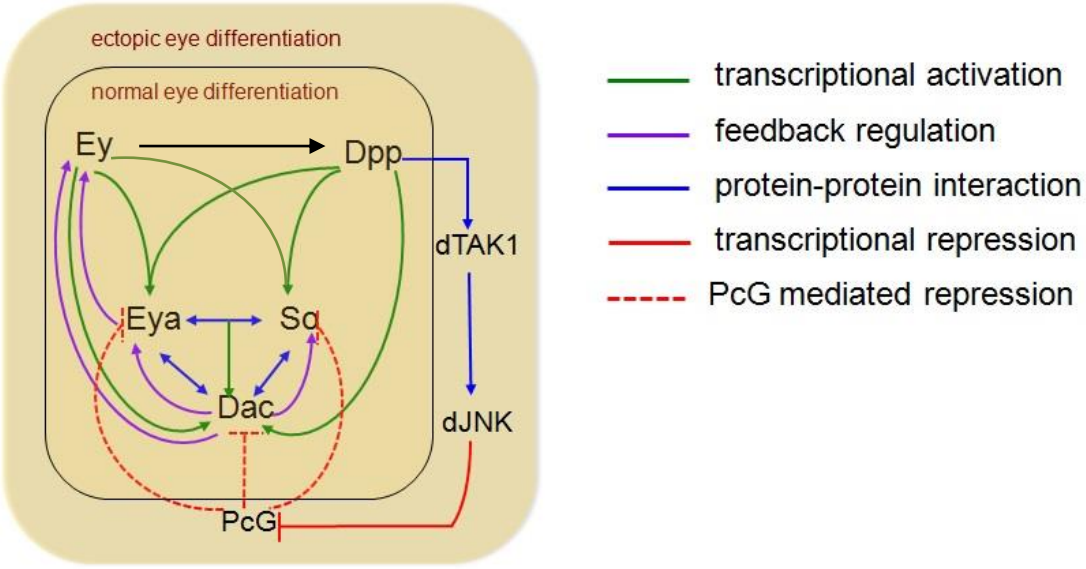

Figure 3.24. Genetic pathway elucidating the role of Dpp signaling in regulating RD gene expression during ectopic eye formation.

specification. Thereby, the morphogen Dpp plays both instructive and permissive roles to activate the expression of its target genes during ectopic eye formation in *Drosophila*. Attenuation of the activities of the members of this newly identified cascade leads to significant drop in RD genes expression and as a consequence drastic reduction in ectopic ommatidial differentiation inspite of canonical Dpp signaling being active. Therefore, it has been inferred that a threshold level of RD genes expression, critical for ectopic photoreceptor differentiation, is not getting attained upon blocking this new cascade. This notion is further supported by the observation that reducing the level of Dac expression to similar extent by alternate means also leads to failure of ectopic photoreceptor differentiation in a comparable manner (Fig.3.17A-E). Summing up, our *in vivo* genetic analysis unravels the employment of a two-tier mechanism by Dpp in modulating the ectopic expression of its target RD genes during eye induction in non-retinal tissue.

Interestingly, attenuating the activities of the all members of this newly identified cascade, the drop in the level of *dac* expression was more as compared to *eya* and *so* despite of the fact that all of these three genes are target of PcG mediated repression. Even though *dac*, like *so* and *eya*, is a target of PcG mediated repression, *dac* transcription can also be regulated by So and Eya (Figure.3.24). Therefore, one of the explainations can be that since *dac* transcription can also be regulated by So and Eya (Fig.3.24) it is possible that the overall reduction in dac expression is an outcome of both the processes. On the other hand, once Dac gets activated, it can also regulate *eya* and *so* transcription by various feedback loops. Therefore, it's very difficult to understand the complex interaction between these RD genes and to define each others involvement in this process. However, this study provides a genetic basis that links Dpp signaling and derepression of RD genes posed by PcG proteins in non-retinal tissues. Therefore, this study further pave the basis for molecular analysis of cis-regulatory elements of these genes involved in PcG mediated repression.

Previous studies during normal as well as ectopic eye development have implicated the involvement of Dpp and Hedgehog morphogens in creating feasible microenvironment to support eye formation in eye and wing imaginal discs of *Drosophila* (Chen et al., 1999; Kango-Singh et al., 2003). These studies have clearly showed that ectopic photoreceptor differentiation only occurs in the domain with high Dpp and Hh activity i.e either in the posterior compartment or along the A/P axis of wing imaginal discs. Overexpression of

Dpp and Hh along with *ey* in the wing discs undergoing ectopic photoreceptors differentiation led to the increase in the number as well as domain of differentiated photoreceptors (Kango-Singh et al., 2003). Contradicting this impression, a recent study found that only subset populations of cells within the Dpp expression domain in wing disc have more developmental plasticity to change their fate to photoreceptors and termed them as hot spots (Salzer and Kumar, 2010). They drove *ey* and others RD gene's expression in various combinations in the wing, leg and eye imaginal discs by different Gal4 drivers and found that only a discrete population of cells were able to change their fate in to eye. However, my results differ from this notion as I observed photoreceptor differentiation in the complete Dpp domain in case of wing discs of *UAS-ey*/+*; Dpp-Gal4*/+ larvae. Even in case of wing discs of *Ser-Gal4*/*UAS-ey* larvae the ommatidial differentiation was restricted to an area in the Dpp expression domain that was beyond the identified hot spot region. However, the concept of hot spots became evident when it was observed that upon attenuating the newly identified Dpp signaling cascade, though there was drastic reduction in photoreceptor differentiation but some cells in the hot spot area were still able to change their fate to photoreceptors. These results suggest that these cells have greater developmental plasticity than other cells of the Dpp domain. The concept of hot spots further became obvious when it was observed that lacZ expression of PcG activator reporter FLW-1 in *Dpp-Gal4* wing discs matched with one of the hot spots identified in the wing disc (Fig.3.20A). Interestingly, upon further analysis, it was found that this correlation was not limited to wing discs but was also present in leg and haltere discs (Fig.3.20B, C). Since lacZ expression of this polycomb reporter line actually reports low PcG activity (Cavalli and Paro, 1998; Lee et al., 2005), these results clearly suggest that the chromatin is in more dynamic open state in the hot spots. Therefore, it explains that more developmental plasticity of these cells in the hot spot region to adopt retinal fate is due to low PcG activity.

Ectopic expression of *ey* by *Dpp-Gal4* leads to ommatidial differentiation along the whole A/P axis, the domain in which Dpp is known to express (Akiyama and Gibson, 2015). Even more interestingly, in case of *Ser-Gal4*/ *UAS-ey* larval wing discs also photoreceptor differentiation gets restricted only to a specific area in the dorsal compartment that overlap with Dpp domain along the A/P boundary, not in entire dorsal compartment where *Ser-Gal4* normally expresses (Yan et al., 2004). Intrestingly, even though ectopic eyeless expression led to Dpp overexpression, the upregulation in the level of Dpp expression was

only limited to the endogenous Dpp domain in both *UAS-ey/+; Dpp-Gal4/+* as well as in *Ser-Gal4; UAS-ey* larval wing discs. Although, upregulation of Dpp in its endogenous domain in *UAS-ey/+; Dpp-Gal4/+* larval wing discs does make sense, as *ey* was driven particularly in endogenous Dpp domain, it is rather intriguing to note that in *Ser-Gal4/UAS-ey* wing discs, despite of *ey* being expressed in the entire dorsal compartment, *ey* could elevate Dpp expression only in its own endogenous Dpp domain along the A/P boundary. Since, the involvement of Hh and Dpp is well established during wing to eye conversion (Kango-Singh et al., 2003); this spatial restriction can be explained in terms of requirement of short range Hh signaling to regulate Dpp expression at the A/P axis.

PcG mediated gene repression play important role in a wide variety of cell biological processes including fate specification, differentiation, maintaining repressed state of Hox genes (Oktaba et al., 2008; Ringrose and Paro, 2004), dosage compensation (Payer and Lee, 2008) and X chromosome inactivation (Heard, 2005). Even during eye development in *Drosophila*, though R1, R6, R7 and R8 photoreceptors originate from the equivalent precursor they adopt different fate by the employment of PcG proteins during the process of fate commitment (Finley et al., 2015). Thereby, PcG are known for maintaining specific gene expression for a specific cell fate. Activation and dynamic regulation of genes repressed by PcG protein complexes can occur at different levels. For instance, during wound healing and regeneration in fragmented wing imaginal disc, JNK signaling downregulates the PcG genes expression (Lee et al., 2005). It has also been demonstrated that only clonal activation of JNK signaling in imaginal disc cells can down-regulates the expression of PcG genes (Lee et al., 2005). In contrast with transcriptional regulation by JNK, PcG proteins activity can also get regulated post translationally in *Drosophila* male germ cells. In testis, testis specific TBP specific factor regulate PcG proteins activity by modulating their nuclear localization (Chen et al., 2005). Furthermore, during myogenesis, activation of p38 leads to the recruitment of activating chromatin factors to the target loci (Rampalli et al., 2007). Nodal, a member of the TGF-family, however uses an entirely different mechanism to revert the repression mark set by PcG proteins in mouse ESCs. In this case, Nodal activated Smad2/3 recruits Jmjd3 (H3K27me3 demethylase) to its target loci to de-repress PcG activity (Dahle et al., 2010). Advancing the concept of PcG mediated genes expression regulation, this work provides the genetic evidence of another interesting mechanism that involves cross talk between TGF-β and JNK signaling pathways to counteract PcG mediated repression by downregulating the expression of PcG

during change in cell fate specification. The downregulation of PcG genes make these cells more susceptible to change by bringing their chromatin in more reprogrammable state.

Importantly, this role of Dpp in de-repressing RD genes is specifically associated with ectopic eye induction as it is not normally observed during normal photoreceptor differentiation. Even mere overexpression of Dpp in the wing disc never lead to downregulation of PcG as revealed by reporter lacZ expression for PcG activity (Fig.3.21I, J). Only during change in cell fate specification Dpp seems to play noncanonical role to modulate epigenetic modifications for altered cell fate. So, the next important step is to find out the physiological conditions present in a cell that trigger the activation of this pathway. However, attenuating this non-canonical signaling never resulted in complete loss of RD genes expression as observed upon blocking the canonical pathway. This in turn indicates the presence of an alternate mechanism, yet to be identified, that is also involved in partial removal of PcG mediated repressionon these RD genes.

Advancing the strategies for fate alteration of adult cells have gained tremendous importance because of their therapeutic application in regenerative medicines. Studies in this direction have shown that such kinds of fate transformations can be carried out by ectopic expression of key transcription factors (Bjornson et al., 1999; Zhou et al., 2008) or even by modulating the levels of morphogens. Hyperactive Wnt signaling converts early lung progenitor cells in to intestinal cells (Okubo and Hogan, 2004) while retionic acid facilitate conversion of regenerating tail in to limbs in case of marblled Balloon frog (Mohanty-Hejmadi et al., 1992). Moreover, such kind of fate switching event is typically associated with various pathophysiological conditions that include Barrett's disease (Yu et al., 2005), myofibroblast transdifferentiation (Mann et al., 2007), wound healing and regeneration (Jopling et al., 2011; Shen et al., 2004). Considering the conserved nature of morphogens and their signaling pathway between flies and vertebrates, it would be further interesting to determine whether morphogen employ similar kind of two tier mechanism to regulate their target genes during these processes of cell fate alterations. This would further help us to design better strategies to induce cell fate switching for therapeutic purposes and to understand cellular plasticity under diseased conditions. Understanding the basis for cell fate commitment and reprogramming could also enhance the possibilities for using somatic stem cells in tissue repair and replacement. Given that the phenomenon of transdetermination is also exhibited by adult stem cells of higher vertebrates, morphogens

might be instrumental in playing a similar role to create an environment that promotes adaptive developmental potential for adult stem cells.

Chapter 4.

Dpp employs dTak1-JNK mediated signaling cascade to downregulate PcG activity to facilitate leg to eye and eye to wing transdetermination in *Drosophila* imaginal discs

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4.1. Introduction

Transdetermination by ectopic gene expression is a very well worked out model for cell fate alteration in developing larval imaginal discs of *Drosophila* (Halder et al., 1995; Maves and Schubiger, 1995; Worley et al., 2012). Apart from wing to eye transdetermination, as mentioned in the previous chapter, the fate of other imaginal discs can also be altered by targeted expression of certain selector genes. For instance, ectopic expression of *eyeless* in other imaginal discs such as the leg disc can also induce ectopic eyes (Salzer and Kumar, 2010). Likewise ectopic expression of *wingless* (*wg*) in the developing leg discs can lead to generation of ectopic wing tissue (Johnston and Schubiger, 1996; Maves and Schubiger, 1995). However, the frequency of these different types of transdetermination events varies from one imaginal disc to another, primarly due to the fact that some transformations are more favoured over the others. This notion is supported by the fact that wing to eye and legs to wing transformations are more favoured than that of eye to wing and wing to leg (Maves and Schubiger, 1999, 2003). Even the frequency of changing the fate of other imaginal discs to wing tissue upon ectopic expression of *wingless* is found to be very less and only restricted to the leg discs (Maves and Schubiger, 1995, 1998). However, ectopic expression of another selector gene *vestigial* (*vg*) triggers ectopic wing generation on leg, antennae and eye imaginal discs with higher frequency.

The gene *vg* encodes a nuclear protein majorly involved in regulating growth and differentiation of wing tissue during normal as well as ectopic wing development (Kim et

al., 1996; Williams et al., 1991). While loss of function of *vg* leads to complete elimination of wing formation (Baena-Lopez and Garcia-Bellido, 2003; Williams et al., 1993), its targeted expression leads to the formation of wings like tissues in to other imaginal discs such as eye, forelegs and antennae (Baena-Lopez and Garcia-Bellido, 2003; Kim et al., 1996). *vg* gene expression is controlled by two enhancer elements, *vg Boundary Enhancer* (*vgBE*) and *vg Quadrant Enhancer* (*vgQE*) during normal wing development (Kim et al., 1996; Williams et al., 1993). Previous studies have demonstrated that the activity of *vgBE* is regulated by Notch signaling along the D/V boundary while the activity of *vgQE* is regulated by Dpp and Wg signaling in the proximal wing blade region (Kim et al., 1996). Vg also regulates its own transcription by regulating the activity of *vgQE* along the D/V boundary through a feed forward auto-regulatory loop and activate the expression of genes such as *cut*, *Scalloped* and *nubbin* that are involved in wing development (Baena-Lopez and Garcia-Bellido, 2003; Kim et al., 1996). Nubbin is a POU-homedomain protein that expresses throughout the wing pouch and is required for normal development and patterning of wing in *Drosophila* (Diaz-Benjumea and Cohen, 1995). Loss of function of *nubbin* leads to complete elimination of wing formation (Cifuentes and Garcia-Bellido, 1997; Diaz-Benjumea and Cohen, 1995). It also acts as a proximal-distal organizing center in the hinge region of developing wing disc independent of D/V and A/P compartment organizing centres (Cifuentes and Garcia-Bellido, 1997).

Importantly, expression of Wg along the Dorsal/ventral and Dpp along the anterior/posterior boundaries of the developing wing disc act as major signaling centres to regulate the expression of various wing specific genes such as *nubbin*, *vestigial* and *scalloped* (Diaz-Benjumea and Cohen, 1995; Kim et al., 1997; Williams et al., 1993) and therefore responsible for proper wing diffentiation. Dpp and Wg play an important role in transcriptionally regulating *vg* expression in the presumptive wing pouch by regulating the activity of *vgQE* along with signaling from D/V boundary (Kim et al., 1997; Kim et al., 1996). Even during ectopic wing formation, it was observed that a high level of Dpp expression was required for *wg* induced leg to wing transdetermination (Maves and Schubiger, 1998). Studies from Schubiger's group have demonstrated that ubiquitous expression of *wg* induced ectopic Vg expression specifically in the dorsal leg disc cells that also expressed high level of Dpp. Interestingly, Dpp and Wg synergistically also activate *vgBE* in the leg cells undergoing ectopic wing differentiation (Maves and Schubiger, 1998) instead of vgQE that is known to get regulated by them during normal wing development

(Kim et al., 1997; Kim et al., 1996). Although, the role of Dpp in triggering the expression of genes involved in wing development have been elucidated, Dpp signaling is equally important to regulate the expression of a completely different subset of RD genes in developing eye disc which are involved in eye development (Chen et al., 1999; Silver and Rebay, 2005). Interestingly, the genes involved in wing development that includes *vg*, *nubbin*, *apterous* are not expressed in eye disc and therefore do not play any role in eye development. Therefore, similar morphogen Dpp evokes the expression of a set of target genes in the developing eye disc that is entirely different from what it does in the developing wing disc. However, during ectopic wing formation in developing eye disc, Dpp needs to activate the expression of genes involved in wing development that otherwise remain repressed in eye disc. Similarly, during leg to eye transdeternination system, Dpp has to activate genes of RD network in leg disc. RD genes normally do not express in leg disc except *dachshund* that play completely different role in leg development (Lecuit and Cohen, 1997; Mardon et al., 1994).

My previous studies have clearly established that morphogen, Dpp plays a two-tier role to regulate the expression of repressed RD genes in wing disc during wing to eye fate alteration in *Drosophila* (Aggarwal et al., 2016). While activation of the canonical pathway of Dpp signaling is essential for the onset of neurogenesis, triggering of newly identified cascade that involves dTaK1 and JNK, Dpp modulates the activities of Polycomb group of genes to remove epigenetic mediated repression on target genes (Fig. 3.24). Therefore, simultaneous activation of both the canonical and dTak1-JNK cascade of Dpp signaling is important for proper execution of wing to eye fate change. Moreover, my results also support the previous studies that established the requirement of high level of Dpp during wing to eye and leg to wing transdetermination in *Drosophila* (Kango-Singh et al., 2003; Maves and Schubiger, 1998). Therefore in this section of my thesis work, I wanted to know whether the identified pathway is specific for wing to eye transdetermination only or is applicable for ectopic eye induction in general. Moreover, I was also curious to check the involvement of this newly identified signaling cascade in other kind of cell fate alteration, for instance, eye to wing transdetermination in *Drosophila*.

4.2. Results

To investigate whether the involvement of Dpp mediated dTak1-JNK signaling cascade during ectopic eye induction, as described in the previous chapter, is applicable for other modes of transdetermination in *Drosophila*, two other independent transdetermination systems in imaginal discs were employed. In one case, ectopic eye were induced in developing leg discs and for the other eye to wing transdetermination system was used.

4.2.1. Ectopic photoreceptor differentiation in the leg disc was restricted to the domain of Dpp over-expresion.

Ectopic eyes were generated in the developing leg imaginal discs by driving ectopic expression of *eyeless* through *Dpp-Gal4*. As revealed by reporter GFP expression, Dpp expression was observed along the anterior/posterior (A/P) boundary with high level of expression in the dorsal part of leg imaginal disc (Fig.4.1A). However, ectopic expression of *eyeless* resulted in the formation of ectopic photoreceptors as revealed by immunostaining with ELAV antibody only in the dorsal part of late third instar larval leg discs (Fig.4.1B). No ELAV positive cells were detected in the ventral compartment of the leg disc. Analysis of adult flies revealed the generation of ectopic eyes at the place of legs (99%; n=300) (Fig.4.1C, D). Upon further investigation, it was observed that as compared to wild type leg imaginal disc where Dpp expresses in a narrow strip of cells along the anterior posterior boundary, ectopic expression of *eyeless* by *Dpp-Gal4* led to overexpression of Dpp majorly in the dorsal cells of leg disc (Fig.4.1E). The expression of Dpp remained unaltered in the ventral compartment. Interestingly, neuronal differentiation was also restricted to this high Dpp expressing domain in the dorsal part of leg disc (Fig.4.1F). These results not only demonstrated that as observed previously for ectopic eye induction in wing discs, conversion of wing disc cells to photoreceptors was also restricted to the domain exhibiting Dpp over expression, but also highlighted the requirement of Dpp signaling for this process.

4.2.2. Attenuating the activities of the members of dTak1-JNK cascade affected ectopic photoreceptor differentiation in leg discs

To investigate the involvement of dTak1 during this process, the expression of *dTak1* was knocked down by coexpressing *UAS-dTak1 (dsRNA)* specifically in the cells where

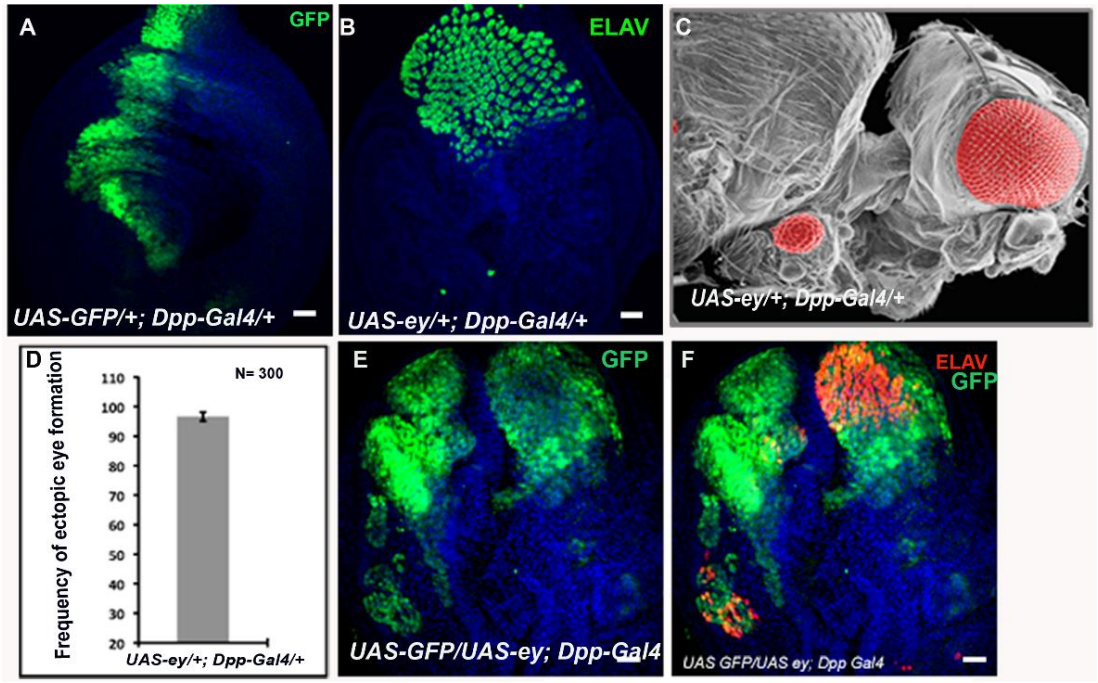

Figure 4.1. Ectopic photoreceptor differentiation in the leg disc was restricted to the domain of Dpp over-expression. For all leg discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A)** Reporter GFP expression for Dpp along the A/P boundary in the leg disc of late third instar larvae. **(B)** Ectopic photoreceptors differentiated as revealed by ELAV expression in the leg discs of *UAS-ey/+; Dpp-Gal4/+* late third instar larvae. **(C)** Scanning electron micrograph image of adult fly showing ectopic eyes at the place of leg magnification (100X). **(D)** Quantitative Graph showing frequency of ectopic eye formation at the places of legs in the *UAS-ey/+; Dpp-Gal4/+*adult flies. **(E)** Ectopic *eyeless* expression led to upregulation in the level of Dpp expression in leg disc of *UAS-ey/+; Dpp-Gal4/+* third instar larvae. **(F)** Ectopic ommatidial differentiation marked with ELAV expression restricted to elevated Dpp domain.

Eyeless was ectopically expressed. Knocking down *dTak1* in these cells led to significant reduction in ectopic ommatidial differentiation in leg discs (Fig.4.2C) when compared to control leg discs of *UAS-ey/+; Dpp-Gal4/+* larvae (Fig.4.2A). Similar reduction in ectopic photoreceptors differentiation was also observed in leg discs of $dTak1^{2}/y$*; UAS-ey/+; Dpp-Gal4/+* larvae (loss of function allele of *dTak1*) (Fig.4.2B). These results established the involvement of dTak1 in leg to eye transdetermination.

Furthermore, to check the involvement of JNK signaling, the activity of *basket* (*bsk*; the fly homolog of JNK) (Agnes et al., 1999; Sluss et al., 1996) was impaired by co-expression of its dominant negative form along with *eyeless* by *Dpp-Gal4* in the leg discs. This resulted in a dramatic reduction in the number of differentiated ectopic photoreceptors (Fig.4.2D). Similar reduction in ectopic ommatidial differentiation was also observed when ectopic eyes were generated in the leg discs mutant for *hemipterous* (*hep*) (Fig.4.2E). Hep is MAPKK homolog in *Drosophila*, which phosphorylates JNK (Glise et al., 1995). In conjunction with these results, It was also observed that over expression of a dominant negative form of dFos as well as knocking down the expression of *kay* in cells that were undergoing fate change prevented these cells from getting differentiated into photoreceptors (Fig.4.2F). dFos or Kay is signal transducer for JNK signaling.

In sum these results of genetic analyses demonstrated the involvement of both dTak1 and JNK signaling during ectopic eye induction in developing leg discs in regulating the number of ectopic photoreceptors.

4.2.3. Expression of puc-lacZ during leg to eye transdetermination was activated by Dpp signaling through dTak1.

Next, to investigate whether the activation of JNK pathway was mediated by dTak1, the ectopic expression of *puckered (puc)* was analyzed which is a direct transcriptional target of the JNK pathway by its reporter puc-lacZ expression (Martin-Blanco et al., 1998). Normally, *puc* expresses in a small set of cells in the dorso-anteromost part (Fig.4.3A) of the developing leg disc. Interestingly, high level of ectopic puc expression was found in the dorsal part of the leg discs of *UAS-ey/+; Dpp-Gal4/+* larvae (Fig.4.3B). In this context it is important to note that ectopic photoreceptor differentiation also takes place only in this dorsal part of the leg disc (Fig.4.1B). To further ascertain whether this ectopic puc-lacZ expression was triggered by Dpp signaling, puc-lacZ expression was checked in

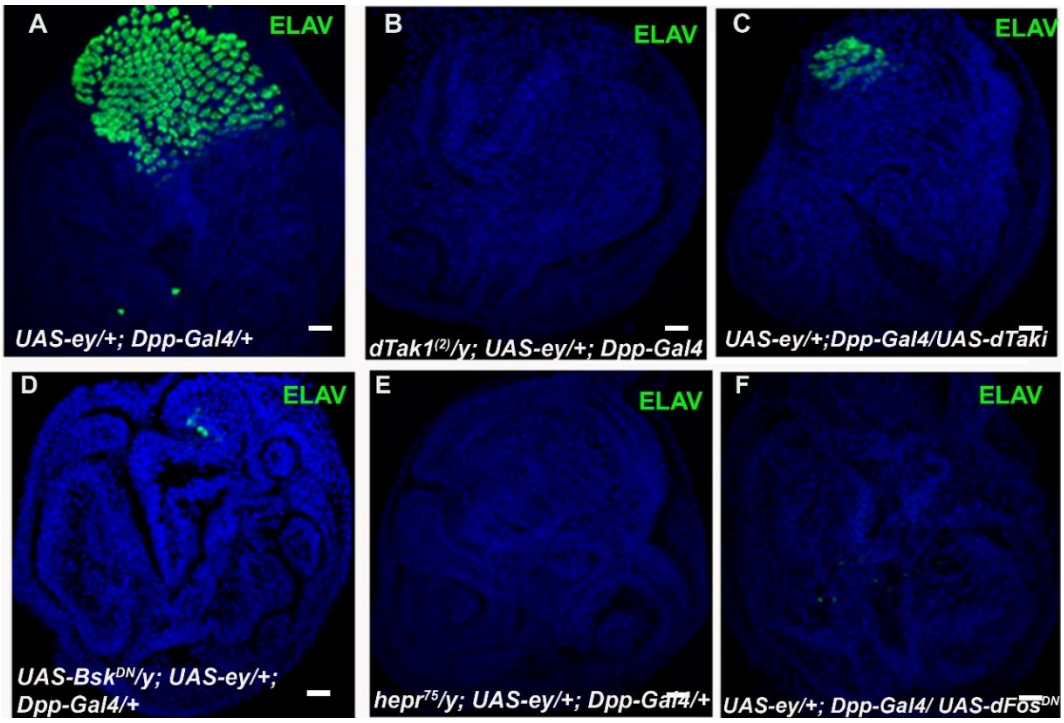

Figure 4.2. Attenuating the activities of the members of dTak1-JNK cascade affected ectopic photoreceptor differentiation in leg discs. For all leg discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(B)** Reduction in ectopic photoreceptor differentiation was seen as shown by ELAV in leg discs of dTak1 mutants as compared to **(A)** *UAS-ey/+; Dpp-Gal4/+* larval leg discs. **(C)** Knocking down the expression of *dTak1* significantly affected the ectopic neuronal differentiation in leg discs of late third instar larvae as shown by ELAV. **(D)** Inactivation of JNK signaling by over expressing a dominant negative form of *basket* drastically suppressed ectopic differentiation of photoreceptor in leg discs of late third instar larvae. **(E)** Impairing JNKK activity by bringing ectopic eye formation in the background mutant for hep^{r75} also resulted in to drastic drop in ectopic photoreceptors induction in leg discs of late third instar larvae. **(F)** Attenuating JNK activity by co-expressing dominant negative form of dFos also resulted in drastic drop in ectopic eye induction in leg discs of late third instar larvae.

the leg discs undergoing ectopic eye induction that were otherwise heterozygous mutant for the *tkv* allele tkv^{7}. In a manner similar to that observed during wing to eye transdetermination, drastic reduction in ectopic puc-lacZ expression was found (Fig.4.3C). Next, to determine the involvement of dTak1, puc-lacZ expression was analyzed in the leg discs heterozygous mutant for the *dTak1* loss of function allele $dTak1^{2}$. A significant reduction in the level of ectopic puc-lacZ expression was observed in $dTak1^{2}$*/y; UAS-ey*/+*; Dpp-Gal4*/+ larval leg discs (Fig.4.3D). With these observations it became evident that the activation of JNK pathway during ectopic eye induction in the developing leg discs was mediated by dTak1.

Furthermore, these results also suggested that during leg to eye transdetermination, elevated levels of Dpp activated dTak1 which in turn was responsible for triggering the JNK pathway and activation of this pathway was essential for ectopic photoreceptor differentiation.

4.2.4. The dTak1-JNK signaling pathway led to downregulation of PcG activity during leg to eye transdetermination.

Next, to investigate the role of Dpp mediated dTak1-JNK signaling, if any, in downregulating the activity of PcG during ectopic eye formation in the leg imaginal discs, PcG activity was checked by employing a reporter lacZ transgenic fly line *FLW-1*. As explained earlier in section 3.2.12, *FLW-1* expresses lacz only when there is low level of PcG activity in a cell (Cavalli and Paro, 1998; Lee et al., 2005).

Very low level of reporter lacZ expression was observed in leg disc upon driving *FLW-1* reporter line with *Dpp-Gal4* (Fig.4.4A). Low level of lacZ expression suggests repression of reporter lacZ expression as an outcome of PcG proteins binding to PRE and as a result not allowing Gal4 to bind to the target *UAS* sequence upstream to *lacZ* gene. Interestingly, significant restoration of lacZ expression was observed after driving *FLW-1* reporter lacZ line in the leg discs of *UAS-ey*/+*; Dpp-Gal4*/+ larvae (Fig.4.4B). Since increase in lacZ expression can only be possible when there is low level of PcG activity in a cell, this restoration in the expression of the reporter lacZ clearly demonstrated that during ectopic eye formation the activity of PcG proteins got down regulated. In sum, these results established that during ectopic eye formation in the developing leg discs, there was downregulation in the level of PcG activity.

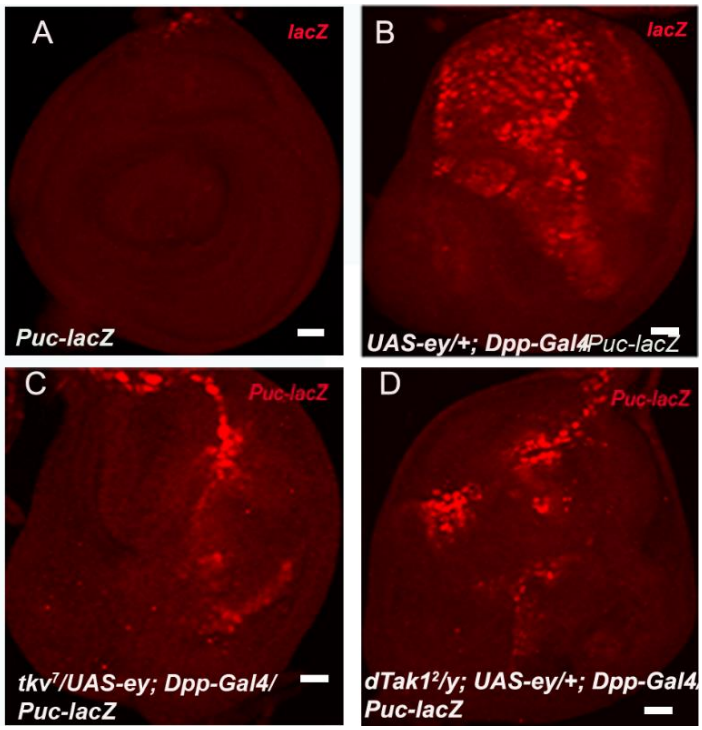

Figure 4.3. Expression of puc-lacZ during leg to eye transdetermination was activated by Dpp signaling through dTak1. For all leg discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. **(A)** No puc-lacZ expression was detected in the pouch region of *puc-lacZ* leg disc. **(B)** Ectopic induction of *eyeless* by *Dpp-Gal4* led to ectopic puc-lacZ expression in the domain that later on differentiated in to photoreceptors. **(C, D)** Sharp reduction in ectopic puc-lacZ expression was observed after attenuating tkv **(C)** and dTak1 **(D)** activity in the leg discs undergoing ectopic photoreceptor differentiation.

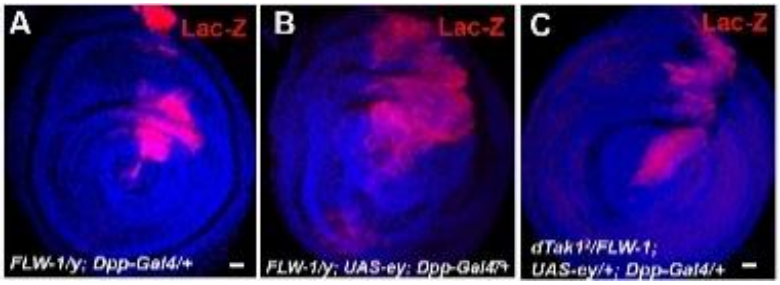

Figure 4.4. dTak1-JNK signaling pathway led to downregulation of PcG activity during leg to eye transdetermination. For all leg discs anterior is to the left and genotypes are as mentioned. Scale= 20µ. Polycomb reporter lacZ expression **(A)** Reduced reporter lac-Z expression was observed in *FLW-1/y; Dpp-Gal4*/+ leg disc of third instar larvae. **(B)** Significant recovery in polycomb reporter lac-Z expression was observed in *FLW-1/y; UAS-ey*/+*; Dpp-Gal4*/+ larval leg disc. **(C)** Reduced polycomb reporter lacZ expression was found upon attenuation of the activity of dTak1 in the leg discs undergoing ectopic photoreceptors differentiation.

To determine the involvement of Dpp mediated dTak1 signaling pathway in downregulating the activity of PcG proteins, the activity of dTak1 was impaired by generating ectopic eyes in leg discs of *dTak1*^{2}*/FLW-1; Dpp-Gal4*/+ larvae and checked for FLW-1 reporter lacZ expression. As evident from Fig.4.4C, significant reduction in the level of lac-Z expression was observed in these cells. Taken together, my genetic and reporter analyses established the involvement of Dpp mediated dTak1-JNK signaling cascade in downregulating PcG activity during leg to eye transdetermination in a manner similar to that observed during wing to eye transdetermination.

4.2.5. Generation of ectopic wings in the developing eye imaginal discs.

Ectopic wings were generated at the place of eyes by ectopic expression of *vestigial* with *Dpp-Gal4*. Vestigial is a bonafide marker for cells undergoing wing differentiation and it has been demonstrated that ectopic expression of *vestigial* is sufficient to generate wing like structure in eyes, legs and antenna (Baena-Lopez and Garcia-Bellido, 2003; Kim et al., 1996; Williams et al., 1991). As revealed by reporter GFP expression, Dpp was found to express along the postero-lateral margins of eye imaginal disc of *UAS-GFP*/+*; Dpp-Gal4*/+ larvae (Fig.4.6A). Apparently, this pattern of Dpp expression contradicts the general conception that suggests Dpp expression along the morphogenetic furrow of developing eye disc (Blackman et al., 1991). This deviation is due to the fact that the driver line *dpp*^{blk} *Gal4 40C.6* does not show Dpp expression along the morphogenetic furrow (Treisman and Rubin, 1995). As evident from Fig. 4.5D, E ectopic expression of *vestigial* in the developing eye disc by *Dpp-Gal4* resulted in the formation of wing like tissues in adult eye**.**

To monitor the differentiation of wing tissue in the developing late third instar eye discs, expression of Nubbin was observed in these discs. Nubbin is an earliest expressed marker for wing fate specification that expresses throughout the wing primodium (Cifuentes and Garcia-Bellido, 1997; Diaz-Benjumea and Cohen, 1995). Importantly, Nubbin does not express in the developing eye imaginal disc (Fig.4.5A), However, ectopic expression of *vg* in the eye disc induced ectopic Nubbin expression. Interestingly, the domain of ectopic Nubbin expression demonstrated a significant overlap with the domain of Dpp expression indicating the involvement of Dpp signaling in inducing ectopic *nubbin* expression (Fig.4.5B, C).

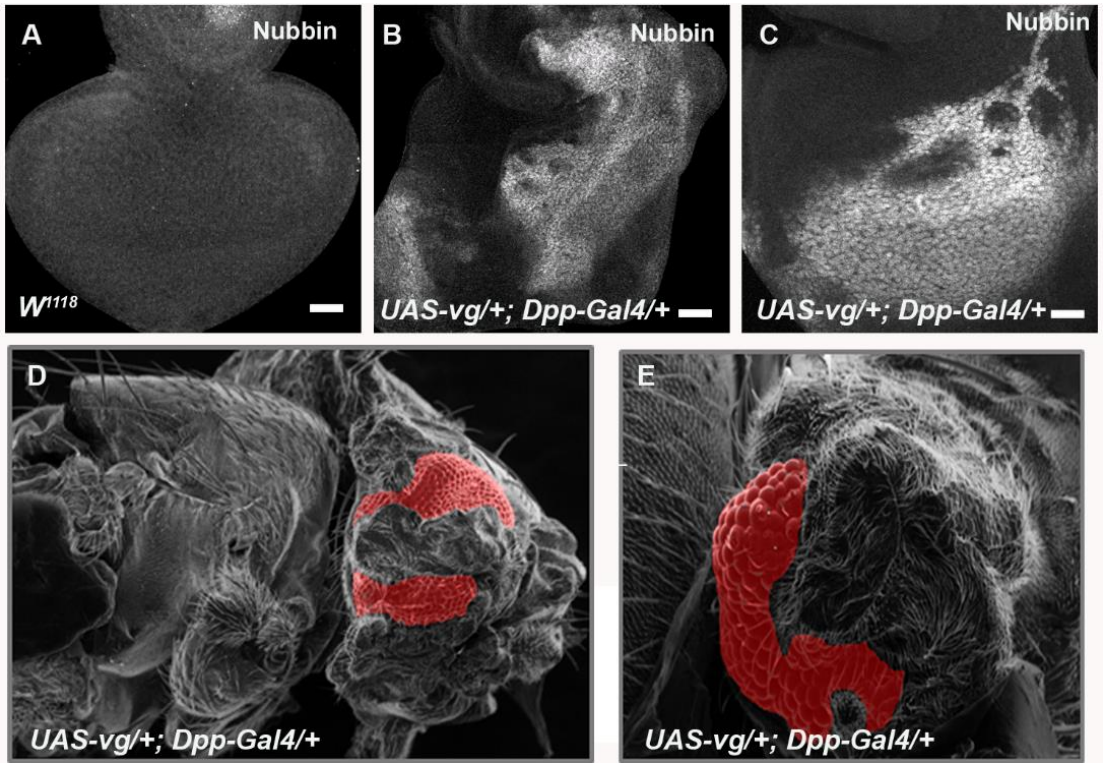

Figure 4.5. Generation of ectopic wings in the developing eye imaginal discs. For all eye discs genotypes are as mentioned. Scale= 20µ. **(A)** No Nubbin expression was detected in the wild type eye imaginal disc. **(B, C)** Targeted expression of *vestigial* induced ectopic Nubbin expression in eye discs of *UAS-vg/+; Dpp-Gal4/+* larvae. **(D, E)** Scanning electron micrograph images of the eye of adult fly showing wings like outgrowths upon ectopic expression of *vestigial* in the eye magnification (100X) **(D)** 270X **(E)**.

Furthermore, it was observed that as compared to wild type eye imaginal disc where Dpp expressed in a small domain along the posterior and posterior-lateral sides of eye imaginal disc (Fig.4.6A), ectopic expression of *vg* by *Dpp-Gal4* led to an expansion in the domain of Dpp expression (Fig.4.6B). Since similar upregulation in the level of Dpp was also observed during wing to eye and leg to eye transdetermination, these results suggested the requirement of high levels of Dpp expression for proper eye to wing transdetermination and prompted me to analyze the role of Dpp signaling during this process.

4.2.6. Involvement of Dpp mediated dTak1-JNK signaling cascade during eye to wing transdetermination.

To investigate the role of Dpp mediated dTak1-JNK signaling cascade during eye to wing fate alteration, the activities of various members of this cascade were impaired in the cells undergoing eye to wing fate change. To start with, the expression of *dTak1* was knocked down by co expressing *UAS-dTak1 (dsRNA)* in the eye disc cells undergoing ectopic wing differentiation. Upon doing so, there was drastic reduction in the level of ectopic Nubbin expression in *UAS-vg/+; UAS-dTak1 (dsRNA)/Dpp-Gal4* larval eye discs (Fig.4.6D) as compared to control eye discs *UAS-vg/+; Dpp-Gal4/+* larvae (Fig.4.5B, C). Similar reduction in the expression of ectopic Nubbin expression was also observed when ectopic wing differentiation was carried out in the eye discs of the larvae heterozygous mutant for *dTak1* (Fig.4.6C). Together, these results demonstrated the involvement of dTak1 in eye to wing transdetermination similar to that observed in case of ectopic photoreceptor differentiation in wing and leg imaginal discs.

Next, the involvement of JNK pathway was checked during this process. For that purpose, the activity of *basket* (*bsk*; the fly homolog of JNK) was inhibited by co-expression of its dominant negative form along with *vg* in the eye disc cells undergoing ectopic wing cells differentiation. This also resulted in dramatic reduction in the ectopic Nubbin expression (Fig.4.6E). In tune with above results, overexpression of dominant negative form of dFos in the cells undergoing eye to wing fate change also resulted in drastic reduction in ectopic Nubbin expression (Fig.4.6F). Together, these results clearly established the involvement of JNK during eye to wing transdetermination.

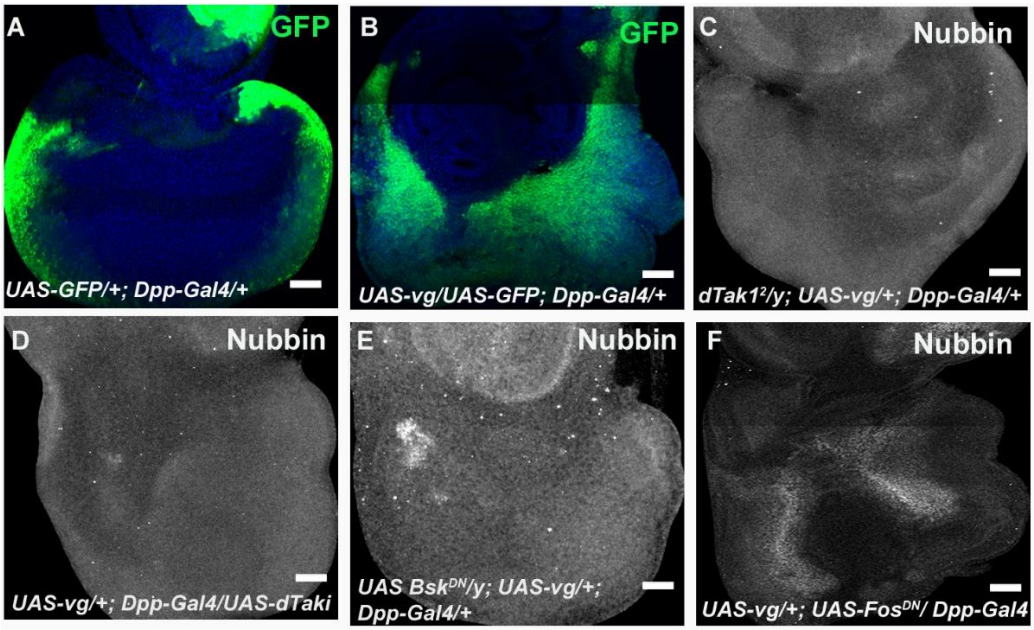

Figure 4.6. Involvement of Dpp mediated dTak1-JNK signaling cascade during eye to wing transdetermination. For all eye discs genotypes are as mentioned. Scale= 20µ. **(A)** Reporter GFP expression in the Dpp domain was observed along the postero-lateral sides in eye disc of third instar larvae. **(B)** Ectopic induction of *vestigial* in *UAS-vg/+; Dpp-Gal4/+* larval eye disc led to higher level of Dpp expression. **(C)** Reduced level of ectopic Nubbin expression was found upon attenuating dTak1 activity by bringing ectopic wing differentiation in *dTak1* mutant's background. **(D)** Knocking down the expression of *dTak1* significantly affected the ectopic Nubbin expression in the eye discs undergoing ectopic wing differentiation. **(E)** Inactivation of JNK signaling by over expressing a dominant negative form of *basket* drastically suppressed ectopic nubbin expression in the eye discs undergoing ectopic wing differentiation. **(F)** Attenuating JNK activity by co-expressing dominant negative form of dFos also resulted in drastic drop in ectopic nubbin expression.

In sum, results of *in vivo* genetic analyses clearly demonstrated the involvement of dTak1-JNK signaling cascade in regulating the expression of ectopic Nubbin during eye to wing transdetermination.

4.2.7. dTak1 downregulates the activity of PcG during eye to wing transdetermination.

In order to determine whether the requirement of activating dTak1-JNK pathway is to repress the expression of PcG genes, activity of PcG genes was checked by employing a reporter lacZ transgenic fly line *FLW-1*. As mentioned earlier, the *FLW-1* trangenic line shows reporter lacZ expression only when there is low level of PcG activity in a cell that in most cases coincide with the identified hotspots (Plese refer to Fig.3.20 and section 3.3).

In tune with previous results during wing to eye and leg to eye transdetermination, very low level of reporter lacZ expression was observed in *FLW-1/y; Dpp-Gal4*/+ larval eye discs (Fig. 4.7A) suggesting that the reporter lacZ expression was repressed due to the binding of PcG proteins to the PRE's. Intrestingly, significant restoration in the reporter lacZ expression was detected after driving *FLW-1* reporter lacZ line in the eye discs of *UAS-vg*/+*; Dpp-Gal4*/+ larvae (Fig. 4.7B). Restoration in the level of lacZ expression suggests drop in the PcG activity. This restoration in the expression of the reporter lacZ clearly demonstrated that during ectopic wing formation the activity of PcG proteins got down regulated.

To determine the involvement of Dpp mediated dTak1 in regulating the activity of PcG proteins, ectopic wings were generated in the eye discs of *FLW-1/y; UAS-vg*/+*; UAS-dTak1 (dsRNA)/Dpp-Gal4* larvae. Significant reduction in the level of lac-Z expression was observed in *FLW-1/y; UAS-vg*/+*; UAS-dTak1 (dsRNA)/Dpp-Gal4* larval eye discs (Fig. 4.7C) as compared to control *FLW-1/y; UAS-vg*/+*; Dpp-Gal4*/+ eye discs (Fig.4.7B). Drop in the level of reporter lac-Z expression exhibited the role of dTak1 signaling in downregulating PcG activity during eye to wing transdetermination. However, no alteration in the level of the reporter Flw-1 lacZ expression in the antennal disc was detected as this region overlaps with the identified hotspot (Salzer and Kumar, 2010).

All these results suggested the involvement of Dpp mediated dTak1-JNK pathway to assist fate alteration during eye to wing transdetermination in a manner similar to its role observed during ectopic eye formation.

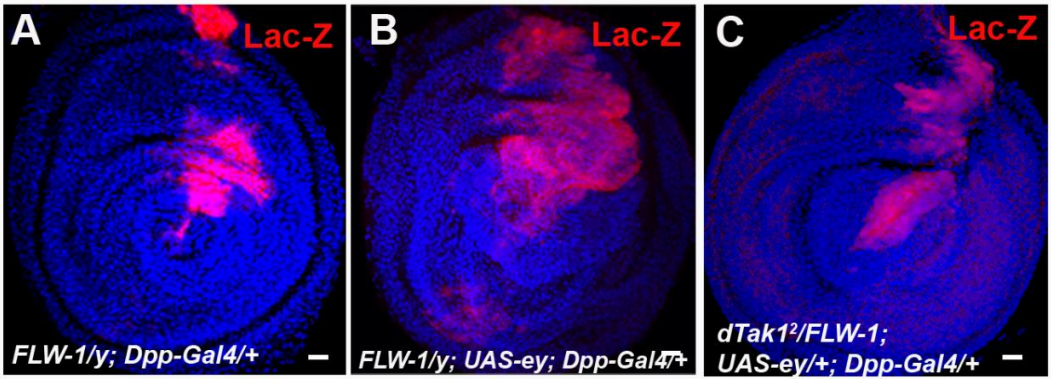

Figure 4.7. The dTak1-JNK signaling pathway led to downregulation of PcG activity during eye to wing transdetermination. For all eye discs genotypes are as mentioned. Scale= 20µ. Polycomb reporter lacZ expression in **(A)** Reduced reporter lac-Z expression was observed in *FLW-1/y; Dpp-Gal4*/+ larval eye disc. **(B)** Significant recovery in lac-Z expression was seen in *FLW-1/y; UAS-ey/+; Dpp-Gal4*/+ larval eye disc. **(C)** Reduced polycomb reporter lacZ expression was detected in the eye disc undergoing ectopic wing differentiation upon attenuation of the activity of dTak1.

4.3. Discussion

In this particular section of thesis, two questions were addressed. First, whether the Dpp mediated pathway identified during ectopic eye induction in wing tissue to derepress the target genes is particularly applicable for a specific tissue or is even involved for ectopic eye induction in any other imaginal discs of *Drosophila* larvae. Second, to know whether this process is associated with only ectopic eye induction or is associated with any other kind of transdeterminations observed in *Drosophila* by ectopic gene expression of selector genes. The idea to investigate this stems from the previous studies that had demonstrated high level of Dpp activity as a prerequisite for any kind of transdetermination process in imaginal discs of *Drosophila*. During eye to wing transdetermination, co-ordinated activity of Dpp is required along with Wg for proper wing fate differentiation (Kim et al., 1997; Williams et al., 1991) and as discussed earlier, previous studies have also established the important role of Dpp signaling during wing to eye and leg to wing transdetermination (Kango-Singh et al., 2003; Maves and Schubiger, 1998). During the study, association of elevated levels of Dpp was observed with the cells undergoing fate change in wing to eye transdetermination in *Drosophila* and its important role in derepressing the expression of target genes essential for wing to eye fate alteration. Similarly, during leg to eye as well as during eye to wing transdetermination, upregulated and more expanded Dpp domain was observed specifically associated with the cells undergoing fate change. Interestingly, Dpp domain was completely defining the domain of cells undergoing fate change, which prompted me to investigate the specific role of Dpp, if any, during such kind of cell fate alteration events. On further investigation, it was found that indeed, in a manner similar to that wing to eye transdetermination, during leg to eye and eye to wing transdetermination also, Dpp involves dTak1-JNK cascade to downregulate the activity of PcG proteins to activate the expression of epigenetically repressed genes. In doing so, Dpp facilitate tissue plasticity to promote such kind of fate alterations. Therefore, the results of my investigation establish that indeed this newly identified signaling cascade that involves the activation of dTak1 and JNK is essential for any kind of transdetermination in *Drosophila* imaginal discs. These results clearly establish the involvement of this newly identified cascade even in two other different transdetermination systems in *Drosophila*. Interestingly, in all these observations, primary role of activation of this pathway is to bring decrease in the expression levels of PcG genes. So, this is a conserved strategy employed by high level of Dpp to create a conducive environmenent to facilitate cell fate

transformation. In this context, it is important to note that just overexpression of Dpp never leads to activation of this pathway (Fig. 3.21I, J). Therefore, these results suggest that the activation of this pathway is context dependent. It only gets triggered during cell fate alteration and upon ectopic expression of selector genes. Although, the mechanism that leads to the specific activation of this pathway is not clear, it would be very interesting to investigate what actually determine the context dependent activation of this pathway.

Genetically modulating the activity of dTak1 in the cells undergoing fate change allowed us to genetically dissect the involvement of dTak1 in this process. TAK1 is a member of MAPKK Kinase family and a key component of various signaling pathways. It activates and phosphorylates MAP2K family members that can further lead to the activation of stress activated MAPK like JNK (Dai et al., 2012; Delaney and Mlodzik, 2006; Sakurai, 2012). While, dTak1 is known to play minor role in embryonic dorsal closure, thorax closure and ommatidial planar polarity in eye disc in *Drosophila* (Mihaly et al., 2001), it does not play any role during normal wing and eye differentiation. My mutant analyses with dTak1 have also established that dTak1 did not have any role in regulating the expression of RD genes as well as ommatidial differentiation during normal eye development in *Drosophila* (Fig. 3.21G). Only during ectopic eye and wing induction, high levels of Dpp employs dTak1 that is not involved during normal eye and wing development to repress the activity of PcG genes involving the activation of stress activated protein kinase (SAPK) JNK. It raises the possibility that whether the cell fate alteration creates a developmental stress like condition that leads to activation to this pathway to provide developmental plasticity for change in cell fate. Therefore, Dpp mediated activation of dTak1-JNK cascade to regulate target genes expression is specifically involved during such kind of cell fate switching events.

For analyzing wing to eye and leg to eye cell fate alteration, expression of *dac* and *eya* was observed to monitor ectopic eye differentiation. Previous studies have also shown that they are the target for PcG mediated repression. For my analyses with ectopic wing induction in the developing eye disc, the expression of Nubbin was investigated. Drastic reduction in the ectopic Nubbin expression was observed upon attenuation of dTak1-JNK signaling. However, since dTak1 is not involved during normal wing development, it has no role in regulating Nubbin expression in normal wing development. Therefore, the regulation of Nubbin expression by dTak1-JNK signaling is specifically involved during generation of

ectopic wings on eye where dTak1-JNK signaling cascade downregulates the activity of PcG genes to remove repression from target genes. As *nubbin* does not get activated during normal development in developing eye discs it is expected that it remains epigenetically repressed there. Therefore, *nubbin* can be activated in eye disc only after removal of PcG mediated repression. Importantly, various studies involving Chip-qPCR and *in silico* analysis have revealed *nubbin* as a potent target for PcG proteins mediated repression (Schwartz et al., 2006; Tolhuis et al., 2006; Zeng et al., 2012). These analyses further support our results that establish the role of dTak1-JNK signaling cascade in downregulating the activity of PcG to remove repression from *nubbin* during ectopic wing formation. Importantly, *nubbin* is not a target of canonical Dpp signalling during wing development, while *dac* and *eya* are targets of canonical Dpp signaling during normal eye development. Therefore, Dpp employs this pathway to regulate the expression of a gene that is involved in wing differentiation but not a target of canonical Dpp signalling.

vg was used as a selector gene for generating wing tissue in the developing eye discs. Though, both *wg* and *vg* are selector genes for wing development, but, Wg requires the activities of other signaling molecules such as Dpp and notch to activate *vg* (Kim et al., 1996; Maves and Schubiger, 1998). Therefore, only the targeted expression of *wg* remains unable to intiate the expression of wing specific genes in eye imaginal discs and ventral part of leg discs. However, Vg along with Scalloped (Sd) (Campbell et al., 1992) form transcriptional activation complex to directly regulate the expression of genes involved in wing development (Halder et al., 1998; Halder and Carroll, 2001; Kim et al., 1996), therefore, *vg* is a more potent inducer for ectopic wing generation in developing imaginal discs in *Drosophila*.

Previous studies have implicated the involvement of different morphogens in cell fate alteration. In the light of all these previous studies, it would be really intriguing to check the involvement of this Dpp mediated dTak1-JNK cascade during such kind of transformations. Given the significant levels of conservation of signaling molecules and pathways between vertebrates and *Drosophila*, these findings also raise the possibility of manipulating the levels of morphogen activity as a therapeutic approach to modulate fate alteration of adult stem cells that has implication in tissue replacement and engineering.

Chapter5.

Employment of Matrix Metalloproteinase-1 by Dpp to regulate Hedgehog activity during ectopic eye formation in *Drosophila melanogaster.*

5.1 Introduction

Matrix Metalloproteinases are multifunctional Zinc endopeptidases normally associated with tissue remodeling and degradation of the extracellular matrix components (Nagase and Woessner, 1999; Sterchi, 2008).They are members of metizincin group of proteases having conserved methionine residue and Zn ion at their catalytic site (Agrawal et al., 2008; Gomis-Ruth, 2009). They are highly conserved endopeptidases and their presence have been documented in vertebrates, invertebrates and even in plants (Bai et al., 2005; Marino and Funk, 2012; Page-McCaw et al., 2003).

As shown in Figure 5.1, all MMPs consist of common structural domains that include signal peptides, prodomain for latency, catalytic domain and hemopexin domain (Glasheen et al., 2009; Nagase et al., 2006).

Figure 5.1. Structure of Matrix Metalloproteinases: MMPs contain a conserved multidomain structure having Singal Sequence, prodomain, catalytic domain and Hemopexin domain.

Most of the MMPs get secreted in inactive form having pro-domain that inhibits catalysis on active site. Signal peptide is required for secretion and localization. They become active only after removal of the pro-domain. Catalytic domain brings about proteolysis of substrate. Hemopexin domain helps in protein-protein interaction that includes substrate recognition, localization and activation of the enzyme.

Based on the primary structure and substrate specificity, MMPs can be classified into two broad categories, either membrane-bound MMPs (7 mammalian MMPs) or secreted MMPs (17 mammalian MMPs). MMPs activity can be inhibited by their endogenous inhibitors known as tissue inhibitor of metalloproteinases (TIMPs).

TIMP sterically and reversibly hinders the active site on MMPs in one-to-one stoichiometry ratio (Gomis-Ruth et al., 1997). MMPs activity can be regulated at various levels such as transcriptional regulation, cleavage of prodomain for their activation, localization and secretion and at the level of inhibitors proteins like TIMP.

Though, MMPs were first discovered as collagenase because of their role in degrading fibrillar collagen in tadpole metamorphosis (Brinckerhoff and Matrisian, 2002), later on, they attained major attention and interest because of their involvement in various pathophysiological conditions like arthritis, inflammation and invasion during metastasis, and cancer. MMPs were found to be upregulated in almost all kinds of cancer. They can be produced by fibroblasts associated with cancer cells or by adjacent stroma or by nonfibroblastic cancer cells (Bhowmick et al., 2004; Vihinen and Kahari, 2002). MMPs can enhance tumor progression by promoting angiogenesis, growth, proliferation and metastasis. MMP-2, MMP-9 and MMP-14 were found to be involved in promoting invasion of cancerous cells by degrading basement membrane (Lakka et al., 2002; Liu et al., 2006). Almost all the members of MMPs family were found to be deregulated in cancerous cells with major involvement of MMP-1, 2, 7, 9, 13 and 14. However, animal studies have also shown that MMPs sometimes have protective roles in cancer development. MMP-9 null mice develop more aggressive skin tumors (Coussens et al., 2000). Similarly, MMP-12 null mice have more tendencies to develop squamous cell carcinoma (Kerkela et al., 2002). MMPs expression has also been documented in synovial membrane, tendons, ligaments, and in cartilage tissues in patients of rheumatoid arthiritis as well as osteoarthritis (Murphy et al., 2002; Murphy and Nagase, 2008). During arthritis, MMPs bring about destruction of these tissues by degrading various ECM components. MMPs also play important role in various challenged conditions like pro-inflammatory responses in tissue injury, regeneration, wound healing (Loffek et al., 2011; Page-McCaw et al., 2007) and in innate immunity by proteolytic activation of various cell adhesion molecules like E-cadherin, CD44 and syndecan-1 (Baena-Lopez and Garcia-Bellido, 2003;

Kajita et al., 2001; Noe et al., 2001), cytokines and chemokines and proteolytic shedding of lipo-polysaccharides receptors like CD14 (Senft et al., 2005).

Analyses of MMPs knockout mutants have revealed that while MMPs don't have any role in early embryonic development, they are involved in later stages of development. Initial characterization of 14 mouse MMP mutants revealed that all the mutants live up to birth (Ducharme et al., 2000; Oh et al., 2004; Rudolph-Owen et al., 1997; Stickens et al., 2004). Dispensability of the MMPs during embryonic development can be explained in terms of enzyme redundancy, compensation and presence of multiple overlapping substrates. However, in later developmental stages MMPs bring about tissue remodeling during bone, vascular tissue and mammary gland development by release of various ECM bound growth factors like insulin growth factor, FGF or TGF-β (Inada et al., 2004; Martignetti et al., 2001; Page-McCaw, 2008; Stickens et al., 2004). Therefore, vast arrays of *in vivo* studies in various model organisms have revealed their roles in various postnatal developmental, physiological and pathological processes.

The various ways by which MMPs can facilitate diverse biological processes (Fig. 5.2)(Vu and Werb, 2000) are as follows:

1. In some instances MMPs degrade ECM to affect cell migration by changing the cells from an adhesive to non-adhesive state. For example, cleavage of laminin-5 and collagen IV results in exposure of cryptic sites that promote migration and collagen-1 degradation by MMP-1 facilitate epithelial cell migration and wound healing in culture models (Giannelli et al., 1997; Keisman and Baker, 2001; Pilcher et al., 1997).

2. Alternately, MMPs can bring about alteration in ECM microenvironment to regulate cell behavior towards proliferation, apoptosis or morphogenesis (Newby, 2006). For instance, Mmp1 regulates proliferation of intestinal stem cells by regulating Egfr signaling in *Drosophila* (Cho et al., 2014).

3. Moreover, MMPs can modulate the activity of biologically active molecules by cleaving or releasing them from the ECM and balance their activities by modulating the activities of their inhibitors (Fowlkes et al., 1995; Wang et al., 2000; Whitelock et al., 1996). They cleave various substrate molecules to produce independent bioactive molecules or ligands that bind to cognate receptors present on cell surface to facilitate diverse cell biological processes.

However, there being 24 members in the MMP family of proteins in vertebrates, it has rather difficult to delineate the role of individual MMPs that show partially overlapping functions (Page-McCaw, 2008). Fortunately, the members of MMP family are evolutionarily conserved, having their homologs in diverse phyla that include plants, various invertebrates and vertebrates. Recent studies have demonstrated the role of MMPs in *Drosophila* tumor invasion (Uhlirova and Bohmann, 2006), anchor cell invasion in *C. elegan* (Sherwood et al., 2005), Hydra regeneration (Leontovich et al., 2000) and in other model organisms such as *Xenopuslaevis* (Harrison et al., 2004; Hasebe et al., 2007), and zebrafish (Bai et al., 2005). Therefore, these invertebrate models provide wonderful opportunities to understand the functions of MMPs. *Drosophila melanogaster* is one of the most developed invertebrate models to study the function of MMPs. Importantly, *Drosophila* has only two conserved MMPs genes *Mmp1* and *Mmp2* (Llano et al., 2002; Llano et al., 2000). Thereby, it is easy to understand the basic questions regarding the activation and regulation of Mmps in a much simpler model organisms like *Drosophila*.

The fly Mmp1 is secretory in nature while Mmp2 is GPI anchored membrane associated protein (Llano et al., 2002; Llano et al., 2000). *Drosophila* has only one Timp that is closely related to TIMP-3 in mammals and can block the functions of both the Mmps *in vivo*. Importantly, fly Timp can also inhibit the activity of mammalian MMP-1, -2, -3 and -14 (Wei et al., 2003) and mammalian TIMP-2 and TIMP-4 can also inhibit fly Mmp1 (Llano et al., 2000) and thereby exhibit the highly conserved nature between mammalian and *Drosophila* MMPs.

Expression studies revealed that while they are expressed during embryonic and larval stages, increased level of *Mmp1* and *Mmp2* transcripts are detected during early pupal stages (Page-McCaw et al., 2003). RNA in situ hybridization revealed *Mmp1* expression at the 13th-15th embryonic stage in the repetitive segments cells at the place of dorsal closure. Mmp2 start expressing in early embryos (stage-10) specifically in the mesoderm, migrating somatogastric nervous system, in the ectoderm as well as in peripheral nervous system. Later on, at stage 14 and 17, Mmp2 also expresses in the central nervous system and brain. In the larval tissues, Mmp1 expression was found in the wing imaginal disc in a band of cells in the region proximal to hinge region that also overlap with tracheal marker *breathless (btl)* while no expression was detected in leg and eye imaginal discs (Page-McCaw et al., 2003).

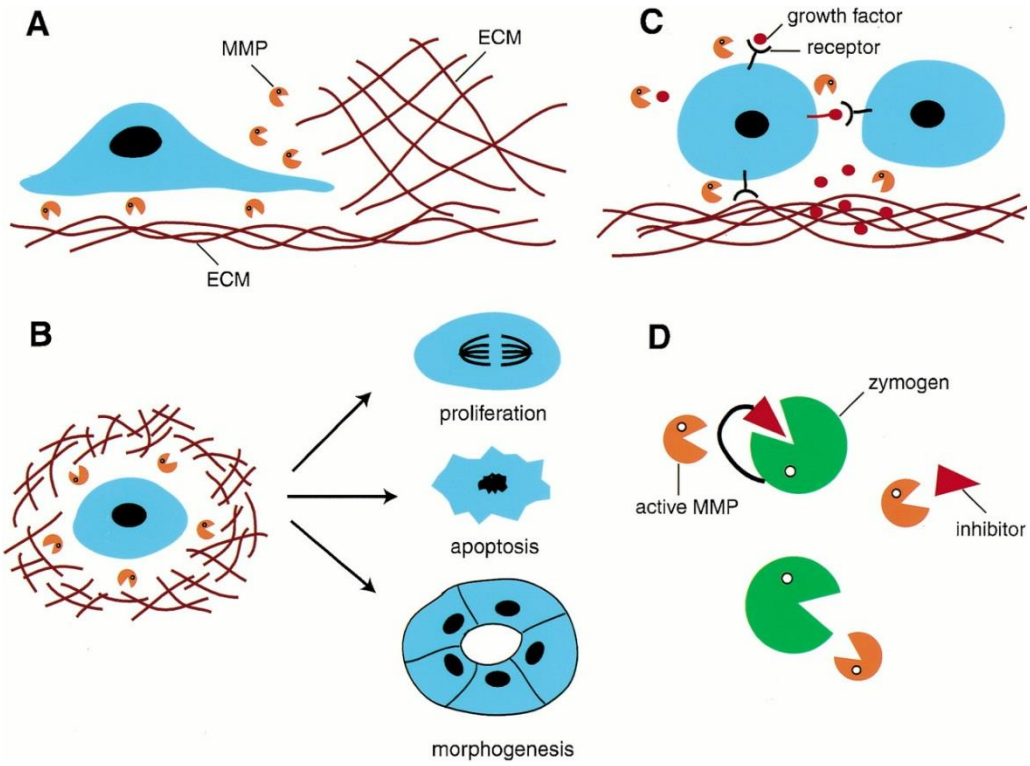

Figure 5.2. Mode of Matrix Metallo-proteinases action. (A) MMPs affect cell migration by degrading ECM. **(B)** MMPs modulate ECM microenvironment to regulate proliferation, apoptosis and morphogenesis. **(C)** MMPs activate various signaling molecules such as growth factors, cytokines by cleavage or by facilititaing their release. **(D)** MMPs regulate proteases activity by modulating their inhibitors (Vu and Werb, 2000).

Though both the Mmps start expressing from the early embryonic state but mutant analyses revealed that both the MMPs do not have any role during embryonic development in *Drosophila* (Page-McCaw et al., 2003). Double null mutants for both *Mmp1* and *Mmp2* can survive through the embryonic stage and even progress up to mid larval stage. Therefore, *Drosophila* Mmps confirm mammalian Mmps mutant results that establish their roles specifically in remodeling processes rather than during intial cell-cell migrations involved during embryonic development (Page-McCaw et al., 2003). Mmp1 has role in tracheal growth that allow oxygen diffusion to tissues during larval period (Glasheen et al., 2010) and in pupal head eversion (Page-McCaw et al., 2003) while Mmp2 plays important role in axon guidance of motor neurons during embryonic development(Miller et al., 2011).

In *Drosophila*, Mmps are known to regulate developmental tissue invasion during eversion of imaginal discs in the pupal stage and tumour invasion by degrading the components of basement membrane (Srivastava et al., 2007). Disc eversion is a process where imaginal discs cells present inside the larval body evade out during metamorphosis to generate respective adult structures. Eversion of disc cells requires degradation of basement membrane components such as collagen IV and Viking lining these cells. Considering that basement membrane remodeling is an essential step during developmental and tumour invasion, it has always been used as a target for cancer therapy.

Moreover, Mmp1 and Mmp2 work co-operatively for fat bodies dissociation during *Drosophila* metamorphosis (Jia et al., 2014). Mmp1 acts on DE-cadherin junction between fat body cells while Mmp2 degrades basement membrane during fat body dissociation. In contrast, both Mmp1 and Mmp2 act independently and reciprocally required to regulate synaptogenesis along neuromuscular junction in *Drosophila* (Dear et al., 2016). They act on Heparan Sulfate Proteoglycans (HSPGs) to regulatetrans-synaptic signaling of the morphogen Wingless. Both the Mmps also play important role in dendritic remodeling during metamorphosis (Kuo et al., 2005).

Apart from developmental processes,expression of Mmp1has been found to be upregulated by JNK signaling upon wounding (Stevens and Page-McCaw, 2012). In this case Mmp1 brings about re-epithelization by reorganizing actin cytoskeleton, promoting cell elongation and basement membrane remodeling to facilitate wound healing. Therefore,

MMPs are not required for embryonic development rather they play various important roles in various other important processes during postnatal developmental processes.

The involvement of MMP in cell fate alteration was first evidenced when results of differential expression profiling of genes associated with transdetermination revealed that both the *Drosophila* MMPs get upregulated in transdetermining leg discs (Klebes et al., 2005). In subsequent years while the involvement of Mmp1 during this process was established but any detectable role of Mmp2 was not observed (McClure et al., 2008). It was observed that the frequency of leg to wing transdetermination by ectopic expression of *wingless* got significantly enhanced in *Mmp1* heterozygous ($Mmp1^{Q112}/+$) mutant background. Activation of ectopic Mmp1 expression was also detected at the site of blastema formation in the dorsal compartment of regenerating /transdetermining leg discs expressing high level of Dpp. However, when they carried out regeneration in Mmp1 mutant background, they found more proliferation only in ventral nonblastema cells while proliferation density in dorsal blastema cells remained unchanged (McClure et al., 2008). More importantly, in Mmp1 mutant background cells of the ventral compartment of the leg disc were able to change their fate. From all these results, they suggested the possibility of a complex interaction between Dpp and Mmp1 in regenerating/transdetermining leg discs. But, the exact role played by Mmps to restrict such kind of cell fate alteration was not clear. Furthermore, the signal that triggers ectopic MMP1 expression during transdetermination was not known.

Alteration in cell fate specification within a given tissue demands complete rewiring of the already existing interactions among various morphogen signalings. As extracellular matrix play important role in defining and shaping morphogen gradients, I was intrigued to know whether Mmp1 plays any role in this process. To address this issue, I employed wing to eye transdetermination system in *Drosophila*.

The specific questions that I was interested to address are as follows:

1. Is Mmp1 also involved in alteration of cell fate during wing to eye transdetermination?
2. How does Mmp1 get activated during wing to eye fate change?
3. What is the role of Mmp1 during this process?

5.2 Results

Ectopic eyes were generated by driving *UAS-eyeless* with *Dppp-Gal4* in the developing wing discs of *Drosophila* to understand the role of Mmps during wing to eye transdetermination. As mentioned in previous chapters, Dpp expresses along the A/P boundary that divides the wing disc into anterior and posterior compartments (Fig.5.3A). As observed earlier, immunostaining of wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae, 136 hrs after egg laying (AEL), led to ectopic photoreceptor differentiation along the entireA/P axis (Fig.5.3B).

5.2.1 Mmp1 negatively regulates ectopic photoreceptor differentiation.

To investigate the involvement of Mmp1 during wing to eye fate alteration, activity of Mmp1 was impaired by various means and its effect on ectopic photoreceptors differentiation was observed. For these analyses, study was resorted to early third instar larval wing discs (124 hrs AEL) to avoid morphological distortions associated with late third instar larval wing discs of these genotypes. Very less number of ectopic photoreceptors were observed in the wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae that were 124 hrs old AEL (Fig.5.3C) as compared to late third instar larval wing discs (Fig.5.3B). To examine the role of Mmp1, its expression was knocked down by co expressing *UAS-Mmp1 (dsRNA)* along with *UAS-ey* by *Dpp-Gal4*. Two independent RNAi lines obtained from two different sources were used for this purpose. Interestingly, 4-5 fold increase in the number of ectopic photoreceptors was observed with *UAS-Mmp1 (B) (dsRNA)* (obtained from D.Bohman lab) (Fig.5.3D, I) while 3-4 fold increase was detected with *UAS-Mmp1 (KK) (dsRNA)* (obtained from VDRC) (Fig.5.3E, I) as compared to control (Fig.5.3C, I). Robust increase of 5-6 folds in the number of ectopic photoreceptors was also observed when ectopic eyes were generated in wing discs with a genetic background mutant for *Mmp1* hypomorphic allele $Mmp1^{Q273}$ (Fig.5.3F, I). Co-expression of the *Drosophila* TIMP (tissue inhibitors of metalloproteases), an endogenous inhibitor of metallo-proteases that occupy active site of the enzyme, in the cells undergoing photoreceptors differentiation also resulted in 4-5-fold increase in the number of ectopic ommatidia (Fig.5.3G, I). In tune with these results, overexpression of Mmp1 led to a drastic drop in the number of ectopic photoreceptors in *UAS-ey/+; Dpp-Gal4 /UAS-Mmp1* larval wing discs (Fig.5.3H, I). To ascertain that the increase in the number of ectopic

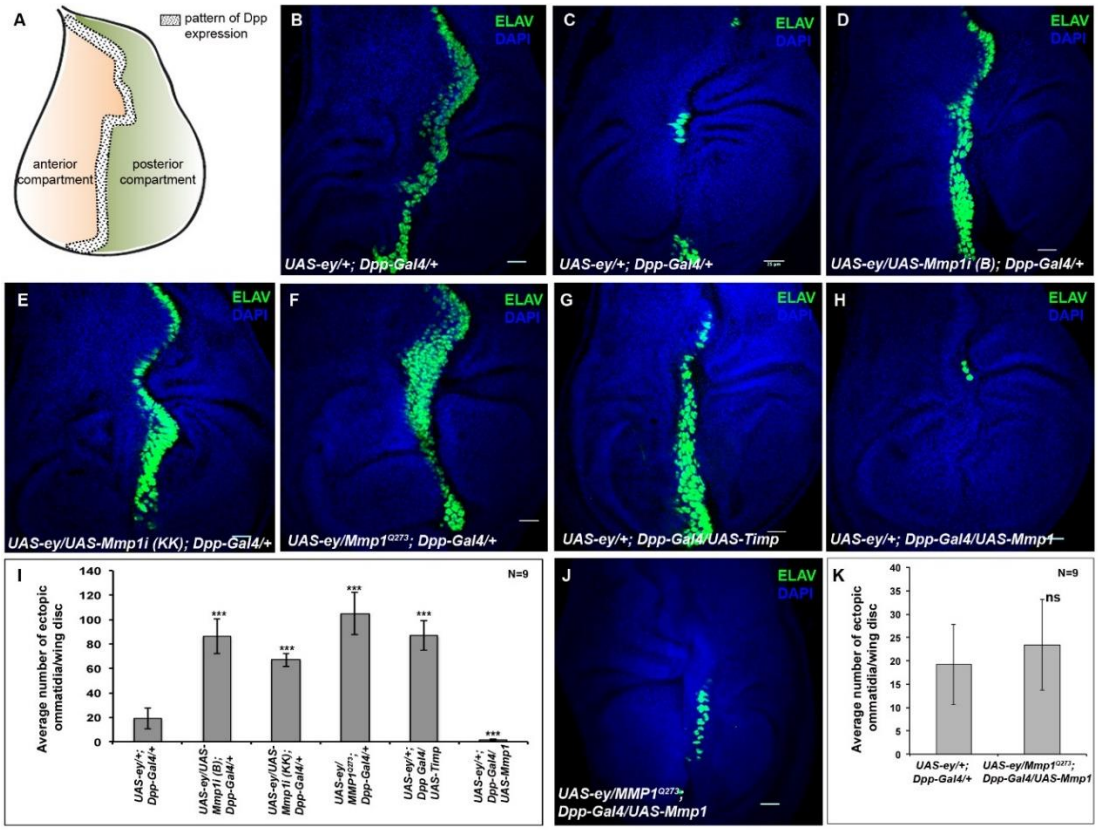

Figure 5.3. Mmp1 negatively regulates ectopic photoreceptor differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A)** Schematic representation of third instar larval wing imaginal disc showing the pattern of Dpp expression along the A/P boundary. **(B)** Ectopic photoreceptors, marked by ElAV expression, differentiated along the whole A/P axis in the wing discs of late third instar larvae (136hours AEL). **(C)** Small number of photoreceptors was detected in the wing discs of mid third instar larvae (124 hours AEL). **(D-G)** Robust increase in ectopic photoreceptors differentiation was observed upon knocking down Mmp1 expression as in **(D)** *UAS-ey/UAS-Mmp1 (dsRNA)(B);Dpp-Gal4/+* **(E)** *UAS-ey/UAS-Mmp1 (dsRNA)(kk); Dpp-Gal4/+* mid third instar larval (124 hours AEL) wing discs as compared to *UAS-ey/+; Dpp-Gal4/+* larval wing discs of similar age. **(F, G)** Similar increase in ectopic photoreceptors number was observed upon attenuating the Mmp1 activity in **(F)** *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/+* **(G)** *UAS-ey/+; Dpp-Gal4/UAS-Timp* mid third instar larval (124 hours AEL) wing discs. **(H)** Drastic drop in the number of ectopic ommatidial differentiation was observed by overexpression of Mmp1 in the mid third instar wing discs of *UAS-ey/+; Dpp-Gal4/UAS-Mmp1* larvae. **(I)** Quantitative estimate of average number of ectopic photoreceptors in the mid third instar larval wing discs of genotypes mentioned (mean ± SD; p value ***<0.0001.). **(J)** The increase in the number of ectopic photoreceptors as observed in *Mmp^{Q273}* mutant background (F) got significantly reduced by over expression of Mmp1 in this background. **(K)** Quantitative estimate of average number of ectopic ommatidia in the mid third instar larval wing discs of genotypes mentioned (mean ± SD; n.s.not significant).

photoreceptor differentiation was specifically due to loss of Mmp1 activity I was interested to see whether the increase in the number of photoreceptors as observed upon attenuating Mmp1 activity in *Mmp1^{Q273}* heterozygous mutant could be reduced by over expressing Mmp1 in this background. Indeed, the increase in the number of ectopic ommatidia upon attenuating Mmp1 activity in the *Mmp1^{Q273}* heterozygous mutant got significantly restored upon over expression of Mmp1 in *Mmp1^{Q273}/UAS-ey; Dpp-Gal4/UAS-Mmp1* larval wing discs (Fig.5.3J, K). Taken together, these results confirm that Mmp1 plays a critical role in limiting the extent of ectopic photoreceptors differentiation in developing wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae.

5.2.2 Dpp activates ectopic Mmp1 expression during wing to eye fate alteration.

The expression pattern of Mmp1 was checked in the wing discs undergoing ectopic photoreceptors differentiation at different larval developmental stages by immunostaining with anti-Mmp1 antibody. Initial expression of Mmp1 was found as an extended web in early third instar larval wing discs (94 AEL) (Fig.5.4B). Upon closer examination, strong Mmp1 expression was observed along the cell boundaries (Fig.5.4B'). No detectable Mmp1 expression was observed in the wild type wing discs of identical developmental stage (Fig. 5.4A). The domain of Mmp1 expression got markedly increased and extended into the pouch region parallel to the A/P axis at later stages. By 114 AEL, while intense ectopic Mmp1 expression was observed like a mesh extending along the A/P axis of *UAS-ey/+; Dpp-Gal4/+* larval wing discs (Fig.5.4D, D'), wild type wing disc was devoid of ectopic Mmp1 expression along the A/P axis (Fig.5.4C).

To have better spatial information of Mmp1 expressing cells, Mmp1 expression was analyzed with respect to Dpp expression along the A/P boundary. Dpp expression was analyzed by reporter RFP expression. As reported earlier, ectopic expression of *eyeless* by *Dpp-Gal4* led to up regulated level of Dpp expression in a broader domain as compared to its expression in wild type wing disc. Co-immunostaining of the discs expressing Dpp RFP with anti Mmp1 antibody revealed a very interesting pattern of Mmp1 expression in the disc proper cells of wing disc. Disc proper cells are a layer of columnar cells that remain juxtaposed to the overlying squamous epithelial cells known as peripodial cells and together these two layers of cells constitute the wing disc. No Mmp1 expression was detected in the apical surface of disc proper cells closer to peripodial membrane; however,

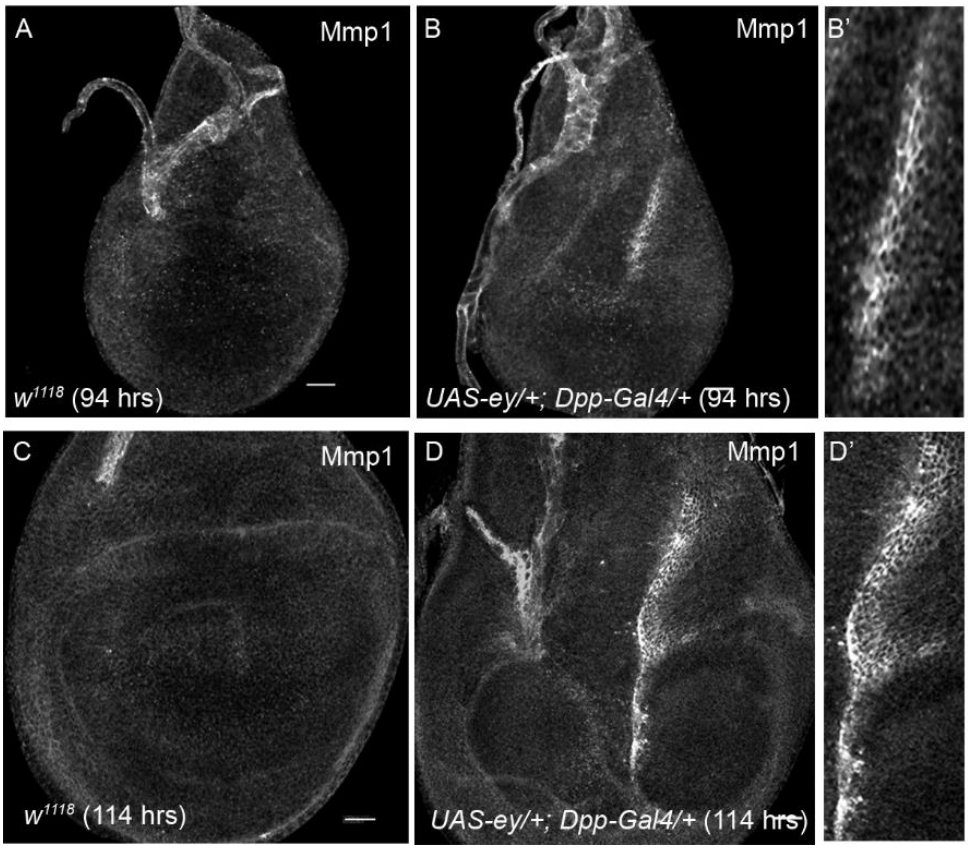

Figure 5.4. Dpp activates ectopic Mmp1 expression during wing to eye fate alteration. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A)** No Mmp1 expression was observed in the pouch region of wild type wing discs of early third instar larvae (94 hrs. AEL). **(B)** Ectopic induction of *eyeless* by *Dpp-Gal4* led to ectopic Mmp1 expression in the wing discs of early third instar larvae (94 hrs. AEL). **(B')** Zoomed in image of the portion of wing disc with ectopic Mmp1 expression. **(C)** Basal level of Mmp1 expression was detected in wild type wing discs of third instar larvae (114 hrs AEL). **(D)** Intense Mmp1 expression was seen along the A/P-axis in the wing discs of *UAS-ey*/+; *Dpp-Gal4*/+ third instar larvae (114 hrs AEL) **(D')** Zoomed in image of the portion of wing disc with ectopic Mmp1 expression in image (D).

these cells did have RFP expression for Dpp (Fig.5.5A). Rather in basal surface of disc proper cells, strong Mmp1 expression was present in cells just adjacent to the broader domain of Dpp expressing cells towards the posterior compartment of wing disc (Fig.5.5A'). Importantly, there was relatively more accumulation of Mmp1 protein adjacent to Dpp domain towards the posterior compartment (Fig.5.5B-B"). Upon careful observation at higher magnification, only few posterior most Dpp expressing cells were found with Mmp1 expression along their membrane (Fig.5.5B-B") and several punctate of Mmp1 expression was detected in between the Dpp expressing cells (Fig.5.5B"). Together these results indicated that the Dpp expressing cells might be producing Mmp1.

However, since there was no complete overlap between Dpp domain and Mmp1 expression, in order to ascertain whether Mmp1 is actually secreted by Dpp producing cells, expression of *Mmp1* was knocked down in the Dpp expressing cells by driving *UAS-Mmp1 (dsRNA)* with *Dpp-Gal4* in the wing discs undergoing ectopic photoreceptors differentiation and checked for ectopic Mmp1 expression. Interestingly, this resulted in a drastic reduction in the expression of Mmp1 (Fig.5.5C) and thereby confirmed that the Dpp expressing cells were actually expressing Mmp1 during ectopic photoreceptor differentiation in wing discs.

5.2.3 Dpp signaling through dTak1-JNK pathway regulates ectopic Mmp1 expression during wing to eye fate alteration.

In the previous sections of the thesis, it was established that Dpp acts through both canonical as well as noncanonical pathway to regulate its target genes expression during ectopic eye formation (Aggarwal et al., 2016) (Fig.3.24). While the canonical pathway involved Mad and Med, the alternate noncanonical pathway involved dTak1-JNK (Fig.5.6A). Therefore, the involvement of both signaling cascades triggered by Dpp was checked for regulating ectopic Mmp1 expression.

The activities of the members of both canonical and noncanonical Dpp signaling was impaired and checked for their effect on Mmp1 expression by immunostaining with anti-Mmp1 antibody. Interestingly, attenuating Dpp signaling by knocking down the expression of one of its receptors *thickveins* (*tkv*) resulted in drastic reduction of Mmp1 expression in these cells (Fig.5.6B). Analogous results were obtained upon generating ectopic eyes in the wing discs heterozygous mutant for *tkv* loss of function allele tkv^{7} (Fig.5.6.C).

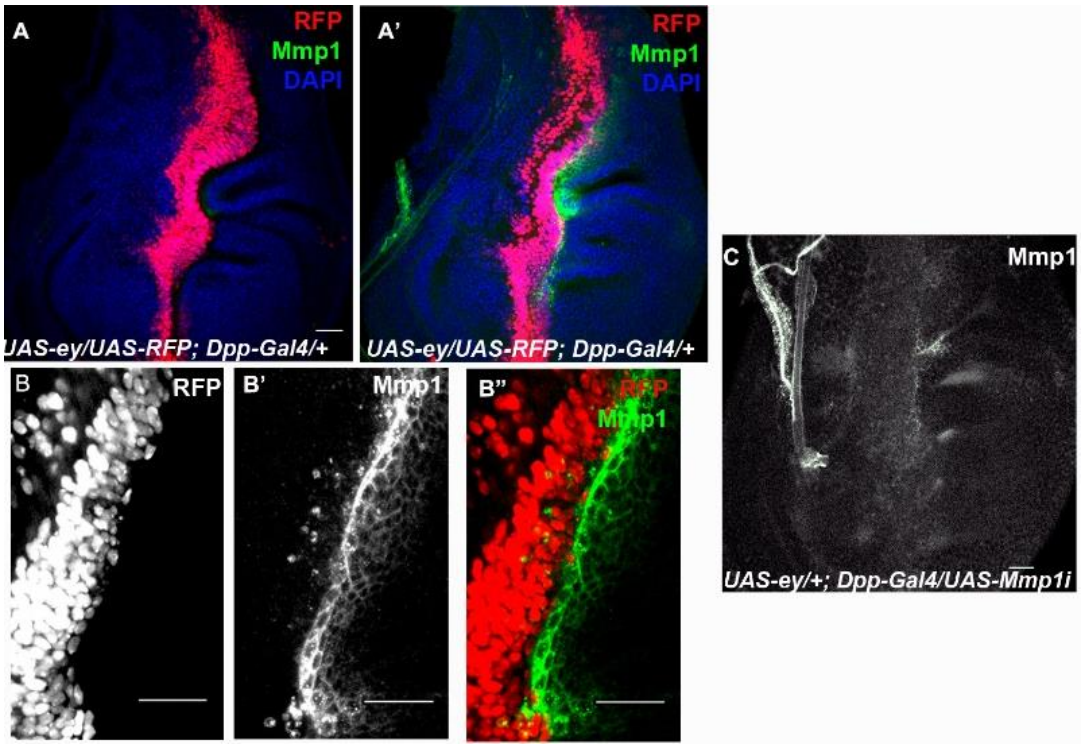

Figure 5.5. Dpp activates ectopic Mmp1 expression during wing to eye fate alteration. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A, A')** Ectopic expression of Mmp1 with respect to high levels of Dpp expression in the third instar larval wing discs undergoing ectopic photoreceptors differentiation no Mmp1 expression with respect to Dpp expression was detected in the apical surface of disc proper cells closer to peripodial membrane **(A)** Strong Mmp1 expression posterior to Dpp expression domain was observed in the basal surface of disc proper cells **(A'). (B-B")** Zoomed in image of the portion of the wing disc of *UAS-ey*/*UAS-RFP; Dpp-Gal4*/+ mid third instar larvae exhibiting Reporter RFP expression for Dpp **(B)** Mmp1 expression **(B')** merged image of reporter RFP and Mmp1 expression **(B"). (C)** Drastic reduction in ectopic Mmp1 expression was seen upon co-expressing UAS-*Mmp1 (dsRNA)* and *UAS-ey* by *Dpp-Gal4*.

Similar reduction in the level of Mmp1 expression was observed when ectopic photoreceptor differentiation was induced in wing discs that were otherwise mutant for *dTak1* (Fig.5.6.D) as well as upon knocking down the expression of *dTak1* (Fig.5.6.E). However, the Mmp1 expression remained unaltered when ectopic eyes were induced in a genetic background with impaired canonical Dpp signaling i.e. in *Mad* mutant (Mad^{12}) (Fig.5.6I) background as well as by knocking down the expression of *Mad* (Fig.5.6J).

These results clearly demonstrate that Mmp1 activation was mediated by Dpp-dTak1 signaling cascade, not by the canonical Dpp signaling involving Mad.

To confirm the involvement of Dpp mediated noncanonical pathway in activating *Mmp1* expression, Mmp1 expression was checked upon compromising the activities of different members of JNK pathway. Importantly, Mmp1 protein level decreased significantly upon inactivation of JNK signaling by co expression of dominant negative form of *Bsk* (JNK in flies) in the transdetermining tissue (Fig.5.6.F). This result showed that Mmp1 expression during ectopic photoreceptors differentiation required JNK activity. Subsequently, the involvement of AP-1 transcription factor formed by dJun and dFos was also investigated for the activation of Mmp1 during ectopic photoreceptor differentiation. Inhibition of dFos activity by co-expression of its dominant negative form as well as knocking down *kayak* expression by expressing *UAS-kayak (dsRNA)* in the transdetermining tissue (Fig.5.6G, H) resulted in significant decrease in the level of Mmp1 expression, that was comparable to that observed upon suppressing Basket activity. These results clearly demonstrated that Mmp1 expression was triggered by JNK signaling during wing to eye fate change.

5.2.4 JNK transcriptionally activates Mmp1 during wing to eye fate alteration.

Further, to ascertain Mmp1 as a transcriptional target of JNK mediated signaling, Mmp1-lacZ transgenic reporter fly line (Fig.5.7.A) (Uhlirova and Bohmann, 2006) was used. This transgenic line harbors a reporter construct containing 4.78-kb fragment spanning first intron in the Mmp1 genomic region having three putative AP-1 binding sites inserted upstream to lacZ coding sequence. Therefore, this construct will express lacZ only if *Mmp1* expression is mediated by AP-1. Interestingly, strong Mmp1-lacZ expression was observed along the A/P boundary (Fig. 5.7B) that completely overlapped with the expression of Mmp1 protein monitored with Mmp1 antibody in wing discs of *UAS-ey*/+*; Dpp-Gal4*/+ larvae (Fig. 5.7.B', B").

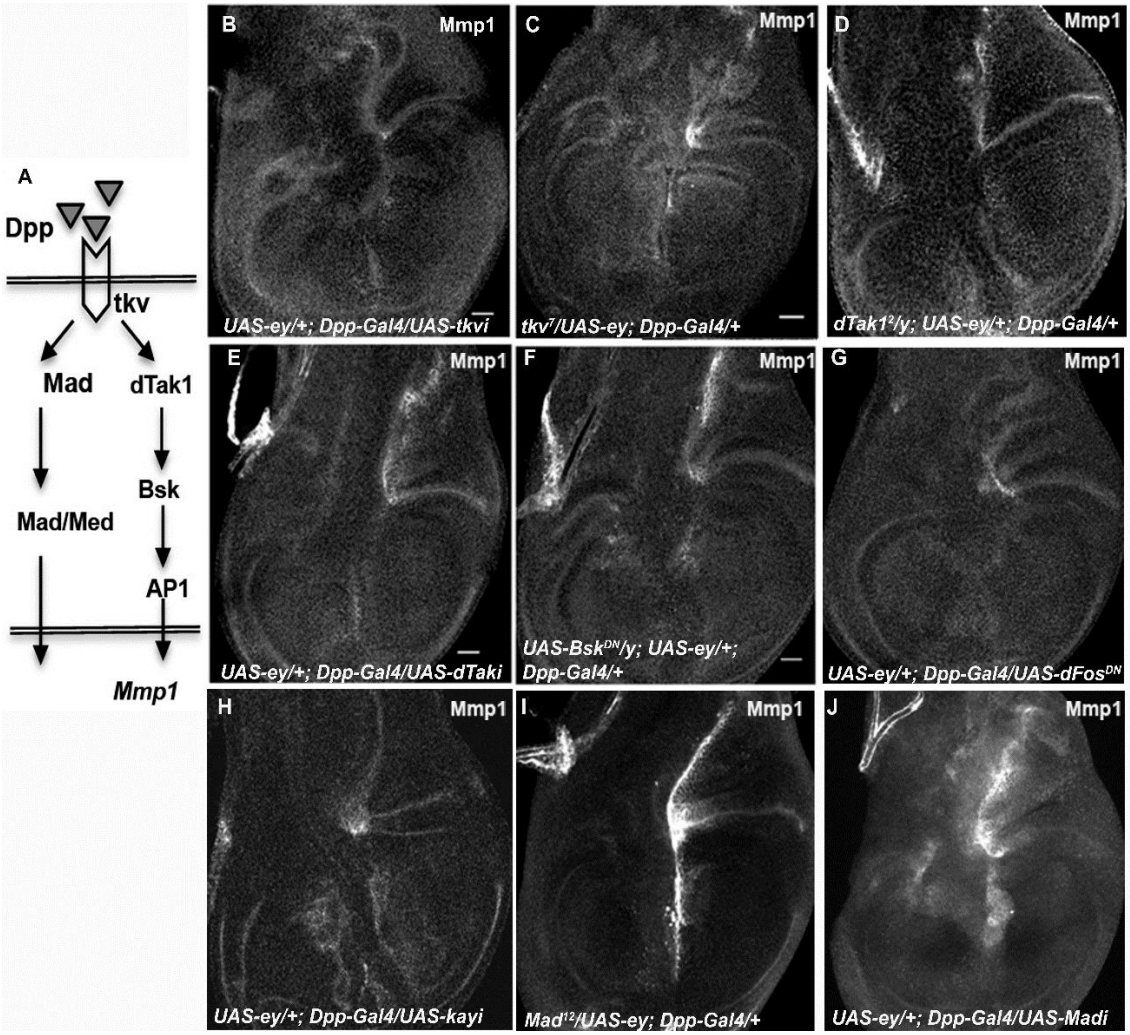

Figure 5.6. Dpp signaling through dTak1-JNK pathway regulates ectopic Mmp1 expression during wing to eye fate alteration. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A)** Schematic representation of the possible Dpp mediated signaling pathways that can lead to ectopic Mmp1 expression during ectopic eye formation. **(B, C)** sharp reduction in ectopic Mmp1 expression was observed either upon impairing the activity of tkv by inducing ectopic eyes in the wing discs of *tkv*^{7} heterozygous larvae **(B)** as well as knocking down the expression of *tkv* **(C). (D, E)** Drastic reduction in ectopic Mmp1 expression was observed either upon Attenuation of dTak1 activity by inducing ectopic eyes in the wing discs of *dTak1*^{2} heterozygous larvae **(D)** as well as upon knocking down the expression of *dTak1* **(E). (F-H)** Significant drop in ectopic Mmp1 expression was seen upon inactivating JNK signaling by co-expressing *UAS-bsk*^{DN} **(F)** and *UAS-Fos*$^{DN.}$ **(G)** and by knocking down the expression of *kay* **(H)**. **(I, J)** Attenuation of Mad activity as well as knocking down the expression of Mad did not affect Mmp1 expression.

In tune with previous results, drastic reduction was found in the level of reporter Mmp1-lacZ expression in these wing cells when they were induced to undergo photoreceptor differentiation in a genetic background heterozygous mutant for *dTak1* loss of function allele $dTak1^{2}$ wing discs (Fig.5.7C). Finally, the transcript level of *Mmp1* was analyzed by QRT-PCR analyses in *UAS-ey/+; Dpp-Gal4/+* larval wing discs. In consistence with the results of immunostaining, significant increase (3-3.5 fold) in the transcript level of *Mmp1* was seen in the wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae as compared to its expression in wild type wing discs (Fig.5.7D). Importantly, this increase in the expression level of *Mmp1* got significantly reduced upon attenuating the activity of dFos in *UAS-ey/+; UAS-*Fos^{DN}*/Dpp-Gal4* wing discs (Fig.5.7D). Taken together, these results confirmed that Dpp mediated JNK signaling transcriptionally regulated the expression of *Mmp1* during ectopic photoreceptor differentiation independent of canonical Dpp signaling.

5.2.5 Morphological changes associated with the wing discs undergoing wing to eye fate alteration.

Larval wing imaginal disc of *Drosophila* is a sac like structure made up of two juxtaposed layers of epithelial cells. A lower layer of elongated pseudo stratified columnar epithelium form disc proper cells. Overlaying disc proper is a layer of squamous epithelium known as peripodial membrane. There were lots of structural changes in the columnar epithelium of wing discs upon ectopic expression of *eyeless* (Fig.5.7B) in contrast to wild type wing discs (Fig.5.7A). To have better insight of the results, studies were shifted to transverse cryosections made through the X-Z plane of wing discs. Phalloidin staining of transverse sections through the wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae revealed the presence of a protrusion of the disc proper layer in a direction opposite to that of the peripodial membrane (Fig. 5.8D, D'). This protrusion was found to be in the middle of the wing disc along the A/P axis. Similar sections through wild type wing disc did not exhibit any folding in the sheet of continuous columnar epithelial cells that constitute the disc proper layer (Fig. 5.8C, C'). To understand the spatial location of the protruded region in the developing wing disc, the expression of Dpp (Shen and Dahmann, 2005) and posterior compartment specific marker protein Engrailed (En) (Strigini and Cohen, 1999) was checked in the transverse sections of the wing discs undergoing ectopic photoreceptor differentiation. As revealed by reporter RFP expression, Dpp was found to express in the

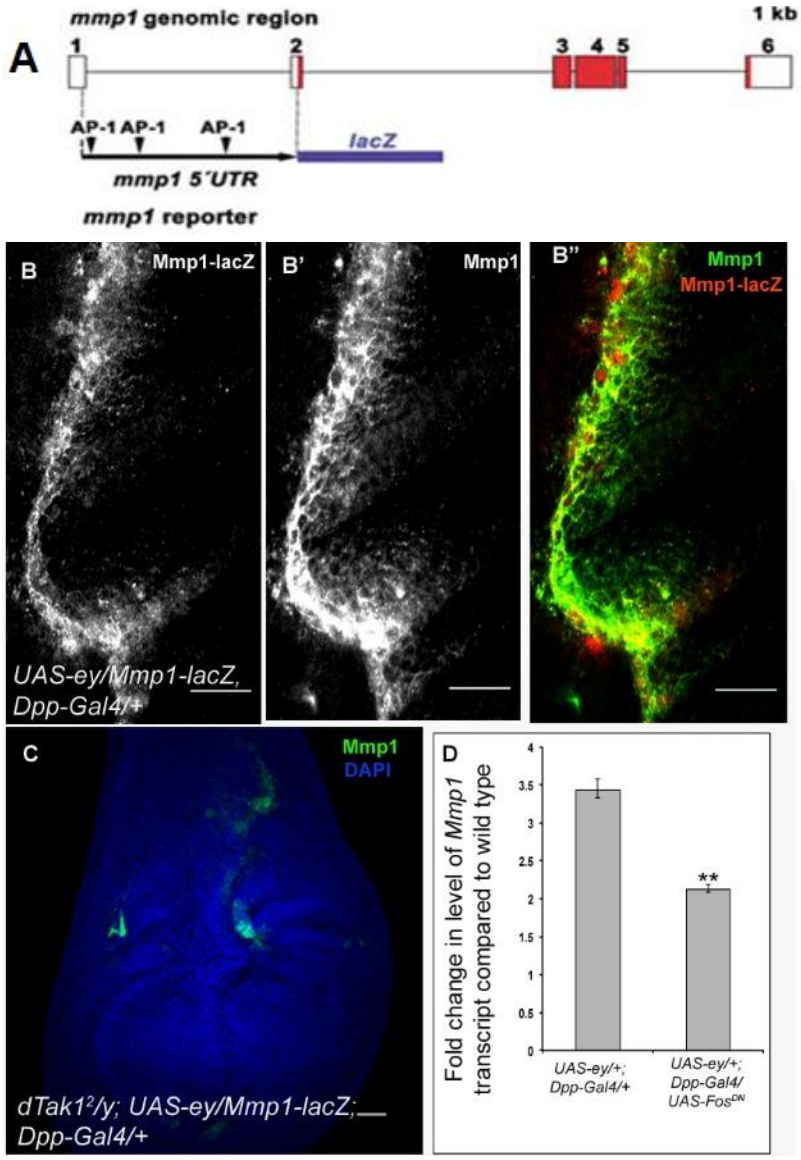

Figure 5.7. JNK transcriptionally activates Mmp1 during wing to eye fate alteration. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A)** Schematic representation of the transgenic construct of Mmp1-lacZ reporter line having three putative AP-1 binding sites in the genomic region (Uhlirova and Bohmann, 2006). **(B, B', B")** Zoomed in image of the region of wing disc showing reporter Mmp1-lacZ expression **(B)** that completely overlaps with ectopic Mmp1 expression as revealed by immunostaining **(B',B")** shows transcriptional activation of *Mmp1* by JNK signaling. **(D)** Mmp1-lacZ expression got significantly reduced upon knocking down the expression of *dTak1* in the wing discs undergoing ectopic photoreceptors differentiation. **(C)** Significant increase in the level of Mmp1 transcript upon ectopic expression of *eyeless* in wing imaginal discs got significantly reduced by impairing the activity of dFos in this background (mean ± SD; p value **<0.001.).

anterior part of the protrusion (Fig.5.8E). Immunostaing with anti-Engrailed antibody showed its expression in other half of protrusion adjacent to Dpp expressing cells (Fig.5.8F). Together these results suggested that the protrusion of the disc proper cells was actually formed along the A/P boundary of the wing disc.

5.2.6 Mmp1 expresses adjacent to eye primordial cells in the wing discs undergoing ectopic photoreceptor differentiation.

As reported earlier, eye development is initiated by the combinatorial activities of members of RD genes network that establish the eye primordial (Firth and Baker, 2009). Ey along with Dpp synergistically activates *eya* and *so* and they finally turn on *Dac* (Curtiss and Mlodzik, 2000; Pappu et al., 2005). Therefore, the expression of RD gene defines or marks the eye primordia. To determine the location of cells undergoing wing to eye fate change in the transverse sections, the expression of one of the RD gene Dac was checked with respect to Dpp expressing cells. As revealed by reporter RFP expression, Dpp expression was observed in the anterior part of the protrusion (Fig.5.9A) and a subset of the Dpp expressing cells towards the posterior compartment was found to express Dac (Fig.5.9A', A"). Next, the position of Mmp1 expressing cells with respect to the domain of Dac expression was analyzed. Intense Mmp1 expression was seen around the boundaries of the cells (Fig.5.9B) just next door to the Dac expressing cells towards the posterior compartment in the protrusion (Fig.5.9B', B").

5.2.7 Mmp1 limits Dac domain to restricts ectopic photoreceptor differentiation

To better understand the role of Mmp1 in ectopic photoreceptor differentiation, Dac and Eya expression was analyzed upon knocking down the expression of Mmp1 by driving *UAS-Mmp1 (dsRNA) (B)* in the wing discs undergoing ectopic photoreceptor differentiation. Interestingly, significant increase in the area of Dac expressing cells as well as in the level of its expression was observed in the transverse sections made through *UAS-ey/+; UAS-Mmp1 (dsRNA) (B)/Dppp-Gal4* larval wing discs (Fig. 5.10B-B") as compared to control *UAS-ey/+; Dpp-Gal4/+* larval wing discs (Fig. 5.10A-A"). Similar increase in the area as well as in the level of Dac expression was detected in *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/+* larval wing discs (Fig. 5.10C-C"). In consistence with increase in Dac expression area, there was an increase in ectopic photoreceptor differentiation in the wing discs of *UAS-ey/+; UAS-Mmp1 (dsRNA) (B)/Dpp-Gal4*

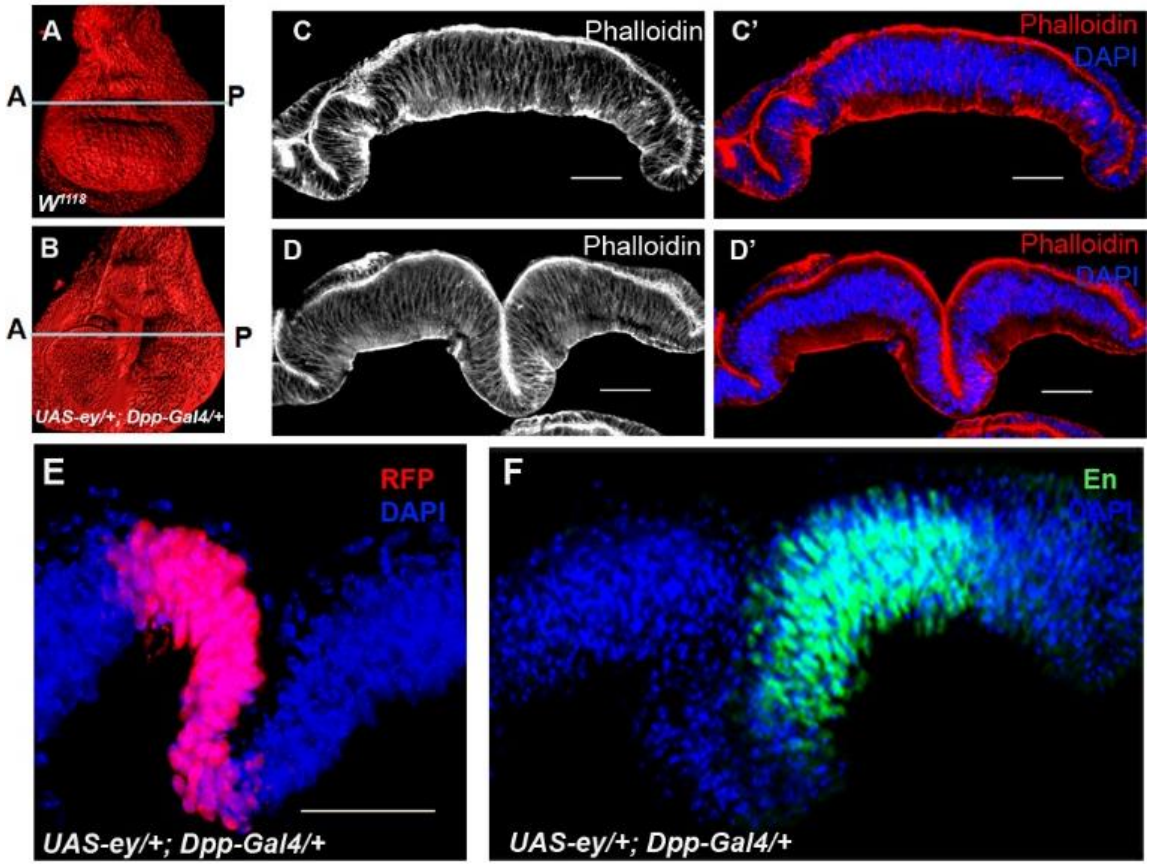

Figure 5.8. Morphological changes associated with the wing discs undergoing wing to eye fate alteration. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A, B)** Grooved wing imaginal disc of *UAS-ey/+; Dpp-Gal4/+* larvae **(A)** as compared to wild type wing imaginal disc **(B)**. Phalloidin staining in the transverse sections of the wing discs made through the axis drawn from anterior to posterior compartment showing **(C, C')** columnar epithelial disc proper cells and squamous epithelial cells of the peripodial membrane in wild type wing disc. **(D, D')** Transverse section through wing disc of *UAS-ey/+; Dpp-Gal4/+*larvae revealed a protrusion in the disc proper cells in a direction opposite to peripodial cells. **(E)** Dpp expression was observed in the anterior most part of protrusion marks A/P boundary. **(F)** Engrailed expression observed in the posterior arm of protrusion.

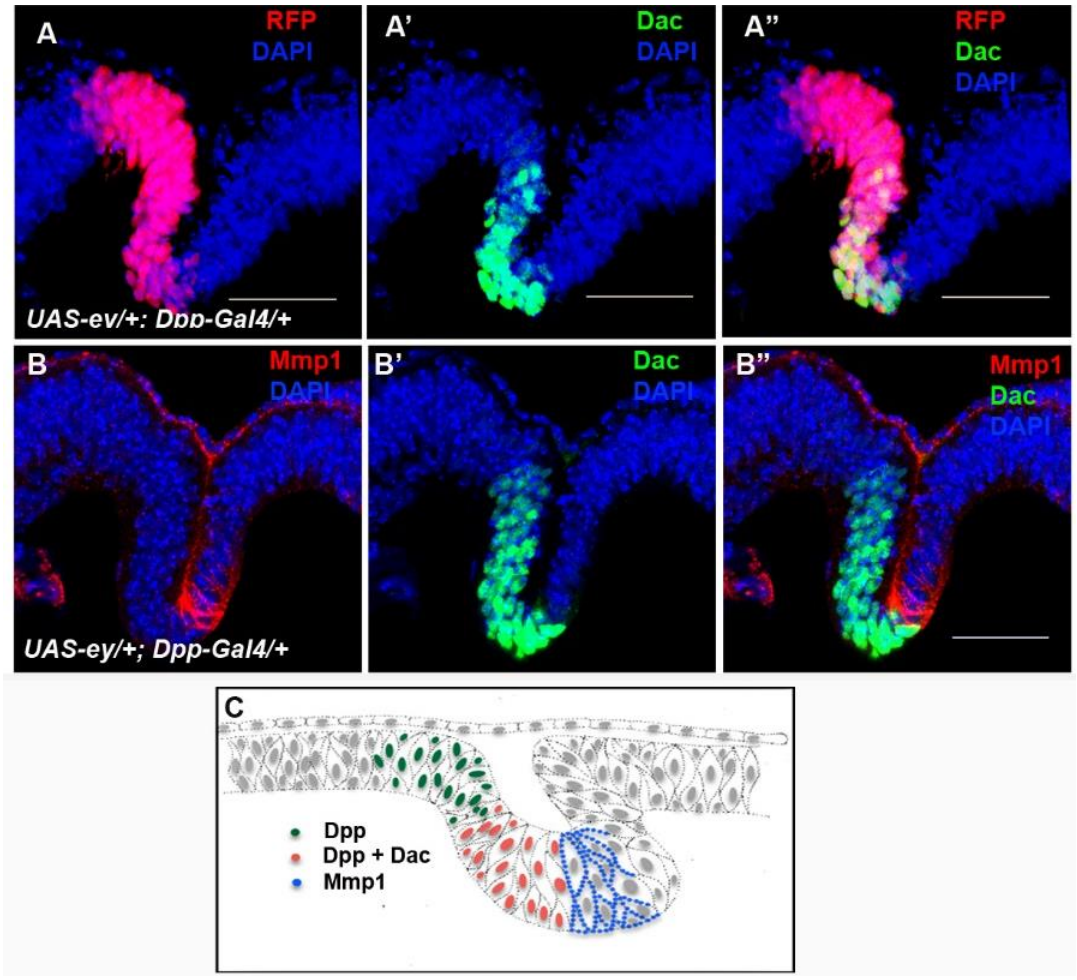

Figure 5.9. Mmp1 expresses adjacent to eye primordial cells in the wing discs undergoing ectopic photoreceptor differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A-A")** Transverse section through wing disc of *UAS-ey/+; Dpp-Gal4/+* larvae showing Dpp expression **(A)** Dac expression **(A')** overlap of Dac and Dpp expression **(A")** showing Dac in the fraction of Dpp expressing cells in the protrusion. **(B-B")** transverse sections through wing disc of *UAS-ey/+; Dpp-Gal4/+* larvae showing Mmp1 expression in the protrusion **(B)** Dac expression **(B')** Mmp1 expression around the boundaries of the cells of posterior arm of protrusion adjacent to Dac expressing cells **(B"). (C)** Schematic representation of protrusion showing expression pattern of Dpp, Dac and Mmp1.

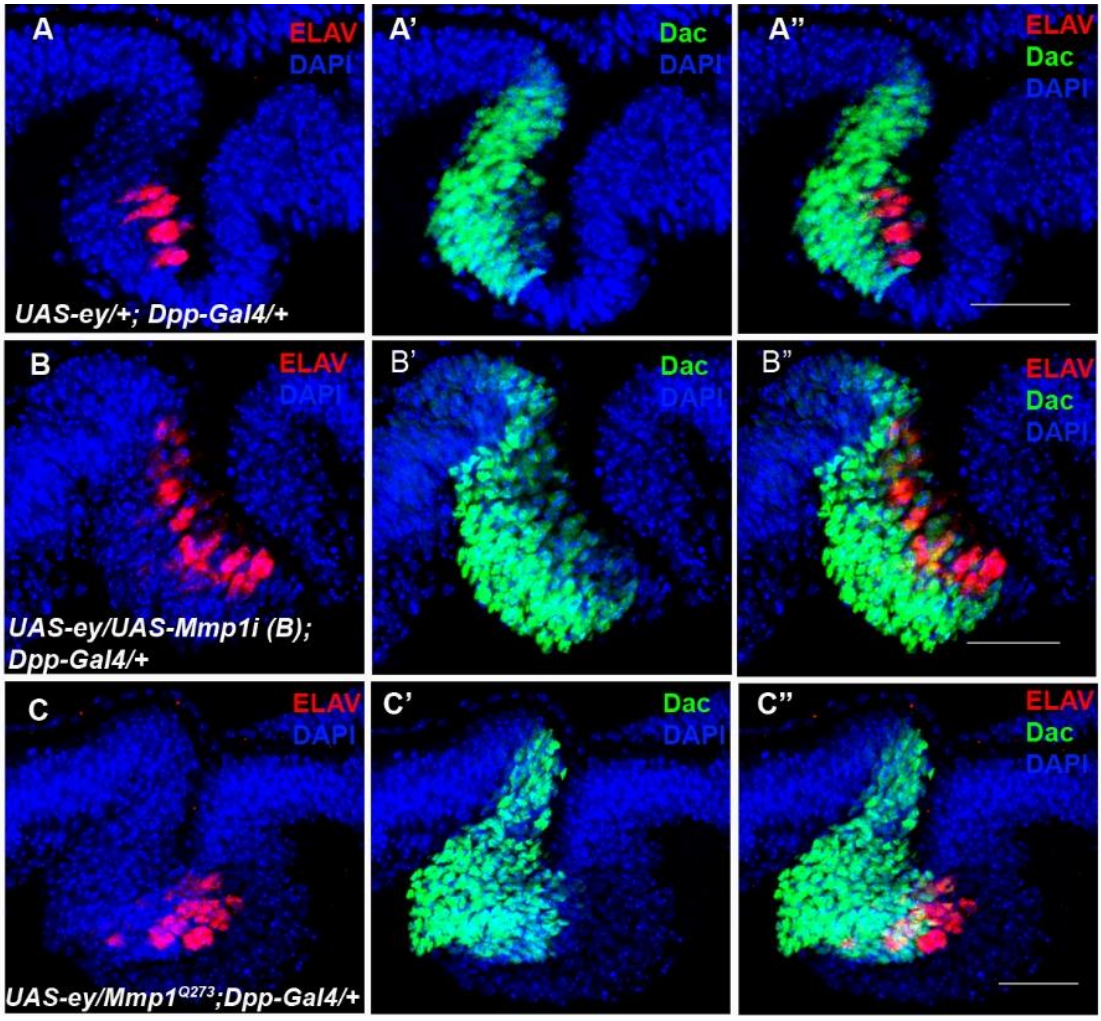

Figure 5.10. Mmp1 limits Dac domain to restrict ectopic photoreceptors differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A-A")** Dac and ELAV expression in the transverse sections made through the wing disc of *UAS-ey/+; Dpp-Gal4/+* larvae ELAV marking ectopic photoreceptors **(A)** Dac expression **(A')** overlap of Dac and ELAV **(A"). (B-B")** Increase in the Dac expression level and a consequent increase in the number of differentiating photoreceptors was observed upon knocking down *Mmp1* expression. **(C-C")** Similar increase in the Dac expression level and in the number of differentiating photoreceptors was observed upon attenuation of Mmp1 activity in the wing dics of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/+* larvae as compared to that observed in the wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae.

and *UAS-ey/Mmp1^{Q273}; Dpp-Gal4*/+ larvae (Fig. 5.10B-B", C-C") as compared to control *UAS-ey*/+;*Dpp-Gal4* larval wing discs (Fig.5.10A-A"). Quantitative analyses revealed almost two fold increase in the area as well as in the intensity of ectopic Dac expression in wing discs of *UAS-ey*/+; *UAS-Mmp1(dsRNA) (B)/Dpp-Gal4* as well as *UAS-ey/Mmp1^{Q273}; Dpp-Gal4*/+ larvae as compared to that observed in wing discs of *UAS-ey*/+;*Dpp-Gal4*/+ larvae (Fig. 5.11A, B).

Similar increase was observed in the area and the level of Eya (Fig. 5.11D) expressing cells upon attenuating Mmp1 activity by generating ectopic eyes in the wing discs heterozygous mutant for Mmp1 hypomorphic allele *Mmp1^{Q273}* as compared to control discs (Fig. 5.11C). There also, almost two fold increases in the area Eya expression (Fig. 5.11F) was observed. Along with increase in the area of Eya domain, a two-fold increase in the intensity of Eya expression was also observed (Fig. 5.11E). Together, these results suggested a role of Mmp1 in restricting ectopic photoreceptor differentiation by limiting the level of expression as well as domain of expression of RD genes Dac and Eya.

5.2.8 Mmp1 regulates cell proliferation to limit the domain of Dac expressing cells.

Increase in the domain of Dac expression prompted me to determine whether the increase in the domain of Dac was an outcome of increased cell proliferation. Therefore, EdU incorporation was performed in the transverse sections of larval wing discs (114hours AEL) in different genetic backgrounds. In transverse sections of wild type wing disc, there was homogenous incorporation of EdU all along the disc proper layer, suggesting that there was no difference in proliferation of disc proper cells present either in anterior or posterior compartment of wing disc (Fig.5.12A-A"). However,very few EdU positive cells were observed in the Dac expressing domain in the anterior part of protrusion as compared to posterior part in the transverse sections made through wing discs of *UAS-ey*/+;*Dpp-Gal4*/+ larvae (Fig.5.12B-B"). In contrast, knocking down the expression of *Mmp1* significantly increased the number of EdU incorporating cells in the Dac expressing domain of *UAS-ey*/+; *UAS-Mmp1 (dsRNA) (B)/Dpp-Gal4* larval wing discs (Fig.5.12C-C"). Similar increase in the EdU incorporation specifically in the domain of Dac expression was observed in *Mmp1^{Q273}* mutant wing discs undergoing ectopic photoreceptors differentiation (Fig.5.12 D-D"). Upon quantification of EdU postive cells in the Dac domain, almost two fold increase was found in the number of cells undergoing proliferation

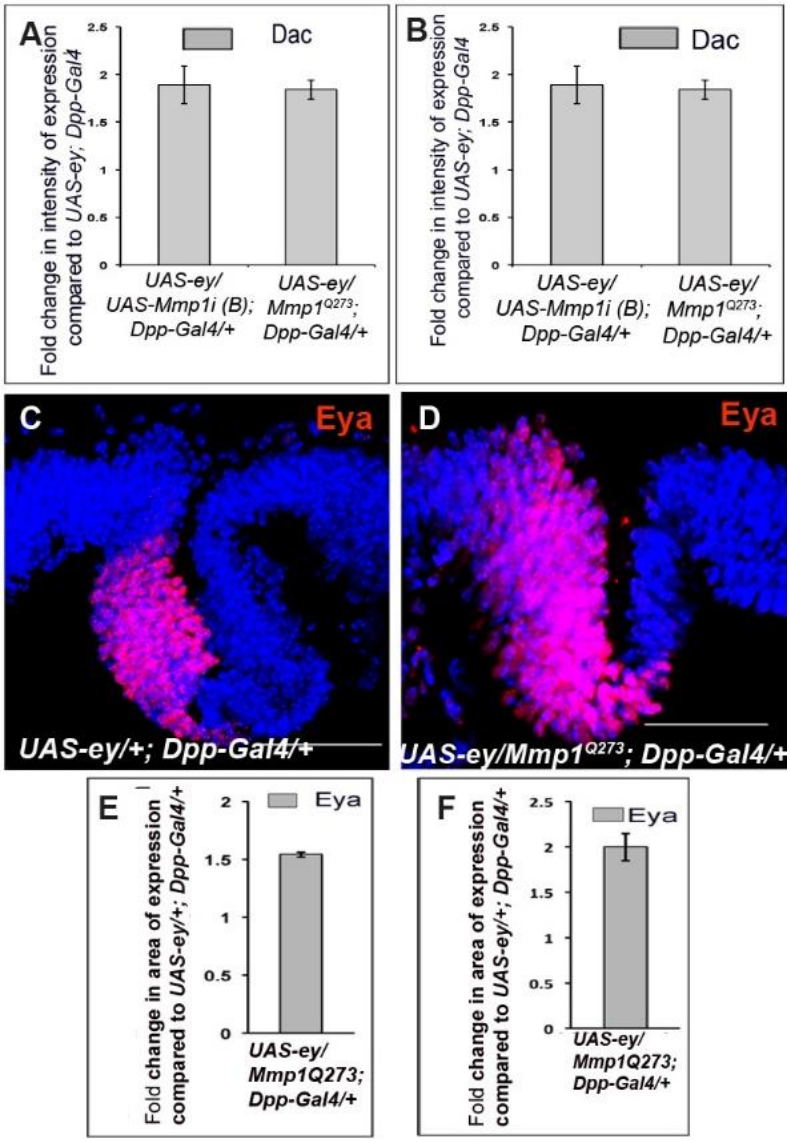

Figure 5.11. Mmp1 limits Dac domain to restrict ectopic photoreceptor differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A)** Quantification of the increases in the area of ectopic Dac expression domain in the larval wing discs of the genotype mentioned when compared to their controls *UAS-ey*/+*; Dpp-Gal4*/+ larval wing discs. **(B)** Quantification of the increases in the fluorescence intensity of ectopic Dac expression per unit area in wing discs of genotypes mentioned when compared to their controls *UAS-ey*/+*; Dpp-Gal4*/+ larval wing discs. **(C, D)** Both the intensity and domain of ectopic Eya expression got enhanced upon generating ectopic eyes in $Mmp1^{Q273}$ mutant background compared to that observed in *UAS-ey*/+*; Dpp-Gal4*/+ larval wing discs. **(E, F)** Quantification of theincreases in the fluorescence intensity of ectopic Eya expression **(E)** as well as in the area of ectopic Eya expression domain **(F)** in the wing discs of genotypes mentioned when compared to their controls *UAS-ey*/+*; Dpp-Gal4*/+ larval wing discs.

in *UAS-ey/+; UAS-Mmp1(dsRNA) (B)/Dpp-Gal4* and *UAS-ey/+; Mmp1^{Q273}/Dpp-Gal4* wing discs as compared to the number of proliferating cells observed in *UAS-ey/+; Dpp-Gal4/+* larval wing discs (Fig. 5.12E).

These results clearly established the involvement of ectopic Mmp1expression in limiting the rate of proliferation within the domain of ectopic Dac expression.

5.2.9 Mmp1 limits the range of Hedgehog signaling in the developing wing discs undergoing ectopic photoreceptor differentiation.

The ectopic photoreceptors differentiation always takes place only in the Dac expressing cells of the anterior compartment adjacent to the A/P boundary of wing disc (Fig. 5.10A-A"). This spatial restriction of photoreceptors towards the posterior compartment can be explained in terms of more availability of Hh to posteriormost Dpp expressing cells. Moreover, as evident from the results, attenuation of extracellular protease Mmp1 activity enhanced more proliferation in Dac domain and consequent increase in Dac domain and ectopic photoreceptors differentiation. Therefore, given the fact that Mmp1 is an extracellular matrix metalloproteinases, it is possible that it might be involved in modulating the activities of different extracellular signaling molecules. Hedgehog is one of the most probable extracellular signaling molecules being released from the posterior compartment of wing disc that can regulate ectopic ommatidial differentiation in the anterior compartment. Previous studies have also established the role of Hh signaling regulating the expression of Eya and Dac during normal eye development (Curtiss and Mlodzik, 2000; Firth and Baker, 2009; Pappu et al., 2003).

Moreover, it has also been demonstrated that co-expression of Hh along with *eyeless* in the anterior compartment of wing disc generates ectopic photoreceptors even in the anterior compartment cells that normally do not respond to ectopic photoreceptors differentiation (Kango-Singh et al., 2003). Thereby, all these previous studies established the major role of Hh in normal as well as ectopic eye formation. Furthermore, Hedgehog is known to regulate cell proliferation in embryonic and adult tissues. Gain and loss of function studies have clearly established the role of Hh signaling in regulating proliferation during wing and eye development in *Drosophila* (Duman-Scheel et al., 2002). Therefore, I was interested to determine whether Mmp1 is limiting Hh signaling from the posterior compartment to restrict proliferation in Dac domain.

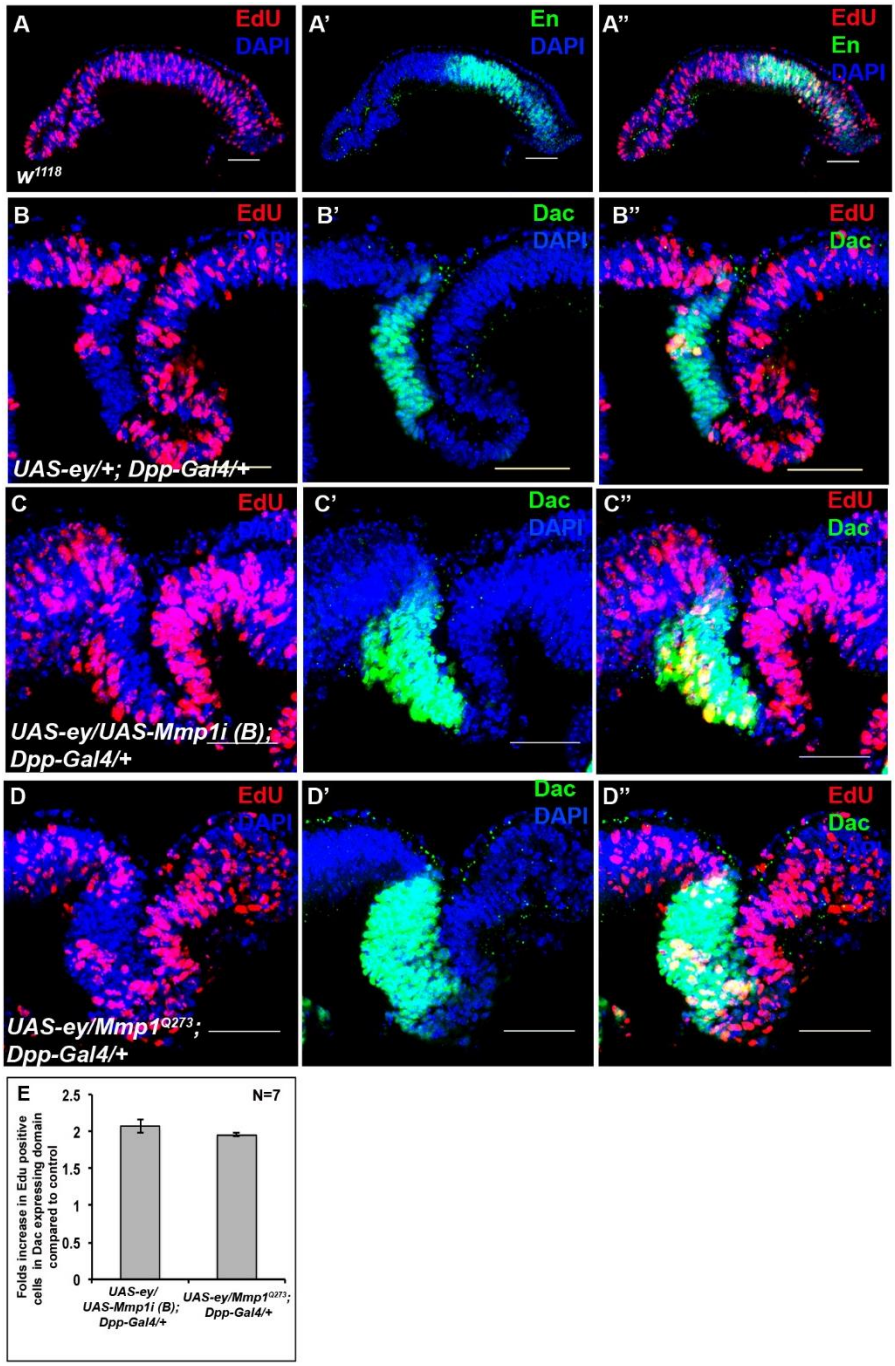

Figure 5.12. Mmp1 regulates cell proliferation to limit the domain of Dac expressing cells. For all wing discs sections anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. Incorporation of EdU marks the cells in S phase of cell division in transverse section of **(A-A")** wild type wing discs **(A)** showing uniform EdU incorporation in both the anterior and posterior compartments posterior compartment marked byEn expression **(A')** merge of both **(A"). (B-B")** transverse sections of *UAS-ey*/+; *Dpp-Gal4*/+ larval wing disc showing EdU positive cells **(B)** Dac expression **(B')** merge of both **(B")**. Increase in EdU positive cells in Dac domain upon knocking down expression of Mmp1 **(C-C")** as well as upon attenuation of Mmp1 activity by inducing ectopic eyes in the wing discs of $Mmp1^{Q273}$ heterozygous mutant larvae **(D-D"). (E)** Quantification of the increase in EdU positive cells per unit area of ectopic Dac domain in wing discs of genotypes mentioned when compared to their controls *UAS-ey*/+; *Dpp-Gal4*/+ larval wing disc.

Hh signaling initiates by binding of Hh to its receptor Patch (Ptch) that directly interacts with another membrane bound protein Smoothened (Smo) (Chen and Struhl, 1996; Huangfu and Anderson, 2006). Upon Hh binding, Ptc undergoes conformational changes and as a result activates Smo (Alcedo et al., 2000). In turn, activated Smo leads to the activation and nuclear localization of Cubitus interruptus (Ci) transcription factor to activate Hh target genes (Huangfu and Anderson, 2006; Ingham and McMahon, 2001). Hh is known to be secreted by the posterior compartment cells of wing disc (Basler and Struhl, 1994; Ramirez-Weber et al., 2000). Therefore, the cells that lie in the vicinity of posterior compartment along the A/P axis receive higher concentration of it and thereby demonstrate high Hh activity. More Hh activity leads to more localization of active Ci to the nucleus. In contrast, the cells that lie distal to the A/P boundary in the anterior compartment receive low levels of Hh thereby demonstrate low levels of Ci accumulation in the nucleus. This process results in the formation of gradient of Ci expression in the anterior compartment of wing disc. Similar pattern of Ci expression was observed in wild type wing discs (Fig.5.13A, C). However, more Ci level was observed along the A/P axis in the wing discs upon ectopic expression of *eyeless* with *Dpp-Gal4*, as compared to wild type wing discs (Fig.5.13B, C). To investigate the role of Mmp1 in regulating Hh signaling, level of Ci protein was checked upon knocking down the expression of *Mmp1* during ectopic eye formation. Interestingly, significant increase in the level of Ci as well as in its domain along the A/P axis was found upon knocking down the activity of Mmp1 as revealed by average intensity profile graph drawn along the axis from anterior to posterior compartment (Fig.5.13D, F). Similar increase in the levels of Ci expression was also observed in $Mmp1^{Q273}$ mutant larval wing discs undergoing ectopic photoreceptors differentiation (Fig.5.13E, F). Therefore, these results suggested the involvement of ectopic Mmp1 in limiting the range of Hh signaling.

5.2.10 Mmp1 limits the range of Hedgehog signaling to restrict proliferation of cells undergoing wing to eye fate change.

To further ascertain the involvement of Mmp1 in limiting Hh signaling to restrict proliferation, I wanted to determine whether the increase in EdU incorporation within the Dac expressing domain as observed in Mmp1 mutants could be suppresed by reducing the dose of Hh signaling. For this purpose, the hh^{AC} allele (Lee et al., 1992) of *hh* was used. It is loss of function allele of *hh*. Indeed, generating ectopic eyes in the wing discs of

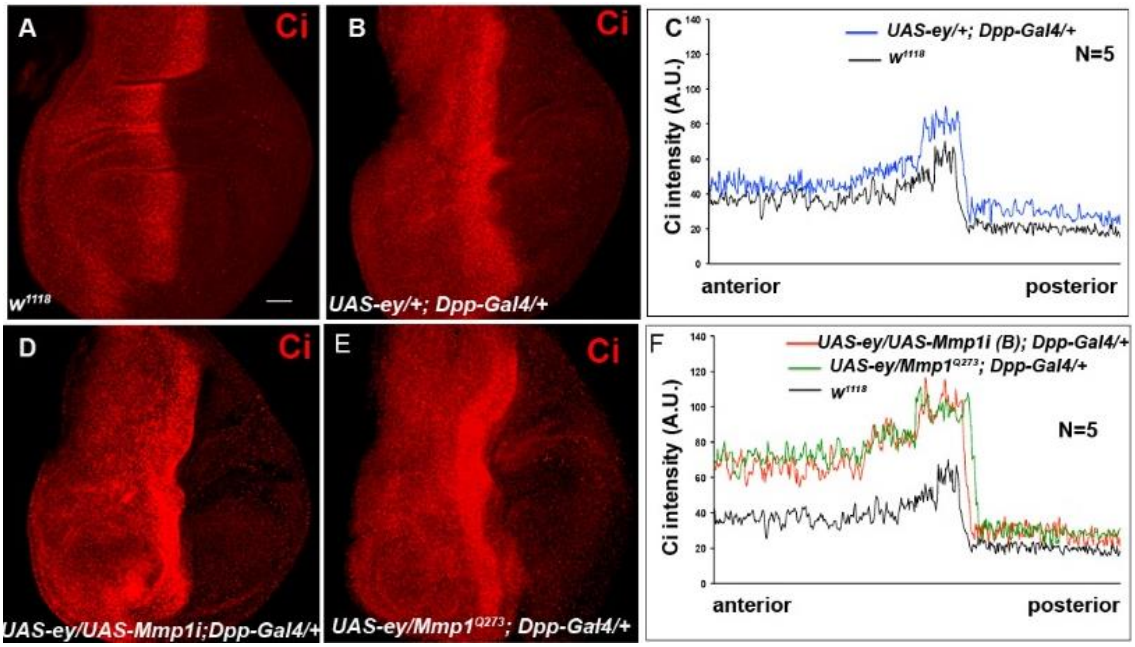

Figure 5.13. Mmp1 limits the range of Hedgehog signaling in the developing wing discs undergoing ectopic photoreceptor differentiation. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A)** Expression pattern of Ci^{act} in wild type wing disc. **(B)** Modest increase in the level of Ci^{act} expression was observed in the wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae. **(C)** Average intensity profile of Ci^{act} expression in the wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae compared to that observed in the wild-type larval wing discs. **(D, E)** Increase in the levels of Ci^{act} expression was seen upon knocking down Mmp1 activity **(C)** as well as impairing Mmp1 activity upon generating ectopic eyes in *Mmp*1^{Q273} heterozygote mutant larval wing disc **(D). (F)** Average intensity profile of Ci^{act} expression in the wing discs of larvae of the genotypes mentioned as compared to that observed in wild-type wing discs.

Mmp^{Q273}*/UAS-ey; hh*^{AC}*/Dpp-Gal4* larvae double mutant for *hh* and *Mmp1*, resulted in significant reduction in the number of cells undergoing proliferation in Dac domain (Fig.5.14A-A", C). Further, the expression of Ci was checked to confirm that whether the reduction in the number of cells undergoing proliferation in the wing discs of larvae double mutant for *hh* and *Mmp1* was due to a drop in the level of Ci expression. Interestingly, the increase in the level of Ci in the wing discs undergoing ectopic photoreceptors differentiation in heterozygous mutant for *Mmp1* got significantly reduced upon attenuating Hh activity in this background (Fig.5.14D). Intensity profile graph drawn along the axis from Anterior to posterior compartment in the wing discs of *Mmp1*^{Q273}*/UAS-ey; hh*^{AC}*/Dpp-Gal4* larvae revealed significant reduction in the level of Ci expression (Fig.5.14E). Thereby, these results further established that the decrease in the number of proliferating cells observed in the double mutant wing discs was an outcome of reduced Ci expression and hence reduced Hh activity.

The drop in the number of proliferating cells in *Mmp1*^{Q273}*/UAS-ey; hh*^{AC}*/Dpp-Gal4* larval wing disc was also associated with a significant reduction in the area and level of ectopic Dac expression (Fig.5.15A-C) in the anterior compartment. As a consequence a drop in the number of ectopic photoreceptors (Fig.5.15D) was also observed. Quantitation of the number of ectopic photoreceptors demonstrated about 70% reduction in the wing discs of *Mmp1*^{Q273}*/UAS-ey; hh*^{AC}*/Dpp-Gal4* larvae (Fig.5.15E) when compared to that observed in the wing discs of *Mmp1*^{Q273}*/UAS-ey; Dpp-Gal4*/+ larvae.

Taken together, all these results established that Mmp1 was instrumental in limiting Hh signaling emanating from the posterior compartment to regulate ectopic photoreceptor differentiation within the anterior compartment during ectopic eye induction in the developing wing discs.

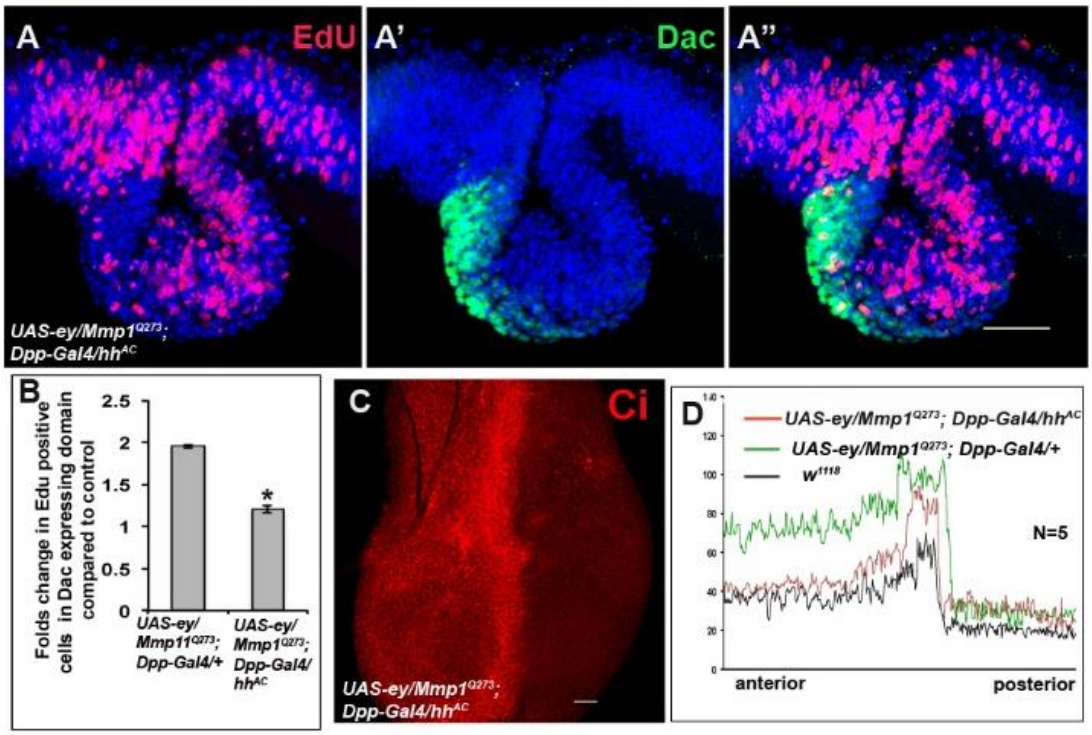

Figure 5.14. Mmp1 limits the range of Hedgehog signaling to restrict proliferation of cells undergoing wing to eye fate change. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A-A")** Reduction in the number of EdU positive cells was observed in Dac domain in *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{Ac}* larval wing disc EdU staining **(A)** reduced EdU positive cells in Dac domain **(A', A").** **(B)** Quantitative analysis showing average number of EdU positive cells in Dac domain in the wing discs of genotypes mentioned (mean ± SD; p value *<0.01.). **(C)** Increase in the expression of Ci^{act} in Mmp1 mutant got significantly reduced upon attenuation of Hh activity in *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{Ac}* larval wing disc. **(D)** Average intensity profile graph showing the levels of Ci^{act} expression in the wing discs of genotype mentioned compared to that observed in wild type wing disc.

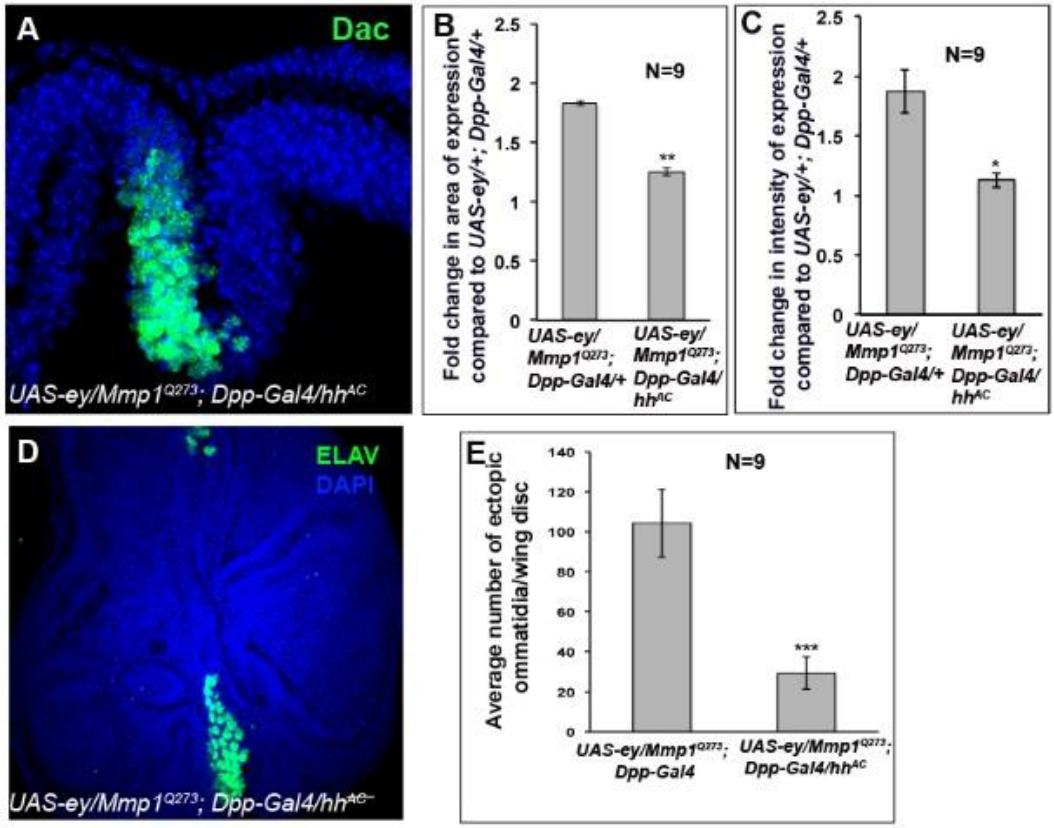

Figure 5.15. Mmp1 limits the range of Hedgehog signaling to restrict proliferation of cells undergoing wing to eye fate change. For all wing discs anterior is to the left and genotypes are as mentioned. The nuclei are marked with DAPI (Blue) Scale=20µ. **(A)** Increase in the area as well as intensity of Dac expression observed in Mmp1 mutant got significantly reduced upon attenuation of hh activity in *UAS-ey*/*Mmp1*^{Q273}*; Dpp-Gal4*/*hh*^{Ac} larval wing disc. **(B, C)** Quantification of the decrease in the domain of ectopic Dac expression **(B)** as well as in the intensity of ectopic Dac expression per unit area **(C)** in the wing discs of *UAS-ey*/*Mmp1*^{Q273}*; Dpp-Gal4*/*hh*^{Ac} larvae when compared to their controls *UAS-ey*/*Mmp1*^{Q273}*;Dpp-Gal4*/+ larval wing discs (mean ± SD; p value**<0.001, *,0.01). **(D)** Reduction in the number of ectopic photoreceptors as evidenced in the wing discs of *UAS-ey*/*Mmp1*^{Q273}*; Dpp-Gal4*/*hh*^{Ac} larvae. **(E)** Quantification of the drop in the number of ectopic photoreceptors in the wing discs of *UAS-ey*/*Mmp1*^{Q273}*; Dpp-Gal4*/*hh*^{Ac} larvae when compared to their controls *UAS-ey*/*Mmp1*^{Q273}*; Dpp-Gal4*/+ larvalwing discs (mean ± SD; p value ***<0.0001).

5.3 Discussion

As evidenced from previous studies in wing imaginal discs, Hh released from the posterior compartment is known to transcriptionally activate and maintain Dpp expression along the cells of the anterior compartment adjacent to A/P boundary and thereby plays an important role in regulating growth and patterning of wing disc (Basler and Struhl, 1994; Masucci et al., 1990; Tanimoto et al., 2000). In *Drosophila* eye development also, Hh is known to direct the wave of photoreceptors differentiation that sweeps from posterior to anterior across the eye primordia. Hedgehog is secreted by the differentiating ommatidia posterior to the morphogenetic furrow. Hedgehog activates Dpp in the morphogenetic furrow that in turn upregulates the expression of Hairy to provide pre-proneuronal state to these cells (Greenwood and Struhl, 1999; Heberlein et al., 1993). Later on, Hh activates Serine-Threonine Kinase Raf that brings about transition from pre-proneuronal cells to proneuronal cells. Raf actually down regulates Hairy and upregulates proneuronal marker Atonal that initiates differentiation of R8 photoreceptor that further organizes differentiation of the other R1-R7 photoreceptors by Raf signaling (Greenwood and Struhl, 1999). Therefore, Hh signaling initiates and regulates the process of photoreceptor differentiation at different levels while Dpp signaling appears to enhance the rate of furrow progression by promoting transition to pro-preneuronal state. Hedgehog is also known to regulate proliferation of the cells arrested in G1 phase of cell cycle in the morphogenetic furrow. By transcriptional activation of *Cyclin E* and *Cyclin D*, Hh promotes entry of G1 arrested cells in to S-phase known as second mitotic wave (Duman-Scheel et al., 2002). Hedgehog also regulates the expression of retinal determining genes *eya* and *dac* (Curtiss and Mlodzik, 2000; Firth and Baker, 2009; Pappu et al., 2003). Therefore, both in wing and eye imaginal discs, expression of Hh is critical for regulating Dpp expression (Basler and Struhl, 1994; Borod and Heberlein, 1998; Heberlein et al., 1993; Masucci et al., 1990; Pappu et al., 2003; Tanimoto et al., 2000). However, in neither of these cases Dpp is unknown to regulate Hh expression (Shen and Dahmann, 2005).

The outcome of this study unravels the interaction between Hh and Dpp signaling during generation of ectopic photoreceptor differentiation in wing disc. Results of my analyses show that Dpp plays an important role in limiting the range of Hh activity in the anterior compartment of the wing during ectopic eye differentiation. In doing so Dpp actually

regulates the rate of proliferation as well as ectopic Dac and Eya expression in the cells of the anterior compartment undergoing ectopic photoreceptors differentiation. While these results reveal the role of Hh signaling in regulating proliferation of eye primordial cells as well as controlling the expression of Dac and Eya during ectopic eye differentiation in a manner similar to that observed during normal eye development, it also unravels an interesting mechanism by which Dpp plays a role in regulating Hh expression by activating Mmp1.

As shown in Fig.5.16, during ectopic eye formation, Dpp triggers a signaling cascade that involves dTak1-JNK to transcriptionally activate *Mmp1*. Mmp1 gets secreted towards the posterior compartment around the Hh producing cells. The results obtained upon expression analyses of Ci suggest that ectopic expression of Mmp1 is involved in regulating the amount of Hh signaling received by the cells of anterior compartment and by doing so, it is playing an important role in regulating the levels of ectopic expression of Eya and Dac as well as the proliferation of eye primordial cells. Together, these results not only unravel the signaling pathway that triggers ectopic Mmp1 expression during transdetermination but also provide evidence about the role of Mmp1 in regulating Hh signaling during this process.

Extracellular matrix present around the cells not only provides structural integrity but also regulate various signaling molecules, gradients of morphogen and morphogen activity (Hynes, 2009). Developmental events like metamorphosis of insects and amphibian tissues, migration of neural crest cells, angiogenesis, tooth and skeletal development, that requires remodeling of ECM are all associated with increased MMP activity (Daley et al., 2008; Fukumoto and Yamada, 2005; Page-McCaw et al., 2007; Zimmermann and Dours-Zimmermann, 2008). In a similar fashion, when some cells of tissue undergo cell fate alteration, modification of ECM with respect to neighboring cells is critically involved. I strongly believe that during ectopic eye formation ECM around the cells undergoing ectopic eye differentiation get altered. As these cells are juxtaposed to the A/P boundary, the altered ECM might facilitate increased level of Hh diffusion from the posterior compartment. An immediate outcome of this process can be the increase in Dpp expression. Turning on Mmp1 by Dpp signaling to regulate the amount of Hh received by the anterior compartment cells might be a strategy by the host cells to maintain the identity of their determined state.

In *Drosophila*, Mmp1 is known to degrade the components of basement membrane during tissue remodeling in larvae as well as adults and also in tumor invasion (Srivastava et al., 2007). Mmp1 also cleaves DE-cadherin mediated cell-cell junctions to dissociate fat body cells during metamorphosis and also acts on HSPGs, Dally and Dally like proteins (Dlp) to modulate Wnt trans-synaptic signaling cascade during synaptogenesis (Dear et al., 2016). Importantly, Dally or Dally like proteins are also known to positively regulate Hh signaling in the anterior compartment of *Drosophila* wing imaginal discs. It has been shown that the transmission of Hh in the receiving cells is facilitated by Dlps that act as co-receptor with Patch to facilitate Hh internalization in to apical surface of the cells (Gallet et al., 2008). However, the ectopic Mmp1 expression observed during wing to eye transdetermination was not present around the cells of anterior compartment (Hh receiving cells). Rather Mmp1 expression was observed around the cells of posterior compartment which are the Hh producing cells. These results suggest that in this process, Mmp1 does not control Hh signaling in the receiving cells by modulating Dlps, instead Mmp1 actually modulate the ECM around the Hh producing cells to regulate the amount of Hh molecules diffusing to the anterior compartment. In this context, it is important to note that the reason for high level of Ci activity in the anterior compartment of wing discs of *UAS-ey/+; Dpp-Gal4/+* larvae might also be an outcome of enhanced Hh expression in the posterior compartment and whether this is getting further enhanced in Mmp1 mutant and knockdown background needs to be explored. But, in any case, these results demonstrate that a morphogen Dpp is employing Mmp1 to regulate the activity domain of another morphogen Hh (Fig.5.16).

Transcriptional activation of MMPs is an area that has been extensively explored in cancer cell lines as well as in vertebrate model organisms. A diverse array of transcriptional activator such as P38, Smad, AP-1, SP-1 has been shown to transcriptionally activate as well as to repress the expression of MMPs and TIMPs genes (Kim et al., 2007; Santibanez et al., 2002; Yan and Boyd, 2007). Even in *Drosophila*, Mmp1 expression has been reported to get activated in response to various stresses like mechanical stress, high ROS, inflammation, aging and by JNK signaling during normal development as well as various pathophysiological conditions (Lee et al., 2012; Srivastava et al., 2007; Stevens and Page-McCaw, 2012; Uhlirova and Bohmann, 2006). JNK activates Mmp1 during developmental tissue invasion for imaginal discs eversion and re-epithelization during wound healing (Srivastava et al., 2007; Stevens and Page-McCaw, 2012). JNK transcriptionally activate

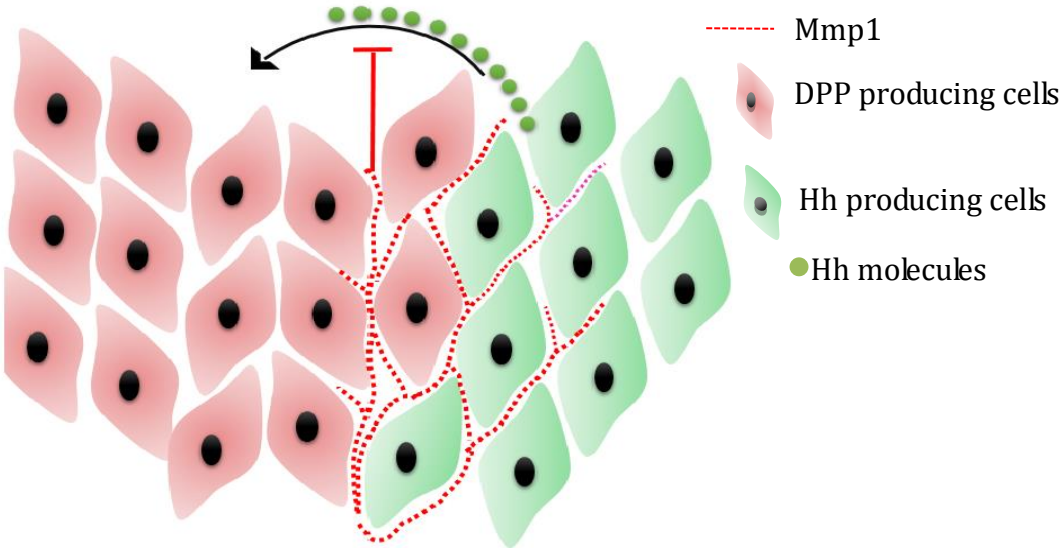

Figure 5.16. A model depicting the role of Dpp in activating matrix metalloproteinase to limit the gradient of Hh.

Mmp1 expression through AP-1 transcription factor during tumour invasion (Uhlirova and Bohmann, 2006). Regulation of MMPs expression by TGF-β signaling in cancer cells is rather complex. In breast epithelial cells, TGF-β induces MMP-2 expression by employing TAK1-p38 MAPK pathway (Kim et al., 2007; Sano et al., 1999). Meanwhile, TGF-β activates MMP-9 expression in keratinocytes by ERK-1, in breast epithelial cells by Rac-ROS-NFkB and in hepatocellular carcinoma cells by activating TAK1-NFkB (Safina et al., 2008; Santibanez et al., 2002; Tobar et al., 2010). Various studies in mammalian cell lines have identified the involvement of JNK activated Jun-Fos (AP-1) family of transcription factors in regulating MMPs genes expression (Chakraborti et al., 2003; Westermarck and Kahari, 1999). Importantly, Smad interacts with the members of AP-1 family to transcriptionally activate MMPs expression (Liberati et al., 1999; Mauviel et al., 1996; Selvamurugan et al., 2004) or directly activates other transcription factors involved in the regulation of MMPs expression that includes AP-1, SP1, NFk-B (Safina et al., 2008; Sano et al., 1999; Santibanez et al., 2002; Tobar et al., 2010; Yan and Boyd, 2007). The outcome of this study provides the first *in vivo* genetic evidence of a complex regulatory mechanism that involves dTak1-JNK signaling cascade that is activated by Dpp, a member of TGF-β family of proteins in regulating Mmp1 expression. This activation of Mmp1 by Dpp might not be very simple as this exhibits spatial restriction. Not all cells that overexpress Dpp may be involved in triggering Mmp1 expression. It is quite possible that the Dpp expressing cells adjacent to the A/P boundary are actually activating Mmp1 expression. Therefore, it would be a real challenge to determine the context dependent activation of Mmp1 in these cells.

Previously, in chapter 3 of this thesis, it has been shown that Dpp simultaneously triggers dTak1-JNK cascade along with Mad dependent signaling cascade. While the later is used for transcriptional activation of target genes, the dTak1-JNK signaling is used to remove PcG mediated repression from the target genes. In doing so, Dpp play both instructive and permissive roles to regulate the expression of RD genes. Along with that, the outcome of this study elucidated that Dpp does also play a vital role in limiting the domain of ectopic photoreceptor differentiation by restricting the activity of Hh. This in turn highlights another facet of Dpp activity in maintaining the identity and determined state of the cells of the wing disc. Taken together, these findings clearly established a dual role of Dpp in regulating cellular plasticity.

Previous studies demonstrated that during leg to wing transdetermination, proliferation and wing differentiation always get restricted to the dorsal blastema cells of the leg disc. Interestingly, ectopic Mmp1 expresses at the boundary of blastema and non-blastema cells and removal of Mmp1 leads to induction of proliferation and wing differentiation even in the ventral non-blastema cells (McClure et al., 2008). From these results, they concluded that this phenotype actually comparable to the phenotype obtained upon ectopic co-expression of both *wg* and *Dpp* in the ventral leg disc cells (Maves and Schubiger, 1998). Therefore, they hypothesized the role of Mmp1 in bringing alteration in the level of Dpp activity. Compared to this, these results revealed that during ectopic eye differentiation Mmp1 plays an important role in regulating Hh signaling and loss of Mmp1 actually causes an increase in the expression of RD genes and proliferation specifically in the cells undergoing ectopic photoreceptor differentiation. Irrespective of the nature of responses observed in two different transdetermination systems, these results, establish a conserved role of Mmp1 in regulating the domain of morphogen activity.

Though cell fate transformation is a rare event in development (Bariety et al., 2003; Patapoutian et al., 1995). But, it is mostly associated with regeneration, wound healing (Echeverri and Tanaka, 2002; Eguchi et al., 1974; Jopling et al., 2011; Shen et al., 2004; Suetsugu-Maki et al., 2012) and various pathophysiological conditions like metaplasia and cancer (Mann et al., 2007; Shen et al., 2004; Yu et al., 2005). It has been documented that the mechanistic basis underlie change in cell fate specification by ectopic gene expression in *Drosophila* strikingly resemble to that observed during regeneration (Schubiger, 1971; Schubiger et al., 2010; Worley et al., 2012). This study highlights the regulatory mechanism imposed by one morphogen (Dpp) to limit activity domain of another morphogen (Hh) specifically along the compartment boundary of wing imaginal disc during ectopic eye formation by ectopic *eyeless* expression. Interestingly, the A/P boundary of the developing wing imaginal disc represents a morphostatic boundary where two morphogens interact. Understanding the phenomenon of interaction between morphogens across the morphostatic boundaries has gained tremendous importance because of its relevance to cancer. As it has been demonstrated that cancer majorly arises from the changes in the microarchitecture defined by morphostatic fields especially at the junction where two morphostatic fields meet (Potter, 2007). Therefore, it would be intriguing to determine whether similar kind of interactions between morphogens as

evidenced in this work is associated with wound healing and regeneration which demands a balace between proliferation and differentiation.

Interestingly, during ectopic eye formation, ectopic Mmp1 expression was observed specifically at the border of Dpp and Hh expressing cells. On further investigation, it was found that Mmp1 is produced as a result of protective measure by the Dpp expressing cells.

Previous studies have identified constitutive activation of Hedgehog signaling associated with tumourous growth and various carcinomas conditions and malignancies (Gonnissen et al., 2015; Rubin and de Sauvage, 2006), Inappropriate activation of Hh signaling leads to different kinds of human cancers including lungs, breast, prostate, gastrointestinal and brain cancer. Hh signaling has also been shown to regulate cancer stem cells and to promote tumor invasiveness. Therefore, inhibition of Hh signaling has always been a potent target for treatment and prevention of cancer (Gonnissen et al., 2015; Rubin and de Sauvage, 2006). Similarly, MMPs have also been found involved in almost all kinds of cancer and majorly known for promoting metastasis and invasiveness (Cathcart et al., 2015; Rundhaug, 2003). Therefore, unraveling the mechanism by which Mmp1 inhibits Hh gradient to limit proliferation would help us design strategies to modulate SHH activity during metastasis. Given the significant conservation of MMPs and TIMP genes between flies and vertebrates and comparatively very less gene redundancy and compensation, this study might provides a mechanistic basis to better understand role of MMPs in cellular plasticity in vertebrates.

Chapter 6.

Conclusion

My work unravels an interesting role of the morphogen, Dpp, in modulating cellular plasticity during the process of cell fate alteration in *Drosophila* imaginal discs. It not only highlights an important aspect of morphogen activity in regulating the expression of its target genes that are otherwise kept epigentically repressed but also sheds light on a mechanism that is involved in rewiring the interaction between two different morphogens during cell fate alteration. The most important outcome of my thesis work is unraveling a two-tier mechanism employed by the morphogen, Dpp, in modulating the expression of its target genes during change in cell fate specification.

Inspite of having conserved signaling cascade, a morphogencan evoke pleotropic responses in different tissue types. This differential response in different tissue types is determined by the repertoire of transcription factors available as well as on the epigenetic landscape already set up during the course of development in that particular cell type. But, during the situation like cell fate alteration, how does a morphogen activate its target genes that were otherwise kept epigenetically silent in that particular cell type and how does the interaction or coordination between different morphogenes get rewired during the process of cell fate alteration were the major fascinating questions that remained unexplored. My work shed light on these issues by identifying a two-tier mechanism employed by the morphogen Dpp in transcriptional activation of its target genes during cell fate alteration. While it employs the conserved canonical signaling pathway that involves Mad and Med to trigger the transcription of the responder genes, by activatinganother signaling cascade that involvedTak1-JNK it derepresses target genes by lowering down the activity of PcG proteins. In doing so, Dpp plays both instructive as well as permissive roles in regulating the expression of the target genes. Interestingly, I found that activation of the otherwise unknown signaling cascade is not specifically associated with one particular kind of cell fate alteration. Rather, my studies clearly demonstrated that this mechanism is employed for different kinds of cell fate alterations associated with ectopic gene expression in the

imaginal discs of *Drosophila*. Together, these results bring about a paradigm shift in our understanding of morphogen activity as it unravels a process that coordinates both epigenetic and transcriptional regulation for target gene expression by a morphogen signaling.

Importantly, Dpp mediated activation of dTak1-JNK signaling cascade to derepress the target genes is specifically involved during cell fate switching events. dTak1 does not repress the activity of PcG genes by involving the activation of stress activated protein kinase JNK during normal eye and wing development. It further suggests that situation like cell fate alteration creates an unique developmental condition where in developmental stress might lead to activation of this pathway to provide developmental plasticity essential for change in cell fate. Although the results of this study unearth the specific signaling pathway that gets activated by Dpp during cell fate alteration in imaginal discs of *Drosophila*, the cell physiological conditions that lead to context dependent activation of this pathway is far from known. Understanding the mechanistic basis of the activation of this signaling cascade, therefore, is an intriguing aspect to explore in future. Due to lack of any specific directionality, as of now, an unbiased approach could be taken to address this issue. In this direction, a loss of function genetic screen can be performed either with deficiency lines or with gene specific RNAi stocks to identify the candidates/cofactors that are responsible for the activation of this pathway. Subsequent genetic epistasis experiments can be done to establish the connections between them. As an alternate, it would be interesting to determine whether ectopic expression of the selector genes leads to activation of genes that code for proteins like TRAF6, TBPs whose interaction with Tak1 is important for its activation. Since Dpp signaling is known to bring about cytoskeleton remodeling, it is even possible that increased level of Dpp signaling brings about changes in cytoskeleton that in turn facilitate recruitment of Tak1 activation assembly proteins. It would be really intriguing to explore this possibility. Gaining insight in any of these plausible mechanisms that lead to specific activation of this pathway can have far reaching implications in wound-healing, regeneration and cancer.

Furthermore, in this study, I have been also successful in unraveling an important role of Matrix-metalloproteinase-1 in regulating the level of Hedgehog signaling to bring a balance between proliferation and differentiation of the cells undergoing cell fate alteration during ectopic eye differentiation in the developing wing imaginal discs of *Drosophila*. My results provide evidence for a very interesting phenomenon where the morphogen Dpp

triggers the expression of *matrix metalloproteinase1* to limit the activity of another morphogen, Hh. Importantly, this activation of Mmp1 is not mediated by the canonical Dpp signaling. Instead Dpp deploys the alternate signaling pathway that involves dTak1 and JNK for transcriptional activation of Mmp1. Unlike previous studies where the role of extra cellular matrix components HSPGs had been elucidated in modulating Hh signaling in the Hh receiving cells (Gallet, 2011; Gallet et al., 2008) my work implicates the role of Mmp1 in modulating the range of Hh signaling by modifying the extra cellular matrix around the Hh producing cells. However, the exact mechanism underlying this process needs to be deciphered. Nonetheless my study has provided the genetic basis of a novel mechanism by which the interaction between two different morphogen activities gets rewired during cell fate alteration.

While in Chapters 3 and 4 of my thesis, I demonstrated the mechanism by which Dpp facilitates cell fate alteration by creating a permissive condition for transcriptional activation of its responder genes that are otherwise kept epigenetically repressed, in chapter 5, I provide evidence of a process by which Dpp triggers the expression of Mmp1 to limit the range of Hh activity specifically in the domain of the wing disc undergoing ectopic eye differentiation. In doing so, Dpp restricts the amount of proliferation and differentiation in the topological area where cells are actually undergoing cell fate alteration. This in turn restricts the amount of cell fate alteration and as a consequence helps the wing imaginal disc cells to maintain their determined state. Taken together, all these results highlight a dual role of Dpp in regulating cellular plasticity. In one hand it helps in creating a conducive condition that promotes cell fate alteration; on the other it also plays a critical role in maintaining the determined state of the cells by restricting their proliferation.

Cell fate alteration, though very rarely observed during normal development, is majorly associated with regeneration, wound healing and various pathophysiological conditions like fibrosis, cancer and metaplasia. A large body of studies has evidenced the association high morphogen activity with change in cell fate specification during wound healing, regeneration and various disease conditions (Adell et al., 2010; Lawrence, 2001; Le et al., 2008; Potter, 2007; Zhang et al., 2009). However, our understanding of the role played by morphogens during these processes is very limited. It is generally perceived that as observed during normal development, during these events morphogens are involved in

transcriptional activation of their target genes. In contrast my work demonstrates that a morphogen actually plays an important role in regulating a plethora of activities associated with cellular plasticity. It would be, therefore, intriguing to determine whether similar kind of interactions between morphogens as evidenced in this work is associated with wound healing and regeneration that demand an intricate balance between proliferation and differentiation. Given the conserved nature of signaling pathways and the fact that the phenomenon of transdetermination is also exhibited by adult stem cells of higher vertebrates, it is quite possible that morphogens might be instrumental in playing a similar role to create an environment that promotes adaptive developmental potential for adult stem cells.

Finally, my observation that the morphogen, Dpp, triggers the expression of *Mmp1* to modulate the extracellular matrix in a fashion that limits the range of activity of another morphogen, Hh along the compartment boundary of wing imaginal disc during ectopic eye formation highlights an interesting mechanism involved in fine tuning the interaction between two morphogens along the morphostatic boundary. From that perspective the outcome of my study has tremendous implicationas it has been demonstrated that cancer majorly arises from the changes in the microarchitecture defined by morphostatic fields especially at the junction where two morphostatic fields meet (Potter, 2007). Moreover, Hedgehog signaling has been majorly associated with tumourous growth and various carcinomas conditions and malignancies (Gonnissen et al., 2015; Rubin and de Sauvage, 2006), Similarly, MMPs have also been found involved in almost all kinds of cancer and majorly known for promoting metastasis and invasiveness (Cathcart et al., 2015; Rundhaug, 2003). Therefore, unraveling the mechanism by which Mmp1 inhibits Hh gradient to limit proliferation has implication in designing strategies to modulate SHH activity during metastasis.

Chapter 7.

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