Noncanonical Decapentaplegic Signaling Activates Matrix Metalloproteinase 1 To Restrict Hedgehog Activity and Limit Ectopic Eye Differentiation in *Drosophila*

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ABSTRACT One of the pertinent issues associated with cellular plasticity is to understand how the delicate balance between the determined state of cells and the extent to which they can transdetermine is maintained. Employing the well-established model of generating ectopic eyes in developing wing discs of *Drosophila* by ectopic *eyeless* expression, we provide evidence for the genetic basis of this mechanism. By both loss-of-function and gain-of-function genetic analyses, we demonstrate that Matrix metalloproteinase 1 (Mmp1) plays an important role in regulating the extent of ectopic ommatidial differentiation. Transcriptional activation of ectopic Mmp1 by the morphogen Decapentaplegic (Dpp) is not triggered by its canonical signaling pathway which involves Mad. Rather, Dpp activates an alternate cascade involving dTak1 and JNK, to induce ectopic Mmp1 expression. Mutational analyses reveal that Mmp1 negatively regulates ectopic eye differentiation by restricting the rate of proliferation and the levels of expression of retinal-determining genes *dachshund* and *eyes absent*. This is primarily achieved by restricting the range of Hedgehog (Hh) signaling. Importantly, the increase in proliferation and upregulation of target retinal-determining genes, as observed upon attenuating Mmp1 activity, gets significantly rescued when ectopic eyes are generated in wing discs of *hh* heterozygous mutants. In conjunction with the previously established instructive and permissive roles of Dpp in facilitating ectopic eye differentiation in wing discs, the outcome of this study sheds light on a mechanism by which Dpp plays a dual role in modulating the delicate balance between the determined state of cells and the extent they can transdetermine.

KEYWORDS *Drosophila*; Mmp1; Dpp; Hh; ectopic eye

IMAGINAL discs are sacs of epithelial cells present within the larvae of holometabolous insects that serve as anlagen for adult appendages such as wing, eye, antennae, genitalia, leg, etc. The study of imaginal discs in the fruit fly *Drosophila melanogaster* has contributed significantly to our understanding of the role of signaling molecules like morphogens in cell

fate specification and pattern formation. For instance, the precise coordination between Hedgehog (Hh) and Decapentaplegic (Dpp) signaling pathways in regulating pattern formation in developing eye imaginal discs of *Drosophila* highlights the role of these morphogens in cell fate induction. Hh, secreted from the differentiating ommatidial clusters, is important not only in regulating cell growth and proliferation by promoting expression of Cyclin E and Cyclin D (Duman-Scheel *et al.* 2002), but is also essential for initiating photoreceptor differentiation by triggering the expression of the retinal-determining (RD) genes, *eyes absent* (*eya*) and *dachshund* (*dac*) (Curtiss and Mlodzik 2000; Pappu *et al.* 2003; Firth and Baker 2009). Furthermore, Hh triggers the expression of *dpp* along the morphogenetic furrow (Heberlein *et al.* 1993) which induces the precursors to

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enter a proneural state (Greenwood and Struhl 1999). In the developing wing discs, on the other hand, Hh signaling from cells of the posterior compartment drives the expression of *dpp* in a stripe of anterior cells along the anterior/posterior (A/P) compartment boundary (Masucci *et al.* 1990; Basler and Struhl 1994). The Dpp protein in turn acts as a long-range morphogen to play a pivotal role in regulating growth and patterning of wing disc (Tanimoto *et al.* 2000; Affolter and Basler 2007).

Drosophila imaginal discs have also served as an excellent experimental model to understand several mechanisms underpinning cell-fate alteration. Regeneration studies, which primarily involved transplanting larval imaginal disc fragments, revealed that these fragments retain their ability to differentiate into appropriate adult structures upon metamorphosis (Gehring 1966). However, in some rare instances, these disc fragments undergo alteration in cell-fate specification by a process termed as transdetermination, to ultimately differentiate into disc-inappropriate adult structures (Hadorn 1968; Schubiger 1971). Studies from different laboratories demonstrated that transdetermination could also be achieved by genetic manipulations of developing imaginal discs. For instance, ectopic expression of *wingless* (*wg*) in developing leg imaginal discs leads to conversion of leg to wing (Maves and Schubiger 1995). Likewise, ectopic eyes can be generated on different parts of adult flies by inducing the expression of RD genes *eyeless* (*ey*), *eya*, and *twins of eyeless* in the developing imaginal discs (Halder *et al.* 1995; Salzer and Kumar 2010).

Given the importance of morphogens during patterning in imaginal discs, it is intuitively obvious that they would also play a pivotal role during transdetermination. Indeed, several studies have demonstrated that high level of Wg expression is specifically associated with cells undergoing leg-to-wing conversion during regeneration in surgically fragmented leg discs (Gibson and Schubiger 1999). Interestingly, during this process, ectopically expressed Wg acts synergistically with higher levels of Dpp to support leg-to-wing transdetermination (Maves and Schubiger 1998; Ing *et al.* 2013). Likewise, ectopically expressed *ey* can induce ectopic eye formation only when it collaborates with high levels of Dpp and Hh (Kango-Singh *et al.* 2003). It is generally perceived that during ectopic eye induction in wing discs, these two morphogens play roles identical to that observed during normal ommatidial differentiation in eye discs. However, since both of these morphogens are equally important for normal wing development, it is not yet known whether the interaction between these two morphogen signaling genes gets reorganized when ectopic eyes are induced in wing discs.

On a separate note, transcriptome analysis of cells undergoing leg-to-wing cell-fate alteration due to ectopic expression of Wg demonstrated a fourfold enrichment in the level of *Drosophila* Matrix metalloproteinase1 (*Mmp1*) (Klebes *et al.* 2005). As a genetic correlate, it was subsequently demonstrated that ectopic expression of *wg* in leg discs of larvae heterozygous mutant for *Mmp1* not only led to significant elevation in the rate of leg-to-wing transdetermination, but was also associated

with ectopic transdetermination in the nonblastema cells that did not generally undergo cell-fate change (McClure *et al.* 2008). Importantly, this phenomenon of transdetermination in multiple sites within the *Mmp1* heterozygous mutant discs was attributed to the failure of these cells in submitting to cell-cycle arrest and a plausible increase in the level of Dpp signaling. Though these findings clearly implicated the importance of *Mmp1* in regenerative proliferation and cellular plasticity, our understanding of the precise role played by *Mmp1* in regulating the process of cell-fate alteration still remains elusive. Likewise, the signaling pathway that triggers ectopic expression of *Mmp1* during transdetermination is yet to be elucidated.

Matrix metalloproteinases (Mmps) belong to the family of zinc-dependent extracellular endopeptidases responsible for tissue remodeling and degradation of extracellular matrix (ECM) across divergent phyla (Page-McCaw *et al.* 2007; Page-McCaw 2008; Marino and Funk 2012). The genome of *Drosophila* codes for two Mmp proteins, *Mmp1* and *Mmp2* (Llano *et al.* 2000, 2002), and one tissue inhibitor of metalloprotease (TIMP) (Wei *et al.* 2003). While *Mmp1* is secretory in nature (Page-McCaw *et al.* 2003), *Mmp2* has a predicted GPI membrane anchor (Llano *et al.* 2002). Importantly, *Drosophila* TIMP (dTIMP) can inhibit mammalian MMP function (Wei *et al.* 2003) and mammalian TIMPs can inhibit fly Mmps (Llano *et al.* 2000), thereby demonstrating that their functions are evolutionarily conserved. Although embryos double mutant for both *Mmp1* and *Mmp2* are viable (Page-McCaw *et al.* 2003), *Mmp2* plays a crucial role in axon guidance of motor neurons during embryonic development (Miller *et al.* 2011). The role of both the Mmps, rather, seems to be more important during later stages of development in processes that demand tissue remodeling (Page-McCaw *et al.* 2003). While the activities of both the Mmps are required for dendritic remodeling (Kuo *et al.* 2005), as well as for dissociation of fat bodies during metamorphosis (Jia *et al.* 2014), they are also independently and reciprocally required for synaptogenesis along neuromuscular junctions (Dear *et al.* 2016). In addition, *Drosophila* Mmps also contribute significantly to reepithelization during wound healing (Stevens and Page-McCaw 2012), as well as to tumor invasiveness in flies (Uhlirva and Bohmann 2006; Beaucher *et al.* 2007). Since it has been evidenced that mammalian Mmps can actively participate in modifying signaling pathways by modulating the ECM proteins (Yu and Stamenkovic 2000; Hamano *et al.* 2003; Lee *et al.* 2005; Zhang *et al.* 2006), we sought to determine whether ectopic expression of Mmp in transdetermining tissue plays any role in controlling morphogen activity.

To address this issue, we resorted to *in vivo* genetic analyses of *Drosophila* larval wing discs undergoing ectopic eye differentiation due to induced expression of the gene *ey*. Our results show that the morphogen, Dpp, triggers the expression of *Mmp1* adjacent to the site of ectopic photoreceptor differentiation. However, instead of the canonical signaling pathway, Dpp employs an alternate signaling cascade involving Tak1 and JNK to activate *Mmp1*. Employing *in vivo* loss-of-function [both

RNA interference (RNAi) and classical mutant] genetic analyses, we establish that enrichment of Mmp1 around the cells producing Hh is actually instrumental in restricting the range of Hh signaling into the zone of ectopic photoreceptor differentiation. In doing so, Mmp1 modulates the rate of proliferation and activation of the genes *eya* and *dac*, two members of the RD gene network, to limit ectopic photoreceptor differentiation.

Materials and Methods

Drosophila stocks and fly husbandry

All stocks were reared on regular corn-flour medium and experiments were carried out at 25°. For details of the fly stocks used, their sources, and the genotypes of the lines generated for this study, please refer to Supplemental Material, Table S1.

Generation of somatic clones

To generate somatic clones of *Mmp1*², *hs-flp/hs-flp; FRT42D, Ubi-GFP, M* flies were crossed to *FRT42D, Mmp1*² flies. Synchronized hatched larvae were administered heat-shock pulses at 37° during the second larval instar. All the larvae were finally reared at 25° till analyses. For generating control mock clones, *FRT42D* flies were crossed to *hs-flp/hs-flp; FRT42D, Ubi-GFP, M* flies and a similar procedure as mentioned above was followed.

Immunohistochemistry and confocal microscopy

All immunostaining experiments were performed at least three times ($n = 15$ per experiment) following the standard protocol. The primary antibodies rat anti-ELAV (7E8A10; 1:100), mouse anti-Dac (mAbdac2-3; 1:20), mouse anti-En (4D9; 1:2), mouse anti-Eya (10H6; 1:2), rat anti-Ci (2A1; 1:3), and anti-Mmp1 (14A3D2, 3A6B4, 3B8D12, 5H7B11 used in a 1:1:1:1 dilution) were obtained from the Developmental Study Hybridoma Bank, Iowa, and anti- β -galactosidase (Z3781, 1:100) was obtained from Promega, Madison, WI. The following secondary antibodies for fluorescent detection were used at a 1:600 dilution: CyTM3-conjugated goat anti-mouse, FITC-conjugated goat anti-mouse, FITC-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories), and Phalloidin Texas Red (Molecular Probes, Eugene, OR). Immunofluorescence images were captured using a Laser Scanning Confocal Microscope (LSM 780; Carl Zeiss, Thornwood, NY) and processed using ImageJ and Adobe Photoshop.

Cryosectioning of imaginal discs

After secondary antibody immunostaining, tissues were refixed with 4% formaldehyde for 30 min, washed, and incubated in 30% sucrose solution (prepared in 1× PBS) overnight at 4°. Wing imaginal discs were oriented in Tissue-Plus O.C.T (optimal cutting temperature) solution (no. 4585; Fisher Healthcare), frozen, and cut into sections of 25 μ m by using a Leica Cryotome (CM3050) instrument. Sections were washed care-

fully with 1× PBS on glass slides, mounted in VECTASHIELD, and imaged.

Quantification of fluorescence intensity

Fluorescence intensity of expression was quantified in terms of Gray values of Dac- and Eya-expressing areas by using ImageJ. Each experiment was repeated three times along with an appropriate control and imaged at similar parameter settings. Ci activity was quantified in terms of pixel intensities within fixed area of wing discs imaged at the same settings by using the plot profile function of ImageJ. The average of the pixel intensities of five imaginal discs was taken to calculate the final Ci intensity profile.

Area measurement

We used Imaris x64 and ImageJ for calculating the area of Dac- and Eya-expressing cells in the wing discs. For each case the calculated areas were normalized with the total disc area. The results shown here are the outcomes of three independent experiments. The ommatidial number was also calculated using Imaris x64.

5-Ethynyl-2'-deoxyuridine incorporation assay

The 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay was performed by using the Click-iT EdU Alexa Fluor 594 Imaging Kit (Molecular Probes). The imaginal discs were incubated in EdU for 40 min. After completion of the procedure, the discs were processed for cryosectioning and imaging. The numbers of EdU-positive cells were calculated in the Dac-expressing domain and represented as number of EdU-positive cells per unit area of Dac expression.

Quantitative analysis of transcripts

Transcript levels of *Mmp1*, *hh*, and *kayak* were analyzed by quantitative RT-PCR (qRT-PCR) on RNA isolated from 50 to 60 wing discs. In all cases, expression levels of these transcripts were normalized with respect to *rp49* expression. Each experiment was repeated three times, using triplicates each time. The list of primers used is as follows:

rp49-forward, 5'-CTAAGCTGTCGCACAAATGGC-3'.
rp49-reverse, 3'-TTCTGCATGAGCAGGACCTC-5'.
Mmp1-forward, 5'-GCGTGTGAAGAACCTCACCT-3'.
Mmp1-reverse, 3'-TCTCCACGAACCTTGATCTCG-5'.
hh-forward, 5'-GCTCCGTCAAGTCAGATTCTG-3'.
hh-reverse, 3'-AGGTTGCGGTCCATGAAGAG-5'.
kayak-forward, 5'-GACCGATACTTCAAGTGCCCA-3'.
kayak-reverse, 3'-TTGAGGTATTTCGCTTGCTG-5'.

Live imaging of cytonemes

Live imaging of cytonemes in the wing discs was performed following the standard protocol (Ramirez-Weber and Kornberg 1999) with slight modifications. In brief, third instar larval wing discs were dissected in Schneider's Insect Medium (S0146, Sigma Chemical, St. Louis, MO) supplemented with

2.5% Phalloidin from *Amanita phalloides* from a stock solution of 1 mg/ml (P2141, Sigma Chemical) to stabilize the F-actin-rich cytonemes emanating from the basal side of the wing imaginal disc. The unfixed imaginal discs were mounted immediately and imaged in a Carl Zeiss LSM 780 Confocal Microscope.

Statistical analyses

Data are expressed as mean $\pm$ SD of values from three independent experiments. Statistical analysis was performed using two-tailed Student's *t*-test. The following *P*-values were accepted as statistically significant: * *P* < 0.01, ** *P* < 0.001, and *** *P* < 0.0001.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Reagents are available upon request.

Results

Mmp1 activity is required to limit differentiation of ectopic photoreceptors in larval wing imaginal discs

For our studies, we induced ectopic eyes in the wings of *Drosophila* by employing the binary Gal4-upstream activation sequence (UAS) system (Brand and Perrimon 1993). We used the Gal4 driver *Dpp-Gal4* to ectopically drive the expression of *UAS-ey* in developing wing imaginal discs. *Dpp* being expressed in the anterior compartment of cells adjacent to the A/P boundary of larval wing imaginal discs (Figure 1A) (Blackman *et al.* 1991), upon driving ectopic *ey* expression by *Dpp-Gal4*, we observed ectopic differentiation of photoreceptors along the A/P axis in late third instar larval wing discs, 136 hr after egg laying (AEL) (Figure 1B).

To investigate the role of *Mmp1*, if any, in modulating the induction of ectopic eyes in developing wing discs, we analyzed the number of differentiating photoreceptors when ectopic eyes were generated in wing discs either with compromised levels of *Mmp1* activity or having *Mmp1* overexpressed. To avoid the gross morphological distortions associated with late third instar wing discs of these genotypes undergoing ectopic eye differentiation, we used wing discs from larvae that were 124 hr old AEL for our analyses. As evident from Figure 1C, at this developmental time point the extent of ectopic photoreceptor differentiation in wing discs of *UAS-ey; Dpp-Gal4* larvae was much less when compared to that observed in discs of late third instar larvae (compare Figure 1C with Figure 1B). Interestingly, compared to the control (Figure 1, C and I), we observed an almost three- to fourfold increase in the number of ectopic photoreceptors upon driving two independent *UAS-Mmp1-RNAi* constructs, obtained from two different sources, by *Dpp-Gal4* (Figure 1, D, E, and I). Likewise, generating ectopic eyes in wing discs that were heterozygous mutant for *Mmp1*^{Q273}, a hypomorphic allele of *Mmp1* (Page-McCaw *et al.* 2003), also yielded a robust increase in the number of ectopic photoreceptors

(Figure 1, F and I). In fact, the increase in number of ectopic photoreceptors observed in these discs was relatively more than that observed upon knocking down *Mmp1*. Overexpression of the sole dTIMP by *Dpp-Gal4* also resulted in an increase in the number of ectopic photoreceptors, comparable to that observed for knocking down *Mmp1* (Figure 1, G and I). In tune with these results, we observed a drastic reduction in the number of ectopic photoreceptors upon overexpressing *Mmp1* by *Dpp-Gal4* (Figure 1, H and I). The involvement of *Mmp1* in regulating ommatidial differentiation, however, appears to be specific for ectopic photoreceptors, as normal ommatidial differentiation remained unaffected in eye discs of *Mmp1*^{Q273} homozygous mutant larvae (Figure S1, A and B, and File S1).

To establish that the observed increase in the number of ectopic photoreceptor differentiation was due to specific loss of *Mmp1* activity, we were interested to determine whether the increased number of ectopic photoreceptors as observed in *Mmp1*^{Q273} heterozygous mutant wing discs could be rescued by overexpression of *Mmp1*. Indeed, driving *UAS-Mmp1* in the wing discs of *UAS-ey/Mmp1*^{Q273}; *Dpp-Gal4* larvae drastically reduced the number of ectopic photoreceptors to a state that was comparable to that seen upon driving *UAS-ey* by *Dpp-Gal4* (Figure 1, J and K). Together, these results clearly established that *Mmp1* activity was required to limit the extent of ectopic photoreceptor differentiation.

Dpp triggers ectopic *Mmp1* expression during wing-to-eye fate alteration

Next we wanted to study the pattern of *Mmp1* expression in wing discs undergoing ectopic photoreceptor differentiation. Detectable levels of *Mmp1* expression in wing discs of *UAS-ey; Dpp-Gal4* larvae were first observed at 94 hr AEL (Figure 2B) in contrast to wild-type wing discs of same developmental stage (Figure 2A). Closer observation revealed the presence of high levels of *Mmp1* along the cell boundaries, creating a web-like pattern (Figure 2B'). As time progressed, we observed an increase in the domain of *Mmp1* expression in wing discs of *UAS-ey; Dpp-Gal4* larvae. By 114 hr AEL, ectopic *Mmp1* expression was observed in an extensive area spanning the A/P axis of *UAS-ey; Dpp-Gal4* wing discs (Figure 2, D and D'). Strikingly, the control wing disc of comparable developmental stage was devoid of any such patterned expression of *Mmp1* (Figure 2C). Even third instar larval eye discs did not exhibit any patterned expression of *Mmp1* (Figure S2, A and A')

To have better spatial information about this topological area of *Mmp1* expression, we analyzed *Mmp1* expression with respect to that of reporter *Dpp-RFP* expression along the A/P axis. As reported earlier (Aggarwal *et al.* 2016), we observed that ectopic expression of *ey* by *Dpp-Gal4* led to higher levels of *Dpp* expression in a relatively wider domain (Figure S2C), as compared to their wild-type control wing discs (Figure S2B). Analyses of *Dpp-RFP* and *Mmp1* expression through the Z-stacks of confocal images revealed a very interesting pattern of *Mmp1* expression. While there was no detectable *Mmp1* expression in the Z-sections along the apical surface of

Figure 1 Mmp1 negatively regulates ectopic photoreceptor differentiation in wing imaginal discs. In all wing discs, anterior compartment is to the left and the genotypes are as mentioned. The nuclei are marked with DAPI (blue). (A) Schematic representation of third instar larval wing disc showing the pattern of Dpp expression. (B) Ectopically differentiated photoreceptor clusters, marked by ELAV expression, adjacent to the A/P boundary in wing discs of late third instar larvae (136 hr AEL). (C) Limited number of ectopically differentiated photoreceptors is detected in wing discs of midthird instar larvae (124 hr AEL). (D and E) Robust increase in ectopic photoreceptor differentiation is seen upon knocking down Mmp1 expression as in D *UAS-ey/UAS-Mmp1(B); Dpp-Gal4* and (E) *UAS-ey/UAS-Mmp1(KK); Dpp-Gal4* midthird instar larval (124 hr AEL) wing discs as compared to *UAS-ey; Dpp-Gal4* larval wing discs of similar age (C). (F and G) Similar increase in number of ectopic photoreceptors is observed upon attenuating the Mmp1 activity in (F) *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* and (G) *UAS-ey; Dpp-Gal4/UAS-Timp* midthird instar larval wing discs as compared to *UAS-ey; Dpp-Gal4* larval wing discs of similar age (C). (H) Overexpression of Mmp1 leads to a drastic drop in the number of ectopic photoreceptors in midthird instar larval wing discs of *UAS-ey; Dpp-Gal4/UAS-Mmp1* larvae. (I) Quantification of the average number of ectopic photoreceptors in the midthird instar larval wing discs of genotypes mentioned. Mean $\pm$ SD; *** $P < 0.001$. (J) The increase in the number of ectopic photoreceptors as observed in *Mmp1^{Q273}* mutant background (F) gets drastically reduced upon overexpressing Mmp1. (K) Quantitative estimate of average number of ectopic photoreceptors in the early third instar larval wing discs of genotypes mentioned (mean $\pm$ SD; ns, not significant). Bars, 20 μ m.

wing disc (Figure 2E), we observed Mmp1 expression in Z-sections that are located basally (Figure 2F). Importantly, the expression of Mmp1 was observed juxtaposed to the wide band of Dpp expression toward the posterior compartment of the wing disc (Figure 2F). When observed under higher magnification, we found that even though the maximum accumulation of Mmp1 was around the cells just next to those expressing Dpp, a fraction of Dpp-positive cells had membranous Mmp1 expression (arrows in Figure 2G' and Figure S2D"). In addition, we also detected the presence of several punctate Mmp1 in between the Dpp-expressing cells (marked by arrowheads in Figure 2, G' and G'' and Figure S2, D' and D''). Taken together, these results indicated that the Dpp-expressing cells might be responsible for the release of Mmp1.

However, in absence of a strong overlap between the expressions of Dpp-RFP and Mmp1, to ascertain whether Mmp1 was actually secreted by Dpp-expressing cells, we resorted to genetic analysis. Employing *Dpp-Gal4*, we ectopically expressed *UAS-Mmp1 RNAi* in wing discs undergoing ectopic photoreceptor differentiation and then checked for ectopic Mmp1 expression. As shown in Figure 2H, this resulted in a drastic loss of Mmp1 expression, thereby demonstrating that the Dpp-expressing cells were indeed responsible for ectopic Mmp1 expression. This induction of Mmp1 expression by Dpp seems to be context dependent as mere overexpression of Dpp does not lead to ectopic Mmp1 expression in wing discs (Figure S2, E and F).

Figure 2 Mmp1 gets ectopically expressed in wing discs undergoing ectopic photoreceptor differentiation. In all wing discs, anterior compartment is to the left and the genotypes are as mentioned. (A) Expression of Mmp1 is not observed in wing imaginal discs of early third instar larvae (94 hr AEL). (B and B') Ectopic induction of *UAS-ey* by *Dpp-Gal4* leads to ectopic Mmp1 expression in wing imaginal discs of early third instar larvae (94 hr AEL). (B') represents the magnified image of the portion marked with a box in (B). (C) Basal level of Mmp1 expression in wing imaginal discs of third instar larvae (114 hr AEL). (D and D') Intense ectopic expression of Mmp1 is seen along the A/P axis in wing imaginal discs of *UAS-ey; Dpp-Gal4* third instar larvae (114 hr AEL). (D') Zoomed-in image of the portion marked with a box in (D). (E and F) Ectopic expression pattern of Mmp1 with respect to high levels of Dpp expression in third instar larval wing discs undergoing ectopic photoreceptor differentiation. While no Mmp1 expression with respect to Dpp expression is detected along the apical region of disc proper cells (E); strong Mmp1 expression posterior to the domain of Dpp expression is seen in Z-sections that are located basally (F). (G–G'') Zoomed-in images of a portion of midthird instar larval wing imaginal disc of *UAS-ey/UAS-RFP; Dpp-Gal4* larvae reveal ectopic expression of Mmp1 with respect to RFP expression in the disc proper cells located basally. Arrowheads in G' and G'' mark the punctate Mmp1 expression in between Dpp-expressing cells and arrows in G'' mark high levels of Mmp1 expression around cells expressing Dpp. (H) Drastic reduction in ectopic Mmp1 expression is seen in wing discs upon coexpressing *UAS-Mmp1i* and *UAS-ey* by *Dpp-Gal4*. Bars, 20 μ m.

Ectopic Mmp1 expression is triggered by dTak1-JNK-mediated Dpp signaling

The canonical Dpp signaling pathway involves binding of Dpp to its receptors, Thickveins (Tkv) and Punt. Activated Tkv, in turn, phosphorylates Mad which forms a heterodimer with Medea (Med) and together they translocate to the nucleus to transcriptionally activate the downstream target genes (Figure 3A) (Hamaratoglu *et al.* 2014). A previous report from our laboratory has demonstrated that besides this pathway, specifically during ectopic eye differentiation, Dpp upon binding to its receptors can also trigger an alternate signaling cascade that involves TGF- β -activated kinase1 (Tak1), leading to activation of JNK (Figure 3A) (Aggarwal *et al.* 2016).

To determine the involvement of Dpp signaling in inducing ectopic Mmp1 expression, we therefore checked for the involvement of both the signaling cascades. To start with, we knocked down the expression of the receptor Tkv in these cells. As evident from Figure 3B, this led to a significant drop in ectopic Mmp1 expression. An analogous result was obtained upon generating ectopic eyes in wing discs heterozygous mutant for the *tkv* allele, *tkv*⁷ (Figure 3C). In contrast, ectopic Mmp1 expression remained unaltered when Mad activity was knocked down (Figure S3A) or when ectopic eyes were generated in wing discs heterozygous mutant for *Mad*¹² (Figure 3D). On the other hand, a significant reduction in ectopic Mmp1 expression was observed when ectopic eyes

were generated in *dTak1* mutant wing discs (Figure 3E), as well as upon knocking down the expression of *dTak1* (Figure 3F). From these results we conclude that activation of Mmp1 expression by Dpp is mediated by dTak1, not by the canonical pathway involving Mad.

To further consolidate this finding, we checked for Mmp1 expression in wing discs undergoing ectopic eye differentiation with compromised levels of expression of members of the JNK pathway. As evident from Figure 3G, a dominant-negative form of *basket* (*bsk*; JNK in flies), when coexpressed with *ey*, resulted in a significant drop in the levels of Mmp1 expression. Likewise, inhibition of dFos activity (one of the transcriptional regulators activated by JNK) by knocking down *kayak* expression (the gene encoding dFos; Figure S3B) resulted in a considerable decrease in the level of ectopic Mmp1 expression (Figure 3H). Similar results were obtained upon coexpression of a dominant-negative form of dFos in the transdetermining tissue (Figure S3C). In tune with these observations, results of our qRT-PCR analyses also revealed that the increase in expression of *Mmp1* transcripts as observed in wing discs of *UAS-ey; Dpp-Gal4* larvae became significantly reduced upon downregulating the activity of dFos (Figure 3I).

The transcriptional regulator dFos is known to form a heterodimer with dJun to form the AP1 complex which binds to its specific DNA binding site and regulates transcriptional

Figure 3 Dpp signaling through dTak1-JNK pathway regulates ectopic *Mmp1* expression during wing-to-eye fate alteration. In all wing discs, anterior compartment is to the left and the genotypes are as mentioned. The nuclei are marked with DAPI (blue). (A) Schematic representation of the possible Dpp-mediated signaling pathways that can lead to ectopic *Mmp1* expression. (B and C) Sharp reduction in ectopic *Mmp1* expression is observed either upon knocking down *tkv* expression (B) or when ectopic eyes are induced in wing discs of *tkv⁷* heterozygous larvae (C). (D) Attenuation of Mad activity does not affect ectopic *Mmp1* expression. (E and F) Drastic reduction in ectopic *Mmp1* expression is detected when ectopic photoreceptors are either induced in wing discs of *dtak1²* mutants (E) or upon driving *UAS-dTak1i* in wing discs undergoing ectopic photoreceptor differentiation (F). (G and H) Similarly, impairing the JNK signaling pathway by expressing *UAS-bsk^{DN}* (G) or by driving *UAS-Kayi* (H) results in a significant drop in ectopic *Mmp1* expression. (I) Quantitative analysis of the fold change in *Mmp1* transcript reveals that the robust increase in the level of *Mmp1* expression as observed in *UAS-ey; Dpp-Gal4* wing discs gets significantly reduced upon coexpressing *UAS-fos^{DN}* along with *UAS-ey* by *Dpp-Gal4*. ** $P < 0.001$. (J–J'') Zoomed-in image of the region of wing disc showing reporter *Mmp1-lacZ* expression (J) that completely overlaps with ectopic *Mmp1* expression (J' and J'') as revealed by immunostaining. (K) Drastic decrease in ectopic *Mmp1-lacZ* expression is observed upon knocking down the expression of *dTak1* in wing discs undergoing ectopic photoreceptor differentiation. Bars, 20 μ m.

activity of the target gene (Kockel *et al.* 2001). We therefore employed a reporter *Mmp1-lacZ* line, which harbors a construct with reporter *lacZ* cloned downstream to the regulatory region of *Mmp1* with three putative AP1 binding sites (Uhlirova and Bohmann 2006), to determine whether the ectopic *Mmp1* expression observed was mediated by AP1. Upon generating ectopic eyes in wing discs of these *Mmp1-lacZ* larvae followed by co-immunostaining with antibodies against *Mmp1* and β -galactosidase, we observed a significant overlap in their expression patterns (Figure 3, J–J''). Furthermore, we observed that knocking down dTak1 activity brought about a drastic drop in the expression of reporter *Mmp1-lacZ* in wing discs undergoing ectopic photoreceptor differentiation (Figure 3K). From all these results, we con-

clude that Dpp, involving a signaling cascade that includes dTak1 and JNK, triggered the transcription of ectopic *Mmp1* during ectopic eye induction.

Ectopic *Mmp1* expression gets spatially oriented toward the posterior compartment

The larval wing imaginal disc consists of two layers of epithelial cells. The disc proper is made up of a layer of columnar epithelial cells, and the apical surface of these cells is juxtaposed with the peripodial membrane made up of squamous epithelial cells. Gross morphological analysis, as assayed by phalloidin staining, revealed that, compared to their wild-type control discs, wing discs undergoing ectopic eye differentiation had a portion of the disc proper cells protruding outward.

Interestingly, this protrusion was always observed toward the basal side. Transverse section of these wing discs further confirmed that the protrusion was indeed formed by the columnar epithelial disc proper cells, more or less midway along the A/P direction of the wing disc (arrowhead in Figure 4, B and B'). Similar sections through wild-type wing discs did not exhibit any such fold in the layer of disc proper cells (Figure 4, A and A'). To have better positional information about this protrusion, we next checked for the expression of Engrailed (En) and Dpp in these wing discs. While cells in the posterior arm of the protrusion were found to express En, a *bona fide* marker for the posterior compartment of wing disc (Figure 4, C and C'), we observed Dpp expression in the anterior arm of the protrusion (Figure 4D). Together, these results demonstrated that the disc proper cells indeed protruded outward basally, midway along the A/P axis, with the A/P boundary around its tip.

To determine the spatial orientation of ectopic Mmp1 expression with respect to these protrusions, we checked for Mmp1 expression in transverse sections, wherein the anterior compartment cells were marked with activated Ci (Ci^{act}) expression. Mmp1 expression was specifically evident around the cells of the posterior compartment adjacent to the posterior boundary of the Ci^{act}-expression domain (Figure 4, E and E'). Results of these expression studies, apart from providing topological information about ectopic Mmp1 expression, also explained the reason for observing Mmp1 expression specifically in Z-stacks of confocal images along the basal surface of wing discs as shown in Figure 2F. Importantly, this kind of directionality of Mmp1 secretion toward the posterior compartment appears to be specific for these wing discs, as it was not evidenced upon overexpression of *Mmp1* by *Dpp-Gal4* in the anterior compartment cells along the A/P boundary (Figure S4, A–A''). Rather, Mmp1 expression was observed both in the anterior and posterior compartments with relatively more in the anterior compartment compared to that in the posterior one.

To account for this directionality in Mmp1 secretion, we further investigated the role of cytonemes, if any. Cytonemes are thin, actin-rich cellular projections involved in signaling between cells, and have been evidenced to play a critical role in patterning in the developing wing discs (Roy and Kornberg 2015). For our analyses, we primarily focused on the cytonemes emanating from the Dpp-expressing cells, as our results indicated the involvement of these cells in secreting Mmp1. We marked the actin-rich cytonemes of the Dpp-expressing cells by driving *UAS-GMA* with *Dpp-Gal4*. The *UAS-GMA* is a chimeric construct that has the actin-binding region of *Drosophila* moesin fused to the C terminus of GFP cloned into the pUAST transformation vector (Bloor and Kiehart 2001). Expression of this construct within a cell allows long-term live imaging of morphogenetic events at a high spatial resolution without affecting the normal behavior of F-actin. In developing third instar larval wing discs, we observed the cytonemes originating from these cells are visibly oriented toward the anterior compartment (Figure 4, F–F'').

In sharp contrast, these cytonemes get majorly oriented toward the posterior compartment in wing discs of *UAS-ey; Dpp-Gal4* larvae (Figure 4, G–G''). However, this kind of change in the directionality of cytonemes was not observed upon overexpressing Mmp1 by Dpp-Gal4 (Figure S4, B–B''). Taken together, these results indicate the possible involvement of cytonemes in specifying the directionality of Mmp1 secretion in wing discs of *UAS-ey; Dpp-Gal4* larvae.

Loss of Mmp1 activity augments ectopic Eya and Dac expression, resulting in an increase in ectopic photoreceptor differentiation

Differentiation of photoreceptors in *Drosophila* is triggered by the activation of a gene-regulatory network that primarily consists of *ey*, *eya*, *sine oculis* (*so*), and *dac* (Heberlein and Treisman 2000; Silver and Rebay 2005). As shown in Figure 5A, while all the members of this network regulate each other's expression by transcriptional activation and feedback loops, the three proteins, Eya, So, and Dac are known to physically interact with one another (Chen *et al.* 1997; Pignoni *et al.* 1997). Moreover, it has also been established that *dac*, along with *eya* and *so*, is a downstream transcriptional target of Dpp signaling (Chen *et al.* 1999; Pappu *et al.* 2005). We therefore analyzed the role of Mmp1 in regulating ectopic photoreceptor differentiation in the light of Dac and Eya expression.

To start with, we checked for the expression of Dac and Mmp1. While ectopic Dac expression was seen in a subset of cells positioned toward the posterior end of the Dpp-expressing domain (Figure 5, B–B''), we observed Mmp1 expression around the cells adjacent to the posterior boundary of the Dac-expression domain (Figure 5, C–C'' and D). Subsequently, we observed that knocking down Mmp1 expression in wing discs undergoing ectopic eye induction resulted in a significant increase in the domain of ectopic Dac-expressing cells (Figure 5F) compared to that observed in wing discs of *UAS-ey; Dpp-Gal4* larvae (Figure 5E), with a concurrent increase in the number of ectopically differentiating photoreceptors (Figure S5, A and B'). An identical increase in the domain of Dac-expressing cells (Figure 5G) and number of ectopic photoreceptors (Figure S5, C and C') was also observed when ectopic eyes were induced in wing discs heterozygous mutant for *Mmp1*^{Q273}. Quantitative analyses revealed that compromised activity of *Mmp1* led to a twofold increase in the domain of Dac expression when compared to that observed in control *UAS-ey; Dpp-Gal4* wing discs (Figure 5H). Interestingly, this increase in the domain of ectopic Dac expression was also associated with an increase in its level of expression. Quantification of fluorescence intensity in the Dac-expression domain revealed an almost 1.8-fold increase in the level of ectopic Dac expression per unit area upon attenuating Mmp1 when compared to that detected in control *UAS-ey; Dpp-Gal4* wing discs (Figure 5I).

Immunostaining of wing discs of *Dpp-Gal4; UAS-ey* larvae with antibodies against the other RD protein, Eya, revealed its expression in the anterior half of the protrusion of disc

Figure 4 Ectopic *Mmp1* expression during wing-to-eye fate alteration is oriented to the posterior compartment. In all transverse-section images of wing discs, anterior compartment is to the left and the genotypes are as mentioned. The nuclei are marked with DAPI (blue). (A and A') Transverse section of wild-type wing discs in the A/P orientation showing the columnar epithelial disc proper cells (dp) and the squamous epithelial cells of the peripodial (pp) membrane. (B and B') Transverse section through wing discs of *UAS-ey*; *Dpp-Gal4* larvae in the A/P orientation reveals the formation of a protrusion of the disc proper cells in a direction opposite to that of the peripodial cells. (C and C') Expression of *En* is observed in the posterior arm of the protrusion. (D) Cells of the anterior arm of the protrusion express *Dpp*. (E and E') Expression of *Mmp1* is detected along the cell boundaries of cells of the posterior arm of the protrusion that are adjacent to the *Ci^{act}*-expressing cells of the anterior compartment. (F–F'') Expression of *UAS-GMA* marking the cytonemes that are projected toward the anterior compartment in wing discs of *UAS-GMA*; *Dpp-Gal4* larvae. (F' and F'') Zoomed-in images of the regions marked in F and F', respectively. (G–G'') Ectopic expression of *ey* in wing discs of *UAS-GMA*; *UAS-ey*; *Dpp-Gal4* larvae leads to a change in the orientation of the cytonemes. As demarcated by the expression of *UAS-GMA*, cytonemes in these wing discs project toward the posterior compartment. (G' and G'') Zoomed-in images of the regions marked in G and G', respectively. Bars, 20 μ m.

proper cells (Figure 5J) in a pattern similar to that observed for ectopic *Dac* expression. Ectopic eyes, when generated in wing discs of larvae that were heterozygous mutant for *Mmp1*^{Q273}, resulted in a significant increase in the domain of ectopic *Eya* expression (Figure 5, K and L) in a manner identical to that observed for ectopic *Dac* expression. This increase in the domain of ectopic *Eya* expression was accompanied with an almost 1.5-fold increase in the intensity of *Eya* expression (Figure 5M). In sum, these results clearly demonstrated that the observed increase in ectopic photoreceptor differentiation in *Mmp1* mutant background or upon attenuating *Mmp1* expression was actually due to an increase in the intensity as well as in the domain of ectopic *Dac* and *Eya* expression.

Loss of *Mmp1* leads to increased proliferation of *Dac*-expressing cells

To ascertain whether the expansion in the *Dac*-expression domain was an outcome of increased cell proliferation, we assessed cell-cycle progression of the *Dac*-expressing cells by

the incorporation of EdU. EdU is a thymidine homolog that gets incorporated in the newly synthesized strand of DNA and thereby marks the cells that are in the S phase of cell division. Uniform incorporation of EdU was observed in both the anterior and posterior compartments of the wild-type wing disc (Figure 6, A–A''); the posterior compartment being marked by *En* expression). However, the pattern of EdU incorporation in wing discs of *UAS-ey*; *Dpp-Gal4* larvae was quite distinct. Although a uniform distribution of EdU incorporation was observed in the transverse section, quite strikingly, a portion in the anterior part of the protrusion exhibited scanty EdU-positive cells (marked by dotted lines in Figure 6B). Interestingly, co-immunostaining of these sections with anti-*Dac* antibody revealed an exact overlap of this region to the domain of ectopic *Dac* expression (Figure 6, B' and B''), thereby indicating limited proliferation of the *Dac*-expressing cells. However, knocking down *Mmp1* activity or generating ectopic eyes in a *Mmp1*^{Q273} mutant background led to a significant increase in the number of EdU-positive cells in the *Dac*-expression domain (Figure 6, C–D''). Quantification of

Figure 5 Mmp1 expression is required to restrict the domain of ectopic Eya and dac expression during wing-to-eye fate alteration. In all transverse-section images of wing discs, anterior compartment is to the left and the genotypes are as mentioned. The nuclei are marked with DAPI (blue). (A) Schematic representation of the gene regulatory network involved in activation of the RD genes during normal photoreceptor differentiation. (B–B'') While all cells of the anterior arm of the protrusion express Dpp (B and B''), a subset of them toward the posterior ectopically express Dac (B' and B''). (C–C'') Expression of Mmp1 (C and C'') is detected along the cell boundaries of cells of the posterior arm of the protrusion that are adjacent to the Dac-expressing cells (C' and C''). (D) Schematic representation of protrusion showing the expression patterns of Dpp, Dac, and Mmp1. (E–G) Both the intensity and domain of ectopic Dac expression get enhanced upon knocking down Mmp1 expression (F) or in Mmp1^{Q273} mutant background (G) compared to that observed in wing discs of *UAS-ey; Dpp-Gal4* larvae (E). (H and I) Quantification of the increases in the domain of ectopic Dac expression (H) as well as in the intensity of ectopic Dac expression per unit area (I) in wing discs of the genotypes mentioned when compared to their control *UAS-ey; Dpp-Gal4* larval wing discs. (J and K) Both the intensity and domain of ectopic Eya expression get enhanced upon generating ectopic eyes in Mmp1^{Q273} mutant background (K) compared to that observed in wing discs of *UAS-ey; Dpp-Gal4* larvae (J). (L and M) Quantification of the increases in the domain of ectopic Eya expression (L) as well as in the intensity of ectopic Eya expression per unit area (M) in wing discs of the genotype mentioned when compared to their control *UAS-ey; Dpp-Gal4* larval wing discs. Bars, 20 μ m.

the above result revealed a twofold increase in the number of EdU-positive cells per unit area of the Dac-expression domain in wing discs with attenuated Mmp1 activity when compared to the control (Figure 6E). Together, these results clearly established that the increase in ectopic Dac- and Eya-expression domain, as observed upon compromising Mmp1 activity, was essentially due to an increase in proliferation within this domain.

Ectopic expression of Mmp1 defines the range of Hh signaling in wing discs undergoing ectopic eye differentiation

Previous studies have demonstrated that, during normal eye development, the differentiating clusters of photoreceptors in the larval eye imaginal disc express the secretory morphogen Hh, which coordinates proliferation of the eye primordial

Figure 6 Mmp1 regulates cell proliferation to limit the domain of ectopic Dac-expressing cells. In all transverse-section images of wing discs, anterior compartment is to the left and the genotypes are as mentioned. The nuclei are marked with DAPI (blue) and the domain of ectopic Dac expression is denoted by dotted line. (A–A'') Transverse section of wild-type wing discs in the A/P orientation showing uniform EdU incorporation in both the anterior and the posterior compartments [posterior compartment is marked by En expression in (A' and A'')]. (B–B'') Transverse section through wing discs of *UAS-ey; Dpp-Gal4* larvae in the A/P orientation reveals EdU incorporation in the protrusion (B and B''). While uniform EdU incorporation is seen in most of the region, very few EdU-positive cells are detected in the domain expressing ectopic Dac (B' and B''). (C–C'') Knocking down Mmp1 expression results in increased level of EdU incorporation (C and C'') in cells of the ectopic Dac-expressing domain (C' and C''). (D–D'') Generating ectopic eyes in wing discs of *Mmp1^{Q273}* heterozygotes also leads to an increase in EdU incorporation (D and D'') in cells of the ectopic Dac-expressing domain (D' and D''). (E) Quantification of the increase in EdU-positive cells per unit area of the ectopic Dac-expressing domain in the wing discs of the genotypes mentioned when compared to their control *UAS-ey; Dpp-Gal4* larval wing discs. Bars, 20 μ m.

cells, and initiates differentiation of photoreceptors, apart from inducing the expression of Dpp in cells ahead of the differentiating clusters (Curtiss and Mlodzik 2000; Duman-Scheel *et al.* 2002; Pappu *et al.* 2003; Firth and Baker 2009). Hh is also expressed by cells of the posterior compartment of wing disc and is known to induce the expression of Dpp in the anterior compartment (Basler and Struhl 1994). Moreover, ectopic photoreceptor differentiation always takes place in Dac-expressing cells adjacent to the posterior compartment. We were intrigued to determine whether the increased proliferation in the domain of ectopic Dac expression, as well as

the increase in intensity of Dac and Eya expression, as observed upon attenuating Mmp1 activity in wing discs of *UAS-ey; Dpp-Gal4* larvae, was an outcome of increased Hh activity.

We therefore analyzed the expression pattern of Ci^{act}, the transcriptional activator molecule of the Hh signaling pathway, in these wing discs (Huangfu and Anderson 2006). In control wing discs, we observed Ci^{act} expression in cells of the anterior compartment with maximum expression along the A/P border which gradually formed a decreasing gradient toward the periphery (Figure 7, A and B). In contrast to this, we detected a modest increase in the level of Ci^{act} expression

Figure 7 Ectopic *Mmp1* expression regulates cell proliferation and levels of ectopic *Dac* and *Eya* expression by restricting the diffusion of *Hh* from the posterior compartment. In all images of wing discs and the transverse sections, anterior compartment is to the left and the genotypes are as mentioned. The nuclei are marked with DAPI (blue). (A) Expression pattern of *Ci^{act}* in wild-type wing discs. (B) Average intensity profile of *Ci^{act}* expression in wild-type wing discs. (C) Modest increase in the level of *Ci^{act}* expression is observed in wing discs of *UAS-ey; Dpp-Gal4* larvae. (D) Average intensity profile of *Ci^{act}* expression in wing discs of *UAS-ey; Dpp-Gal4* larvae compared to that observed in wild-type wing discs. (E and F) The level of *Ci^{act}* expression gets drastically enhanced either upon knocking down *Mmp1* (E) or upon generating ectopic eyes in wing discs of *Mmp1^{Q273}* heterozygotes (F). (G) Average intensity profiles of *Ci^{act}* expression in wing discs of larvae of the genotypes mentioned compared to that observed in wild-type wing discs. (H) Quantitative analysis of the fold change in *hh* transcript reveals that compared to wild-type wing discs, the level of *hh* expression remain unaltered in *UAS-ey; Dpp-Gal4* and *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* wing discs. (I and I') Expression of *Ci^{act}* in wing discs with wild-type mock clones. Absence of GFP in I' marks the clone. (J and J') A marginal increase in the level of *Ci^{act}* is observed in somatic clones of *Mmp1²*. The clonal area is marked by the absence of GFP in (J'). (K) Average intensity profiles of *Ci^{act}* expression in clones of *Mmp1²* compared to that observed in wild-type clones. (L) The level of

in the anterior compartment of wing discs of *UAS-ey; Dpp-Gal4* larvae (Figure 7, C and D). Interestingly, knocking down *Mmp1* expression in wing discs of *UAS-ey; Dpp-Gal4* larvae resulted in a robust increase in the level of Ci^{act} expression (Figure 7, E and G). Not only was the intensity of Ci^{act} expression along the A/P boundary higher, but even the gradient of Ci^{act} expression in the anterior compartment exhibited a significant level of increase. Analogous results were observed in wing discs of *Dpp-Gal4; UAS-ey/Mmp1^{Q273}* larvae (Figure 7, F and G). Together, these results clearly demonstrated that loss of *Mmp1* activity led to increased Hh signaling in the anterior compartment of wing discs undergoing ectopic eye induction.

To account for the increase in the gradient of Ci^{act} expression in wing discs of *UAS-ey; Dpp-Gal4* larvae, we then checked for Hh expression. However, our qRT-PCR analyses did not reveal any detectable increase in the level of *hh* transcripts in wing discs of *UAS-ey; Dpp-Gal4* larvae when compared to that observed in control wing discs (Figure 7H). Likewise, we also found that the level of *hh* expression remained unaltered even in wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* larvae (Figure 7H), which demonstrated a robust increase in the gradient of Ci^{act} expression in the anterior compartment (Figure 7, F and G). Therefore, our results demonstrated that the increase in the gradient of Ci^{act} was not an outcome of increased *hh* expression. Rather, these results suggested that loss of *Mmp1* might have allowed a greater amount of Hh to diffuse into the anterior compartment of the wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* larvae to account for the increase in the level of Ci^{act} . These results suggested that ectopic *Mmp1* expression in wing discs of *UAS-ey; Dpp-Gal4* larvae might be playing a role in restricting the diffusion of Hh molecules in the anterior compartment of the wing disc.

To ascertain whether *Mmp1* plays a similar role in restricting Hh signaling in the anterior compartment during normal development, we generated somatic clones of *Mmp1²* [a null allele of *Mmp1* (Page-McCaw et al. 2003)] in the wing disc by

employing a flip-FRT technique to check for Ci^{act} expression. As shown in Figure 7, J and J', we observed a marginal increase in Ci^{act} expression in somatic clones of *Mmp1²*, when compared to that in wild-type mock clones (Figure 7, I and I'). This increase is further evident in the plot profile analysis, as shown in Figure 7K. A similar increase in the level of Ci^{act} expression in the anterior compartment was also observed when we knocked down the expression of *Mmp1* along the A/P axis by *Dpp-Gal4* (Figure S6, A–C). From these results, we conclude that, though marginally, *Mmp1* is instrumental in limiting Hh activity in the anterior compartment of wild-type wing discs.

We then argued that if this holds true, then we should be able to rescue all the phenotypes observed in wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* larvae by genetically manipulating the amount of Hh molecules being produced. For that purpose, we adopted genetic means to reduce the amount of Hh expression in the posterior compartment of wing discs by employing heterozygous mutants for *hh^{AC}*, a null allele of *hh* (Lee et al. 1992). As evident from Figure 7L, wing discs of *UAS-ey/Mmp1^{Q273}; hh^{AC}/Dpp-Gal4* larvae demonstrated a 30% reduction in the level of *hh* expression as compared to the *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* control. Consistent with this, when these wing discs were checked for the expression of Ci^{act} , they demonstrated a drop in expression along the A/P border as well as in its gradient in the anterior compartment (Figure 7M). Quantitative analysis of the intensity profile revealed the level of Ci^{act} expression was comparable to that observed in wing discs of *UAS-ey; Dpp-Gal4* larvae (Figure 7N).

To determine whether this drop in Hh signaling was able to rescue the increase in cell proliferation of the *Dac*-expression domain of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* larvae, we analyzed the pattern of EdU incorporation in wing discs of *hh^{AC}/Dpp-Gal4; UAS-ey/Mmp1^{Q273}* larvae. As evident from Figure 7, O–O", an appreciable drop in the level of EdU incorporation was detected, suggesting that the cells in the *Dac*-expressing domain were indeed dividing less. Quantification of the number

hh expression gets reduced in wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{AC}* larvae. (M) Compared to that observed in *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* wing discs (F), a drop in the level of Ci^{act} expression is observed in wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{AC}* larvae. (N) Average intensity profiles of Ci^{act} expression in wing discs of larvae of the genotypes mentioned compared to that observed in wild-type wing discs. (O–O") Amount of EdU incorporation in the domain of ectopic *Dac* expression gets reduced in wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{AC}* larvae. (P) Quantification of the relative change in EdU-positive cells per unit area of the ectopic *Dac*-expressing domain in wing discs of the genotypes mentioned when compared to their control *UAS-ey; Dpp-Gal4* wing discs. ** $P < 0.001$. (Q) Transverse section through the wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{AC}* larvae demonstrating reduction in both the intensity and domain of ectopic *Dac* expression. (R and S) Quantification of the decrease in the domain of ectopic *Dac* expression (R) as well as in the intensity of ectopic *Dac* expression per unit area within the domain (S) in wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{AC}* larvae when compared to their control *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* wing discs. ** $P < 0.001$. (T) Transverse section through the wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{AC}* larvae demonstrating reduction in both the intensity and domain of ectopic *Eya* expression. (U and V) Quantification of the decrease in the domain of ectopic *Eya* expression (U) as well as in the intensity of ectopic *Eya* expression per unit area within the domain (V) in wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{AC}* larvae when compared to their control *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* wing discs. ** $P < 0.001$. (W) Reduction in the number of ectopic photoreceptors as evidenced in the wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{AC}* larvae. (X) Quantification of the drop in the number of ectopic photoreceptors in wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4/hh^{AC}* larvae when compared to their control *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* wing discs. Mean $\pm$ SD; *** $P < 0.0001$. (Y) Schematic representation of the regulation of Hh activity by *Dpp* in wing discs undergoing ectopic eye differentiation. Transcriptional activation of *Mmp1* by *Dpp* signaling through a pathway involving *Tkv*, *Tak1*, and *JNK* leads to release of *Mmp1* outside the cell. Modification of the ECM by *Mmp1* restricts the amount of Hh molecules that diffuse to the anterior compartment. Expression of the target genes of Hh signaling that include *cycE*, *dpp*, *dac*, and *eya* depend upon the level of Ci^{act} generated in the anterior compartment cells. Bars, 20 μ m.

of EdU-positive cells per unit area of the domain of Dac expression in these wing discs exhibited a significant drop when compared to that observed in wing discs of *UAS-ey/Mmp1^{Q273}; Dpp-Gal4* larvae (Figure 7P). We subsequently checked for the expression of the RD genes Dac and Eya. For each of them, we observed a significant drop both in their domain as well as in their level of expression (Figure 7, Q–V). Finally, as a functional correlate of these results, we observed that reducing the amount of Hh signaling resulted in a significant drop in the number of ectopic photoreceptors in wing discs of *UAS-ey/Mmp1^{Q273}; hh^{AC}/Dpp-Gal4* larvae (Figure 7, W and X). In sum, results of our expression studies, in conjunction with that of *in vivo* genetic analyses, corroborated the hypothesis that the altered phenotypes observed in *UAS-ey/Mmp1^{Q273}; hh^{AC}/Dpp-Gal4* wing discs was an outcome of decreased Hh signaling in the anterior compartment. These results further established that in wing discs of *UAS-ey; Dpp-Gal4* larvae, Mmp1 was playing an important role in limiting the range of Hh signaling in the anterior compartment.

Discussion

Our results unravel an otherwise unknown mechanism by which Dpp signaling regulates the range of Hh signaling (Figure 7Y) during ectopic eye differentiation in wing discs of *Drosophila*. We show that by employing a signaling cascade that involves Tak1 and JNK, Dpp activates the expression of *Mmp1*. The role of secreted Mmp1 seems to regulate the diffusion of Hh molecules into the anterior compartment and, in the process, restricts proliferation and the level of ectopic *dac* and *eya* expression. In doing so, Mmp1 limits the amount of ectopic photoreceptor differentiation. With these findings we have been successful not only in unraveling the developmental signaling cascade leading to ectopic Mmp1 expression, but also in providing evidence to establish the role played by Mmp1 during ectopic photoreceptor differentiation. From a broader perspective, this entire regulatory mechanism (Figure 7Y) unearths a unique phenomenon where one morphogen, Dpp, regulates the activity domain of another morphogen, Hh, by employing Mmp1, a known modulator of the ECM.

Several studies have outlined the regulation of Dpp signaling by Hh in both eye and wing imaginal disc development (Heberlein *et al.* 1993; Basler and Struhl 1994). However, regulation of Hh activity by Dpp has not yet been evidenced (Shen and Dahmann 2005). We provide the first *in vivo* genetic evidence for the regulation of Hh signaling by Dpp. The mechanism involved does not lead to any transcriptional regulation of Hh signaling. Rather, Dpp achieves this regulation through activation of the JNK signaling cascade that elicits ectopic expression of Mmp1. Mmp1, in turn, limits the range of Hh signaling. Importantly, our results indicate that this kind of restriction of Hh diffusion by Mmp1 also happens, although marginally, in developing wild-type wing discs, as revealed by an increase in the level of Ci^{act} in anterior compartment cells that are homozygous mutant for *Mmp1*, as

well as upon knocking down the expression of *Mmp1* along the A/P boundary. Since the glypican, Dally-like protein (Dlp), is a potential target of Mmp1 and Dlp facilitates the transmission of Hh signaling by acting as coreceptor for Patch in the Hh receiving cells of the anterior compartment (Gallet *et al.* 2008), it is quite possible that in these cases the increase in Ci^{act} in anterior compartment cells might be an outcome of an increased level of Dlp. However, even though the molecular basis of regulation of Hh by Mmp1 during ectopic eye differentiation is yet to be made evident, we believe it to be independent of Dlp, primarily because we observe ectopic Mmp1 expression around the Hh-releasing cells of the posterior compartment. Since no increase in Mmp1 expression is observed in cells of the anterior compartment, it is quite evident that during this process, Mmp1 does not control Hh signaling in the receiving cells by modulating Dlps. Rather, Mmp1 actually modulates the ECM around the Hh-producing cells to regulate the diffusion range and accessibility of Hh molecules to its cognate receptors present on cells of the anterior compartment by an alternate mechanism yet to be made clear.

Our results clearly demonstrate that even though the domain of Dpp expression gets significantly increased in wing discs of *UAS-ey; Dpp-Gal4* larvae, ectopic expression of Dac and Eya is restricted only to the posterior part of the Dpp-expression domain. Consistent with this, ectopic photoreceptor differentiation is also observed in this domain with ectopic *dac* and *eya* expression. Despite Ey and Dpp being expressed in the entire Dpp-expressing domain, this restriction of ectopic *dac* and *eya* expression toward the posterior part of this domain highlights the requirement of Hh signaling in ectopic eye differentiation. Interestingly, restriction of ectopic *dac* and *eya* expression in cells juxtaposed to the A/P boundary which have a high expression of Ci^{act}, suggests threshold-dependent activation of these genes by Hh. This notion becomes further endorsed as we find that increase in expression of Ci^{act} in *Mmp1* mutant wing discs leads to a concomitant increase in ectopic *dac* and *eya* expression and subsequent photoreceptor differentiation. Importantly, this increase in the level of ectopic *dac* and *eya* expression can be rescued by lowering the amount of Hh signaling from the posterior compartment. Likewise, Hh signaling from the posterior compartment also regulates proliferation in the Dac-expressing domain. Lowering Mmp1 levels allows more Hh signaling to induce the Dac-expressing cells, which were otherwise nonproliferating, to proliferate further. Interestingly, the increase in proliferation occurs toward the posterior part of the Dac-positive region with the cell-cycle arrest still persisting anteriorly. To a certain extent, this bears similarity to the progression of the morphogenetic furrow by Hh signaling, as seen during ommatidial differentiation in developing eye discs (Borod and Heberlein 1998). With increased Hh signaling, the region of cell-cycle arrest moves anteriorly and the cells entering S phase in the posterior part of the Dac-positive region might resemble the second mitotic wave. As a consequence, new rows of photoreceptors differentiate anteriorly.

A striking phenotype associated with ectopic ommatidial differentiation is the protrusion formed by disc proper cells around the region undergoing cell-fate alteration in wing discs of *UAS-ey; Dpp-Gal4* larvae. This process appears to be independent of *Mmp1* activity, as the formation of the protrusion is not affected in *Mmp1* mutant or in an *Mmp1* knock-down background. Within this protrusion, ectopic *Mmp1* expression is specifically directed toward the posterior compartment. Based on our results, we speculate that altered orientation of the cytonemes emanating from the Dpp-expressing cells, resulting from prominent remodeling of disc proper cells, might be responsible for this directionality. Since this kind of change in the orientation of cytonemes as well as directionality in *Mmp1* secretion is not seen when *Mmp1* is overexpressed or upon ectopically expressing another RD gene, *eya* (results not shown), in all likelihood, ectopic expression of *ey* is responsible for this structural remodeling by a mechanism yet to be revealed. The expansion in the domain of Dpp expression in anterior compartment cells observed in these wing discs might also result from more Hh molecules diffusing from posterior to anterior compartment as a result of local alteration in ECM due to these structural changes. However, since the cells of the wing imaginal discs are in a determined state, it is quite possible that activation of *Mmp1* by Dpp signaling might be a measure adopted by these cells to limit Hh diffusion, so as to counteract (or outcompete) the extent of fate alteration, and in the process maintain their determined state. In this context, it is important to note that previous studies have demonstrated that Dpp signaling elicits both instructive and permissive roles to regulate the ectopic expression of RD genes critical for ectopic photoreceptor differentiation (Chen *et al.* 1999; Curtiss and Mlodzik 2000; Pappu *et al.* 2005; Aggarwal *et al.* 2016). The outcome of this current study, in conjunction with those results, clearly establishes a dual role of Dpp signaling in cellular plasticity. While on one hand it facilitates cell fate alteration by creating a micro-environment conducive for the process, simultaneously it also restricts the proliferation and differentiation potential of the cells undergoing fate alteration. By balancing these two events, Dpp plays a pivotal role in maintaining tissue homeostasis.

Studies in the recent past, primarily involving *in vitro* analyses of cancer cells, have implicated the involvement of JNK, Wnt, NF- κ B, and TGF- β signaling pathways in transcriptional activation or repression of *Mmp* and *TIMP* genes (Yan and Boyd 2007; Fanjul-Fernandez *et al.* 2010). Transcriptional regulation of MMPs in cell lines by TGF- β signaling, however, is rather complex. While binding of Smad to the TGF- β inhibitory element negatively regulates transcription of MMP-1 and MMP-7 (Yuan and Varga 2001), Smads play an important role in altering MMP expression (Verrecchia *et al.* 2001) by interacting with AP1, the transcriptional regulator of JNK pathway. Although the involvement of JNK signaling has also been evidenced in transcriptional activation of *Mmp1* in flies (Uhlirva and Bohmann 2006), the involvement of TGF- β signaling in triggering *Mmp1* expression was not known. Here we present *in vivo* genetic evidence of an otherwise complex regulatory mechanism that involves cross talk be-

tween Dpp (a member of the TGF- β family of proteins) and JNK signaling in the transcriptional activation of *Mmp1* in a Mad- (the fly homolog of Smad) independent manner. Of note, involvement of the TGF- β -TAK1 signaling cascade in activating mammalian MMP-2 has also been demonstrated in preneoplastic MCF10A human breast epithelial cells (Sano *et al.* 1999; Kim *et al.* 2007), providing evidence for the evolutionary conservation of this signaling module.

Aberrant activation of the Sonic hedgehog (Shh; the vertebrate counterpart of Hh) signaling pathway has been implicated in initiation, progression, and metastatic invasion of multiple cancer types (Evangelista *et al.* 2006; Song *et al.* 2016). While deregulated Shh signaling upregulates levels of different components of cell-cycle machinery leading to unrestricted proliferation of cancer cells (Kenney and Rowitch 2000; Mille *et al.* 2014), Shh signaling also promotes metastasis through induced expression of MMP-2 and MMP-9 (Wang *et al.* 2015). MMPs, on the other hand, play a dual role in tumor growth and metastasis processes. Although numerous studies have demonstrated tumor-specific upregulation of MMPs together with their involvement in tumor invasion and metastasis in various cancer types, growing evidence suggests that some MMPs also have tumor-inhibiting functions in a context-dependent manner (Kessenbrock *et al.* 2010; Gialeli *et al.* 2011). It would be really intriguing to find, in the future, whether mammalian MMPs play any role in limiting Shh signaling, as demonstrated in this study, to limit uncontrolled proliferation and cancer progression.

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