Inhibition of Drosophila Wg Signaling Involves Competition between Mad and Armadillo/β-Catenin for dTcf Binding

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Abstract

Precisely regulated signal transduction pathways are crucial for the regulation of developmental events and prevention of tumorigenesis. Both the Transforming Growth Factor β (TGFβ)/Bone morphogenetic protein (BMP) and Wnt/Wingless (Wg) pathways play essential roles in organismal patterning and growth, and their deregulation can lead to cancers. We describe a mechanism of interaction between Drosophila Wg and BMP signaling in which Wg target gene expression is antagonized by BMP signaling. In vivo, high levels of both an activated BMP receptor and the BMP effector Mad can inhibit the expression of Wg target genes. Conversely, loss of mad can induce Wg target gene expression. In addition, we find that ectopic expression in vivo of the Wg transcription factor dTcf is able to suppress the inhibitory effect caused by ectopic Mad. In vitro binding studies revealed competition for dTcf binding between Mad and the Wnt effector β-catenin/Armadillo (Arm). Our in vivo genetic analyses and target gene studies support a mechanism consistent with the in vitro binding and competition studies, namely that BMP pathway components can repress Wg target gene expression by influencing the binding of Arm and dTcf.

Introduction

Wnt/Wingless (Wg) and TGFβ/BMP are two distinct families of secreted ligands that employ different signaling components to exert their biological effects. These pathways play essential roles in the growth and patterning of most tissues across species from worms to humans (reviewed in [1,2]). Therefore, any insight that can be gained into their interactions may reveal important mechanisms of regulation that participate in fine-tuning signaling and achieving proper differentiation. In addition, mutations in components of these pathways can have dire consequences for organismal development and survival. Thus, identifying regulatory networks can provide insight into aberrant development and cancers that result when regulation is disrupted.

Wg signaling is mediated by the DNA-binding transcription factor, dTcf, and its co-activator β-catenin/Armadillo (Arm) [3]. In the absence of Wg signaling, cytoplasmic Arm levels are kept low through continuous proteasome-mediated degradation, which is controlled by a complex of Zw3, APC and Axin [4,5,6,7]. Wg signaling results in down-regulation of Zw3 kinase activity, which allows Arm to escape degradation and accumulate in the cytoplasm. Subsequently, Arm proceeds into the nucleus where it forms a complex with dTcf, a member of the lymphoid enhancer factor 1 (Lef)/T-cell factor (Tcf) family of transcription factors. Arm and dTcf regulate the transcriptional activation of numerous target genes [3,8,9]. These genes, such as achaete (ac), senseless (sens) and distal-less (dll) organize the D/V boundary of the wing and contribute to adult features such as the wing margin [10,11].

The best-characterized TGFβ ligand in Drosophila is Decapentaplegic (Dpp), a member of the BMP family of ligands (reviewed in [12]). Upon Dpp ligand binding and receptor oligomerization, the type II receptor Punt activates the serine/threonine kinase activity of the type I receptor Thickveins (Tkv). Tkv then phosphorylates the receptor-regulated Smad, Mothers against Dpp (Mad), which then allows it to bind the co-Smad Medea (Med), translocate to the nucleus and bind DNA to activate gene expression.

The Drosophila wing has served as a very amenable tissue in which to dissect the roles of signaling molecules in development. Adult wing patterning is initiated in epithelial cells of the larval imaginal discs. In wing disc development, the Wingless (Wg) member of the Wnt family organizes the dorsal/ventral (D/V) axis, while BMPs are required to pattern the anterior/posterior (A/P) axis (reviewed in [13]). Both pathways regulate the expression of target genes that influence wing shape, size and patterning. Consistent with these diverse roles in the wing, mutations in the two pathways result in distinct adult wing phenotypes. Reduced Wg signaling leads to loss of the entire wing.
blade, or loss of D/V wing margin tissue, resulting in variable notching [14]. Reduced BMP leads to the formation of wings that are typically larger than wild-type and have vein patterning defects (reviewed in [15]).

Crosstalk between Wnt/Wg and TGFβ/BMP signaling pathways has been extensively described in numerous systems. These interactions involve both cooperation and antagonism between these pathways to modulate gene expression. This cross regulation has generally focused on the role of both pathways in regulation of transcription. It has been previously shown that vertebrate Lef/Tcf proteins can associate with Smads and synergistically activate transcription of Xnx [16,17] and mouse c-My [18]. In Drosophila, the expression of several genes is regulated coordinately by Dpp and Wg, including Ubx and dpp [19,20,21,22,23]. Furthermore, a mutual antagonism between Wg and Dpp pathways that results in transcriptional repression of the two ligands, Wg and Dpp, has been well described in the Drosophila leg imaginal disc. Ectopic Dpp signaling in leg discs leads to reduced Wg ligand expression and phenotypes indicative of loss of Wg signaling. Conversely, ectopic Wg expression leads to a loss of Dpp ligand and phenotype associated with loss of Dpp signaling [24,25,26]. However, it has been noted in numerous studies that a different mechanism exists in the wing disc, as altering the individual pathways does not affect the ligand expression [24,25]. Ectopic Dpp signaling in the wing pouch does not result in changes in Wg expression, yet it can cause phenotypes indicative of loss of Wg signaling, such as wing notching [27].

Another example of their different roles in wing development compared to the leg is seen with the regulation of Dll. In the leg, Dpp signaling can mimic Wg loss of function [28,29], while in the wing is seen with the regulation of Dll. In the leg, Dpp signaling can mimic Wg loss of function [28,29], while in the wing is seen with the regulation of Dll. In cell culture experiments we find that Arm and Mad can compete for association with dTcf. While ectopic expression of dTcf does not enhance the output of the Wg pathway [3], we have found that ectopic expression of core components of the BMP pathway with various Gal4 drivers also causes similar defects and phenocopped loss of Wg signaling. Expression of UAS-Mad with either wg-Gal4 (Fig. 1C) or vg-Gal4 (Fig. 1E) or UAS-Mad with vg-Gal4 (Fig. 1D), or the activating regulator of BMP, UAS-Sara, with vg-Gal4 (Fig. 1G) in wing discs all caused variable notching of the wing. Bennett et al. (2002) also described increased wing notching as a consequence of progressively higher levels of BMP signaling [27]. Others have also found that Mad and Mad induce such phenotypes upon misexpression, and these effects may involve enhanced stabilization of endogenous Mad [32].

To further investigate whether Dpp can influence the signaling capacity of the Wg pathway, genetic interaction studies were undertaken. We observe that co-expression of vg>Sara and Tcf can suppress posterior notches caused by expression of vg>Sara alone (Fig. 1F). Consistently, we found that the vg>Sara-induced notching (Fig. 1G) was enhanced by heterozygosity for dTcf [31] and suppressed by heterozygosity for the Wg inhibitor vg>Mi-1 (uz3/GSK3β) (Fig. 1H). These interactions suggest the vg>Sara-induced notching was due to reduced Wg signaling, and that elevated BMP can inhibit endogenous Wg signaling. This effect is distinct from what is observed in the leg disc (see Introduction) and is not due to the suppression of vg, as ectopic BMP signaling does not affect vg ligand expression in the wing pouch (Fig. 2H) [24,25].

Dpp loss of function has phenotypes associated with Wg gain of function

To further characterize the inhibition of Wg by BMP pathway components, we determined whether dpp loss of function mutants display any phenotypes suggestive of elevated Wg signaling. We found that dpp<Δ/dpp<Δ flies displayed ectopic bristles along the L3 vein with 47% penetrance (n = 122; arrow in Fig. 1K). Ectopic bristles were also seen upon expression of activated UAS-ArmS10 with T93-Gal4 (Figs. 1L) and these are known to be caused by elevated Wg signaling [14,33]. In addition, rare homozygous dpp<Δ flies had tiny wings lacking most vein tissue that displayed patches of ectopic bristles suggesting elevated Wg activity (arrows in Fig. 1N, O).

Wg target gene expression is inhibited by Dpp signaling

We next examined the expression of four Wg targets, nemo (nmo) (Fig. 2A), dill (Fig. 2B), sens (Fig. 2C) and ac (Fig. 2D), in wing discs where the Dpp pathway was activated [10,14,34,35,36]. We wanted to determine whether the observed adult wing phenotypes and genetic interactions reflected changes in Wg target genes. The flip-out clone technique was used to express either UAS-Mad (Fig. 3D, J) or an activated form of the receptor UAS-TkvQD [3A, M, P] in GFP-marked clones [34,37]. We obtained similar results from both transgenes, indicating that in this context, expression of high levels of Mad can lead to high levels of BMP pathway activity. In all cases, flip-out clones showed reduced Wg target gene expression (Fig. 3C, F, L, O, R). Expressing UAS-TkvQD in the dpp expression domain also suppressed Dll protein expression (Fig. 3G–I). Consistent with this data, we observed that surviving adults from flip-out UAS-TkvQD crosses displayed margin notching, confirming that reduction of target gene expression in larval imaginal discs results in Wg loss of function adult phenotypes (Fig. 3S, T).

Reduced BMP signaling leads to elevated Wg signaling

We then sought to demonstrate that an elevation of Wg signaling output is observed upon reduction of BMP signaling. mad<Δ clones were induced in a Minute background and examined for Dll expression. In clones located outside the endogenous Dll domain, in regions of the wing disc exposed to low levels of Wg, a cell autonomous induction of Dll was observed upon loss of mad (Fig. 4B–H). Clones within the endogenous Dll domain did not show elevated
Dll staining, likely due to saturation of Wg signaling within the Dll domain. Furthermore, as described above, the adult wing phenotypes observed after mad^0 clone induction (Fig. 4I, K) closely resemble phenotypes observed with ectopic stabilized Arm (Fig. 4J, L). These observations reveal that in the absence of Mad, Wg target gene expression can be elevated. Thus both increased and decreased Mad signaling can modulate the extent of Wg pathway activity.

In vitro competition affects Wg-dependent gene expression

Our genetic interaction studies suggest an inhibitory interaction in the wing between the signaling effectors of the Wg and BMP pathways. Specifically, elevating the levels of BMP signal through the ectopic expression of Mad or activated Tkv led to diminished expression of Wg targets. Since it has been shown previously in vertebrate as well as Drosophila that members of the Lef/Tcf family of proteins can associate with Smads, we sought to investigate the possibility that sequestering of dTcf by Mad in the wing could lead to a reduction in Wg signaling output.

To further characterize the mechanism of Wg inhibition by BMP signaling, biochemical studies were performed with dTcf, Arm and Mad. Immunoprecipitations (IPs) were performed from HEK293 cells transfected with Drosophila expression constructs. These experiments showed an interaction between Mad and dTcf (Fig. 5A), but not between Mad and Arm (Fig. 5B). Next, Mad and

Figure 1. Modulation of BMP signaling in the wing results in wg-like phenotypes. (A) Wildtype adult wing. (B) Inhibition of Wg signaling by expression of vg>dTcf^DN caused extensive wing notching. Ectopic expression of omb>Mad (C), vg>Medea (D), vg>Mad (E) and the positive regulator of Dpp, Sara, in vg>Sara (G) resulted in wing notching. Co-expression of Mad and dTcf with vg-Gal4 suppressed the wing notches (F). Heterozygosity for dTcf^DN enhanced the vg>Sara notching (H) while heterozygosity for sgg^M1-1 suppressed the notching (I). (J) The wildtype distal portion of the third longitudinal vein (L3). Loss of function transheterozygous alleles of dpp, dpp^Dpp/dpp^vas show ectopic bristles (K) that phenocopy ectopic Wg signaling seen in T93>Arm^16 (L). (M) An enlarged view of a wildtype proximal anterior wing margin, showing the normal pattern of bristles. (N) Wing from a dpp^0 adult that lacks most veins, and displays ectopic bristles (arrow). (O) A higher magnification of the patch of extra bristles (arrow) seen in (N).

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dTcf binding domains were mapped using truncation constructs (Fig. 5C). Mad truncations were made in which the two conserved MH1 and MH2 domains were deleted. The MH1 domain contains the DNA binding domain, while the MH2 domain is involved in protein-protein interactions and transcriptional activation (reviewed in [38]). dTcf can bind full length Mad and MadMH1, but not MadMH2 or Mad-linker, thus dTcf binds the MH2 domain of Mad (Fig. 5D). Mad binds two C-terminal truncations of dTcf, but not a deletion of the HMG domain (Fig. 5E), indicating that Mad binds the DNA-binding HMG domain of dTcf.

To address whether the binding of Mad and dTcf affects the Arm/dTcf complex, protein binding was examined in cells triply transfected with Mad and dTcf and increasing amounts of Arm. dTcf precipitated both Mad and Arm when the Arm amount was relatively low (Fig. 5F, lanes 2 and 3), while increasing amounts of Arm blocked the binding of dTcf and Mad in a dose-sensitive manner (Fig. 5F, lane 4). Reciprocally, cells were transfected with dTcf, Arm and increasing amounts of Mad (Fig. 5G). Mad, dTcf and Arm were co-immunoprecipitated under conditions in which the Mad amount was relatively low (Fig. 5G, lanes 2 and 3), but higher levels of Mad blocked the Arm/dTcf complex (Fig. 5G, lane 4). Since dTcf can bind both Mad and Arm, we examined whether the proteins form a heterotrimeric complex. When lysates from cells expressing all three proteins were immunoprecipitated (IP), a Mad IP (anti T7) failed to pull down Arm (Fig. 5H) and an Arm IP (anti HA) failed to pull down Mad (Fig. 5I), suggesting that the precipitates seen in Fig. 5F, G (lanes 2, 3) represent mutually exclusive complexes of dTcf/Arm and dTcf/Mad.

High levels of Mad can inhibit Wg-dependent gene expression in vitro

To study the effect on transcription of Mad/dTcf binding, the Tcf-responsive Topflash reporter was used (Fig. 5J, K) [39]. Co-transfection of Arm and dTcf abundantly induced Topflash (Fig. 5J, K). Co-transfection with full length Mad caused a dose-sensitive inhibition (Fig. 5J). Transfection of MadΔMH1 or the Mad linker did not inhibit Topflash expression, showing that binding between Mad and dTcf was required for the inhibition (Fig. 5K). MadΔMH1 could inhibit Topflash, but not to the degree that full length Mad could, indicating that some inhibitory function is retained in the MH1 domain. Thus, expression of forms of Mad that can bind dTcf resulted in a decrease in Wg-dependent gene expression.

In vivo competition

To test the hypothesis that excess Mad can saturate dTcf in vivo, Wg target gene expression was monitored in wing discs clones ectopically expressing Mad and dTcf. Our prediction would be that Mad inhibits Wg targets by competing with Arm for dTcf binding. Thus, if excess dTcf is provided, it should alleviate the
repressive effect of Mad and allow dTcf/Arm-driven transcription to proceed. Ectopic dTcf in flip-out clones showed no change in Sens expression (Fig. 6G–I), consistent with the lack of phenotype seen with 

g>

dTcf expression. Ectopic expression of dTcf does not lead to a modulation of transcription as members of the Lef/Tcf family of transcription factors are abundantly expressed and bound to DNA and must rely on association with co-factors to activate gene transcription [reviewed in [1]]. On the other hand, as shown previously in Fig. 3, flip-out Mad clones showed suppressed Sens expression (Fig. 6A–C). Simultaneous expression of dTcf in such clones blocked the inhibition caused by Mad and the normal expression pattern was seen (Fig. 6D–F). Similar results were obtained for the expression of Dll and nmo (data not shown). Thus, enhanced levels of dTcf could suppress the negative effects of ectopic Mad on Wg transcriptional output. These observations strengthen our model in which ectopic Mad competes with dTcf and leads to a reduction in Wg signaling output. By expressing even higher levels of dTcf, we effectively were able to titrate the suppressive effects of elevated Mad protein.

To determine if the effect we observed was specific to Wg target genes, we examined the expression of the Mad target gene spalt major (salm) [40]. Flip-out Mad clones showed ectopic Salm protein (Fig. 6J–O). This gene activation was not suppressed by the simultaneous expression of dTcf (Fig. 6P–R) suggesting that the interaction of Mad and dTcf specifically blocks dTcf-dependent transcription.

Discussion

In this study, we show that Wg-dependent gene expression can be modulated in vivo by elevated BMP signaling due to activated receptor or high levels of Mad. We find that the molecular basis for this effect arises through Mad/dTcf complex formation, which can inhibit the binding of Arm with dTcf and block Wg-dependent gene expression in vitro. We propose that Mad and Arm compete for binding of dTcf, and that ectopic nuclear Mad inhibits Wg signaling through direct binding with dTcf. In support of this model, overexpression of dTcf inhibits Mad-dependent suppression of Wg target gene expression in vivo. Thus elevated Dpp signaling can inhibit Wg signaling both in vitro and in vivo. We also show that loss of BMP signaling can result in elevated Wg target gene expression, suggesting the interaction between the two pathways normally acts to fine-tune the Wg response.
Figure 5. Mad and dTcf form a complex that competes with Arm-dTcf binding and blocks dTcf-dependent gene expression. (A) Binding of Mad and dTcf. pCMV-T7-Mad and pCMV-Myc-dTcf were co-transfected into HEK293 cells. Cell lysates were immunoprecipitated (IP’d) with anti-Myc, anti-T7 or IgG (control). Immunoblotting (IB) was performed with anti-Myc and anti-T7 antibodies. (B) Mad and Arm do not associate directly. pCMV-T7-Mad and pCMV-HA-Arm were co-transfected into HEK293 cells. Cell lysates were IP’d with anti-HA, anti-T7 or IgG (control). IB was performed with anti-HA and anti-T7 antibodies. (C) A schematic map of the dTcf and Mad truncation constructs and indication of their ability to bind the other. (D) dTcf binds the MH2 domain of Mad. HEK293 cells were transfected with Myc-dTcf and the indicated T7-Mad constructs. Cell lysates were IP’d with anti-Myc or IgG (control). IB was performed with anti-Myc and anti-T7 antibodies. (E) Mad interacts with the HMG domain of dTcf. HEK293 cells were transfected with T7-Mad and Myc-dTcf constructs. Cell lysates were IP’d with anti-Myc or IgG (control), IB was performed with anti-HA, anti-T7 or IgG (control). IB was performed with anti-HA, anti-T7 antibodies. (F) Increasing concentrations of Arm can inhibit the Mad/dTcf complex. 1.5 mg of T7-Mad, 1.5 mg of Myc-dTcf and increasing amounts of HA-Arm were co-transfected into HEK293 cells. Cell lysates were IP’d with anti-Myc. IB was performed with anti-HA, anti-Myc and anti-T7 antibodies. (G) High concentrations of Mad can inhibit Arm/dTcf complex formation. 500 ng of HA-Arm, 800 ng of Myc-dTcf and increasing amounts of T7-Mad were co-transfected into HEK293 cells. Cell lysates were IP’d with anti-Myc. IB was performed with anti-
Figure 6. In vivo competition between dTcf and Mad affects Wg target genes. Flip-out clones (positively marked with GFP) were generated to express Mad and/or dTcf. (A–C) Sens expression was suppressed in Mad misexpression clones (arrows in C). (D–F) No reduction of Sens was seen in double flip-out clones expressing Mad and dTcf (arrows in F). (G–I) Flip-out dTcf clones showed no reductions in Sens (arrows in I). (J–L) Flip-out Mad clones induced ectopic expression of the Dpp target Salm. (M–O) Double flip-out clones expressing Mad and dTcf did not show suppression of the ectopic induction of Salm.

Consistent with our findings, Takaesu et al. (2005) describe that expression of a dominant negative human Smad4 construct in Drosophila wings leads to elevated Wg signaling and target gene expression [41]. The molecular mechanism of this interaction is not yet known, but may involve mutant Smad4 titrating endogenous Mad protein, thus mimicking our mad loss of function studies.

We and others [27,32] have shown that ectopic expression of Mad or Med generates wing margin notches, which mimic a loss of Wg phenotype. Adachi-Yamada et al. [1999] reported that overexpression of a constitutively active version of Tkv leads to activation of the JNK apoptotic pathway and the consequence is loss of wing tissue [42]. We cannot exclude the possibility that either vg>Mad or vg>Med may activate the cell death pathway directly. However, the observation that the notchig phenotype can be enhanced by loss of dTcf and rescued by gain of Wg, as seen with the zw3+/− heterozygous mutant, supports the hypothesis that vg>Mad and vg>Med suppress Wg signaling activities, therefore leading to the terminal consequence: apoptosis in the wing margin. Indeed, reduction of Wg signaling leading to activation of the JNK apoptotic pathway has been elegantly illustrated by Giraldez and Cohen [2003][43].

We have shown that both dpp loss of function mutants and mad somatic clones display ectopic bristles phenotypes on the wing blade, a phenotype indicative of elevated Wg signaling. The relatively weak adult phenotype can be explained if Dpp attenuation of Wg signaling plays a fine-tuning role in a specific developmental time window. Likely the damage caused by loss of dpp and mad can be compensated for by later development events. During early larval development, Dpp and Wg execute their global patterning function in organizing the A/P and D/V axes. In this stage, Dpp signaling antagonizes Wg signaling at the A/P-D/V intersection. In the late 3rd instar larval and pupal stages, Wg functions in wing margin organization, which requires repression of dpp expression [44]. Consequently, in this stage Dpp signaling is not active in the wing margin. Therefore, suppression of the Dpp pathway does not lead to a severe loss of the wing margin, and only rare cases of ectopic bristle phenotype were observed in the Dpp signaling mutants. However, overexpression of Dpp signaling in this stage can cause loss of the margin (Figure 1C–F, Fig 3 S, T). In support of the notion, we found that in the early larval stage, Dpp and Wg domains intersect, suggesting a possible crosstalk of the two pathways (Fig 2E–G). In the late 3rd instar, Dpp expression is suppressed when the A/P and D/V boundaries intersect (Fig 2I–K; [44]). Taken together, we postulate that the mechanism of Dpp inhibiting Wg signaling is temporal and likely functions in the early larval stage to fine-tune the global patterning of the wing disc.

Nevertheless, we observe that altering the levels of BMP signaling is sufficient to modify Wg target gene expression. We observed ectopic Dll in mad mutant clones, and suppression of Dll, nmo, Sens, and Ac expression upon overexpression of mad and in activated tkv flip-out clones.

Several studies in vertebrate have shown that association between Smad and Lef/Tcf plays significant roles in controlling certain developmental events. In Xenopus, Smad interacts with Lef1 to synergistically activate Xtwn transcription [16,17]. In mice, a Smad- Lef/Tcf complex is implicated in transcription of c-myc, Emx2, MSX2, and gastrin [18,45,46,47]. These studies suggest a general molecular mechanism that not only is the Smad-Lef/Tcf complex required, but also the promoter specific cis-elements are needed for the synergistic activation of the target genes. For example, activation of Xtwn requires Smad3 binding to the Smad binding elements (SBEs); c-myc activation needs Smad4 binding to the Lef/Tcf binding elements 1 (TBE1). Our studies in the fly wing unveil a novel mechanism of interaction of the Smad-Tcf complex, in which it exerts an antagonistic role on Wg target gene expression, both in vivo and in vitro. The antagonism is cis-element independent, as evidenced by the finding that expression of Mad in vitro can inhibit Tcf-response in Topflash assays, where reporter gene expression is controlled solely by Tcf binding sites. These in vitro findings are consistent with the modified levels of targets we observe in the wing, namely Dll, nmo, Sens and Ac. Our
biochemical studies suggest a more general molecular mechanism for the Smad-Lef/Tcf interaction in the wing, in which Mad and Arm compete for the binding of dTcf. Excessive Mad can inhibit the association of Arm/dTcf in vitro. The suppression of Wg target genes by ectopic Mad can be rescued by replenishing the dTcf pool (co-expression of dTcf in zebrafish). Although a different molecular mechanism is proposed here, the binding domains between Smad and Tcf are conserved between Drosophila and vertebrate. Similar to the vertebrate study [16], our study indicates that the MH2 domain of Mad associates with the HMG domain of dTcf. Previous research showed that the amino terminus of dTcf binds to Arm [3]. The fact that Mad and Arm interact with different domain of dTcf independently would not exclude the possibility of competition for binding, due to conformational changes upon protein/protein interaction. It is intriguing to speculate that such a Smad-Lef/Tcf antagonism is also conserved in vertebrates.

Distinct tissue-specific mechanisms of interaction between Wg and BMP signaling have evolved. For example, in contrast to the mutual repression of Wg and BMP seen in leg discs, elevated Dpp and BMP signaling have evolved. For example, in contrast to the mutual repression of Wg and BMP signaling in the wing pouch through competition between Mad and Arm for dTcf binding, we propose that the different expression domains, tissue specific regulators and temporal patterns of activation will determine the specificity of the different modes of regulation. The interaction we observe in the wing represents a novel mechanism of interaction between Wg and BMP signaling and highlights the importance of cross regulation of signaling pathways during development.

Materials and Methods

Fly strains

The following fly strains were used: nmo-lacZ (nmoISO) [43], UAS-lacZ, UAS-Mad, UAS-Med, UAS-Tks [37], UAS-Sara [1]. This form of Sara leads to elevated signaling by blocking receptor downregulation [27], ey-Gal4 (expressed in the wing pouch), Chip-Gal4 (expressed along the D/V boundary), T93-Gal4, UAS-Amt [49], UAS-dTcf, dTcf and UAS-dTcfAN [3], Ay-Gal4:25-145, UAS-

GFP, S63T, dppIE; dppIE, sggtM1d and madSO [50].

Flip-out clones, somatic loss of function clones and antibody staining

Flip-out ectopic expression cloning and staining were generated as described in [34,51]. For each genotype, at least 30 clones were examined. Somatic mad clones were generated by crossing hsflp.22/Y; M(2)21AB 1 GFP FRT40A/CyO females. Embryos were collected for 24 hours and heat-shocked at 38°C for 90 minutes at 72-96 hours after egg laying. madSO clones located outside of the endogenous Dll domain were examined and ectopic Dll was observed in 39% (n = 33).

The following antibody dilutions were used: rabbit anti-β-βgal (1:2000), rat anti-Dll (1:500), mouse anti-Dll (1:400) [52], mouse anti-Ac (1:50), rabbit anti-GFP (1:1000), rabbit anti-Salm (1:600) and guinea pig anti-Sens (1:1500), anti-Wg (1:100).

Expression vectors

pCMV-HA-Arm and pCMV-Myc-dTcf were generated by D. Bessette (D.B. and E.M.V., unpublished), dTcf constructs were generated in pCMV-Myc. The dTcfAC1 construct encodes amino acids (aa) 1–522 (full length dTcf is 751 aa); dTcfAC encodes aa 1–394; dTcfAMHG encodes aa 1–244. Mad constructs were generated from pCMV-T7-Mad [46]. MadAMH1 encodes aa 157–455; MadAMH2 encodes aa 1–256; Mad linker encodes aa 157–256. More details on construct generation can be supplied upon request. Co-IPs were performed using standard protocols.

Topflash reporter assay

HEK293 cells were cultured in 6 well plates and transiently transfected by using Polyfect (Qiagen). The renilla luciferase pRL-CMV served as an internal control. Transfections contained 1 mg of pTOPFLASH reporter, 0.1 mg of pRL-CMV and others as described in Figs. 3J. K. pCMV empty vector was used to add to a total of 1.15 mg per well. Luciferase assays were performed with the Dual Luciferase Reporter assay system (Promega) according to the manufacturer’s instructions and as described in Korinek et al. (1997). Each experimental condition was examined three times and the results were standardized against the internal controls.

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Author Contributions

Conceived and designed the experiments: YAZ MR SW WL EMV. Performed the experiments: YAZ MR SW WL EMV. Analyzed the data: YAZ MR SW WL EMV. Wrote the paper: YAZ MR EMV.
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