R-Smad Competition Controls Activin Receptor Output in Drosophila

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Abstract
Animals use TGF-β superfamily signal transduction pathways during development and tissue maintenance. The superfamily has traditionally been divided into TGF-β/Activin and BMP branches based on relationships between ligands, receptors, and R-Smads. Several previous reports have shown that, in cell culture systems, “BMP-specific” Smads can be phosphorylated in response to TGF-β/Activin pathway activation. Using Drosophila cell culture as well as in vivo assays, we find that Baboon, the Drosophila TGF-β/Activin-specific Type I receptor, can phosphorylate Mad, the BMP-specific R-Smad, in addition to its normal substrate, dSmad2. The Baboon-Mad activation appears direct because it occurs in the absence of canonical BMP Type I receptors. Wing phenotypes generated by Baboon gain-of-function require Mad, and are partially suppressed by over-expression of dSmad2. In the larval wing disc, activated Baboon cell-autonomously causes C-terminal Mad phosphorylation, but only when endogenous dSmad2 protein is depleted. The Baboon-Mad relationship is thus controlled by dSmad2 levels. Elevated P-Mad is seen in several tissues of dSmad2 protein-null mutant larvae, and these levels are normalized in dsmad2; baboon double mutants, indicating that the cross-talk reaction and Smad competition occur with endogenous levels of signaling components in vivo. In addition, we find that high levels of Activin signaling cause substantial turnover in dSmad2 protein, providing a potential cross-pathway signal-switching mechanism. We propose that the dual activity of TGF-β/Activin receptors is an ancient feature, and we discuss several ways this activity can modulate TGF-β signaling output.

Introduction
TGF-β superfamily signaling controls a multitude of metazoan cellular behaviors. Much of the signaling activity can be explained by the canonical Smad pathway, wherein extracellular ligands direct formation of active receptor complexes that phosphorylate and activate R-Smad transcription factors [1]. Multiple levels of regulation are known, ranging from extracellular ligand processing to diverse post-translational modification of Smad proteins [2–3]. In addition, a number of Smad-independent signaling pathways have also been demonstrated for various receptors and in different cell types [4].

Two functional subfamilies in TGF-β signaling are generally recognized based on genetic phenotypes and molecular studies: the TGF-β/Activin branch and the BMP branch. Phylogenetic analysis indicates that the TGF-β and BMP branches are present in all metazoans [5], suggesting that a robust TGF-β signaling network is an ancient feature of multicellular animals. The key difference between the two branches is that they activate different classes of R-Smads and thus induce different transcriptional responses in target cells. The TGF-β/Activin ligands work through Smad2/3 (dSmad2 in Drosophila) and BMP ligands work through Smad1/5/8 (Mad in Drosophila). These selective outputs are orchestrated by the Type I receptors, which bind to specific extracellular ligands and phosphorylate specific subsets of R-Smads.

Although genetic and molecular biology studies have consistently indicated that TGF-β/Activin and BMP signals use different R-Smads, there have been several reports of activation of BMP R-Smads by TGF-β ligands. This cross-talk activity was observed in several types of mammalian cells, but conflicting mechanisms were put forth on how this is achieved. Goumans et al. [6] described phosphorylation of BMP R-Smads upon TGF-β ligand exposure in human endothelial cells, and they concluded that TGF-β and BMP Type I receptors are both required for the reaction. Another report suggested that TGF-β phosphorylation of BMP R-Smads in...
human epithelial cells is accomplished by a BMP receptor [7]. In that model, the receptor signaling complex again includes two Type I receptors from different signaling branches: a TGF-β/Activin receptor that both acts as a scaffold for the ligand and phosphorylates TGF-β/Activin R-Smads, and a BMP receptor that phosphorylates BMP R-Smads. However, two other studies demonstrated that a human TGF-β/Activin receptor can phosphorylate BMP R-Smads directly, eliminating any role for a BMP receptor in some cell types [8–9]. Activin-to-BMP cross-talk in Drosophila cell culture has also been reported [10], but the receptor directly responsible for the Activin-stimulated phosphorylation of Mad, the mechanisms by which this phosphorylation are regulated, and whether such cross-pathway signaling occurs in vivo at endogenous levels of the various signaling components are not known.

In this report, we further explore these issues using both cell culture and in vivo methods. We conclude that phosphorylation of the Drosophila BMP R-Smad, Mad, by Baboon, encoded by the only Type I TGF-β/Activin family receptor, is direct and is an evolutionarily conserved signaling event. Furthermore, we find that competition between R-Smads has a profound influence on Baboon-to-Mad phosphorylation activity and that R-Smad pathway switching can be promoted by loss of dSmad2.

Results

Activation of Baboon by mutation or by ligand stimulation leads to phosphorylation of both Mad and dSmad2

In order to define the molecular nature of cross-talk, we employed a cell-based signaling assay that we have used to study other aspects of TGF-β signaling [11]. Expression of a constitutively active form of Baboon (Babo*) in Drosophila S2 cells resulted in phosphorylation of both the Activin and BMP R-Smads, dSmad2 and Mad (Figure 1A). To ensure that this phosphorylation of Mad is a property of the Baboon receptor itself and is not due to an artifact of the activating mutation in Babo*, we exposed S2 cells to the Activin-like ligand Dawdle (Daw), which signals through Baboon [12]. These cells, which express only endogenous Baboon, also responded to Daw by robustly phosphorylating both dSmad2 and Mad (Figure 1A). These results demonstrate that an Activin ligand can stimulate the BMP pathway in Drosophila S2 cells.

Mad phosphorylation by Baboon is independent of BMP Type I receptors

As described above, a key mechanistic question is whether Baboon can directly phosphorylate Mad or if the traditional BMP receptors are involved. We addressed this point by conducting the signaling assay in cells lacking specific TGF-β/family receptors due to experimental RNA interference (RNAi). Similar to data shown in Figure 1A, control cells whose receptors were not targeted by RNAi accumulated phosphorylated Mad in response to the BMP-type ligand Decapentaplegic (Dpp) or the Activin-type ligand Daw (Figure 1B). Reducing expression of Punt, the only Type II BMP receptor expressed in S2 cells, by RNAi eliminated the response to Dpp and Sax BMP Type I receptors (B, right half). In contrast, Daw signaling required the Punt Type II receptor and the Tkv and Sax BMP Type I receptors (B, right half). In contrast, Daw signaling to Mad required Baboon and Punt, but not Tkv and Sax.

 Saxophone and Thickveins RNAi treatments were validated for their ability to essentially eliminate expression of these proteins (see Figure S1). These results indicate that 1) BMP Type I receptors are neither necessary nor sufficient for Activin-induced phosphorylation of Mad in S2 cells, and 2) Baboon is the lone Drosophila Type I receptor that is necessary and sufficient for Activin-to-BMP cross-talk.

Cross-talk is a conserved property of TGF-β receptor intracellular domains

Recent work has uncovered functional differences among isoforms of the sole Activin receptor in Drosophila. The three isoforms have divergent extracellular domains and identical kinase domains, and only one isoform, Babo, is activated by the Daw ligand in S2 cells [12]. We wondered if the isoform differences might influence the ability to phosphorylate Mad, perhaps by directing different receptor complexes to form via extracellular interactions. We tested whether the other isoforms of Baboon, Babo and Babos, were also able to stimulate cross-talk. When over-expressed in S2 cells, both of these isoforms displayed activity in the absence of exogenous ligand, and induced phosphorylation of both dSmad2 and Mad (Figure 2A). This result is consistent with studies suggesting that the kinase domain’s L45 loop—a portion of the protein identical in Baboon isoforms—is primarily responsible for substrate recognition [13]. Extracellular differences among isoforms do not control cross-talk activity, supporting the
smallest model of direct interaction between Mad and the kinase domain of Baboon.

Many reports have demonstrated that members of the same TGFB-family pathway are interchangeable across species. For example, expression of a human BMP ligand can rescue mutation in Drosophila BMP. When transfected into S2 cells, the constitutively active forms of mammalian Activin receptors Alk4 and Alk7 (Alk4* and Alk7*), like Babo*, induce phosphorylation of dSmad2 and Mad in S2 cells. Arrowhead indicates that the displayed image contains two portions of one blot. (B) Constitutively active versions of mammalian Activin receptors Alk4 and Alk7 (Alk4* and Alk7*), like Babo*, induce phosphorylation of Drosophila Mad in S2 cells.

dSmad2 directly influences Baboon’s phosphorylation of Mad

In support of this concept, we found that over-expression of dSmad2 suppressed the blistered wing phenotype of Babo* (Figure 3G), whereas dSmad2 over-expression alone did not affect wing size or vein formation (Figure 3D). This result rules out a general influence of dSmad2 on Mad activity and instead indicates that dSmad2 dosage specifically modifies the substrate choice of Baboon.

An in vivo Baboon gain-of-function phenotype is primarily mediated through Mad and not dSmad2

Given the demonstrated ability of Baboon to phosphorylate Mad and dSmad2 in cell culture, we looked for evidence that Baboon can signal through Mad in vivo. Previous studies have shown that moderate expression of Babo* in developing wings can generate enlarged but normally patterned wings [15]. We found that Babo* expression in the imaginal wing using the vg-GAL4 driver led to visible adult wing defects (Figure 3B, C). To determine whether activation of Mad or dSmad2 caused the phenotype, we used RNAi to remove one R-Smad at a time in S2 cells. We were curious if transfection of the constitutively active forms of mammalian Activin receptors Alk4 and Alk7, like Babo*, could induce phosphorylation of both dSmad2 and Mad (Figure 2B and not shown). This interaction highlights the strong evolutionary conservation of the described cross-talk phenomenon.

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studies comparing vertebrate TGF-β/Activin Smad2/3 and BMP Smad1/5/8 identified two residues in the L3 loop of the MH2 domain as key for targeting different Smads to the L45 loop of either TGF-β/Activin or BMP receptors [18]. We changed the R446 and T449 residues of dSmad2 to H and D, respectively, to give dSmad2 a Mad-like L3 loop. We also made a variant of dSmad2 that harbors the E300K substitution in the MH2 domain found in the dSmad2MB388 allele [19].

We compared the relative phosphorylation levels of wildtype (WT) and mutant forms of dSmad2 versus Mad in response to Baboon. In the experiment shown in Figure 4B, the steady-state P-dSmad2(WT) signal was greater than the P-Mad signal, and the E300K and HD variants had reduced P-dSmad2. Importantly, the P-Mad signal increased as the P-dSmad2 signal decreased, which is consistent with the substrate competition model. This behavior was confirmed in a Drosophila time-course experiment for dSmad2(WT) and dSmad2(HD). During the first hours of signaling, phosphorylation of the HD variant was dramatically reduced compared to WT. P-Mad accumulation, modest over this time range when forced to compete with dSmad2(WT), was enhanced when pitted against the less effective dSmad2(HD) protein (Figure 4B).

dSmad2 restricts Baboon-Mad cross-talk activity in vivo

These signaling assays in S2 cells demonstrate that the Baboon kinase can use Mad as a substrate and that dSmad2 levels modulate the phosphorylation of Mad by Baboon. We next sought to determine if these two phenomena occur in vivo. We chose the wing disc as a platform to manipulate Baboon because the normal P-Mad pattern is well characterized and, as described below, seems to be independent of canonical dSmad2-Baboon signaling. Wing discs from third-instar larvae have a well-defined pattern of P-Mad that critically depends on the Dpp ligand and the BMP Type I and Type II receptors Tkv and Punt, respectively. baboon is expressed in the wing disc, but baboon mutants display a normal P-Mad pattern ([13] and Figure 4E). Similarly, dSmad2 is expressed in discs, but removal of dSmad2 in the wing pouch by RNAi did not significantly alter the P-Mad pattern (Figure 4D, compare to 4C). The lack of an aberrant P-Mad pattern after reduction of either Baboon or dSmad2 is consistent with a lack of appreciable cross-talk activity in the larval wing disc at this developmental time under endogenous conditions.

Against this backdrop, we manipulated Baboon signaling in wing discs and used P-Mad staining patterns as an indicator of activity. When expressed in the L3 wing disc blade using a vg-GAL4 driver, constitutively active Babo* altered the P-Mad pattern. Curiously, the P-Mad signal was reduced in the wing blade, disrupting the normal gradient pattern (Figure 4F). This resembled a loss-of-function condition for Thickveins (compare to Figure 4I) that is likely caused by titration of Punt away from BMP signaling complexes. Indeed, when Punt was co-expressed with Babo*, P-Mad was detected throughout the vg-GAL4 pouch region (Figure 4H). The Punt expression line used for this experiment did not induce ectopic P-Mad in the wing pouch by itself (not shown). It is likely that Babo*-Punt complexes are active towards dSmad2, but we could not directly verify this because no P-dSmad2 immunohistochemistry (IHC) detection assay is available. However, we were able to show that dSmad2 impacts the output of Babo* by simultaneously expressing Babo* and knocking down endogenous dSmad2. This combination produced strong ectopic P-Mad throughout the vg-Gal4 expression domain (Figure 4G). Once again, we interpret these data as reflecting a competition between dSmad2 and Mad proteins for binding to and activation by Baboon. Reinforcing the result seen in S2 cells, this in vivo scenario implicates dSmad2 as the preferred substrate for Baboon since endogenous dSmad2 protein can prevent over-expressed Baboon from phosphorylating Mad.

We also tested if Babo* phosphorylation of Mad can occur independently of the relevant BMP Type I receptor in the wing disc. Simultaneous Babo* expression and dSmad2 RNAi led to ectopic P-Mad even with effective knockdown of dSmad2 (Figure 4K).
Figure 4. Baboon phosphorylation of Mad is inhibited by dSmad2. (A) Phospho-Smad accumulation upon exposure to Daw ligand. S2 cells transfected with Flag-Mad were treated with dsRNA for dSmad2 or GFP, and transfected with Flag-dSmad2 as indicated. Phospho-Smad and Flag signals were assayed on samples before Daw exposure and after 1 and 3 hours of incubation. Note that there is a gel artifact affecting the appearance of the Flag bands in several lanes. Quantified P-Mad band intensities are plotted to illustrate that accumulation of P-Mad depends on the level of dSmad2. The experiment was repeated with several batches of reporter cells, and the relative signals for the 3 hour time point were always observed in the same order. The 1 hour time points were near background detection and are less reliable due to noise. (B) Mutated dSmad2 proteins with varying phosphorylation efficiency modulate the rate of P-Mad accumulation. Babo* stimulation revealed the steady-state levels of P-dSmad2 and P-Mad. Daw exposure for 1 or 4 hours showed the difference in response to short-term signaling between the WT and HD forms of dSmad2. In both conditions, P-Mad levels were inversely correlated to P-dSmad2 levels. (C–K) Wing imaginal discs from third instar larvae were stained to detect P-Mad.
and imaged by confocal microscopy. For each condition at least three discs were imaged, and a representative Maximal Intensity Projection encompassing the wing blade is shown (6 sections @ 3 micron interval for C,D,F–H; all sections @ 3 micron interval for E; 5 sections @ 2 micron interval for I–K). Anterior is to the left and the scale bar shown in panel C applies to C–K. The normal P-Mad staining pattern is shown for vg-GAL4 alone (C, scale bar = 100 microns). Expression of a UAS-dSmad2 RNAi construct did not alter the P-Mad pattern or the shape of the wing disc (D). Wing discs from babo* and dSmad2 heterozygotes showed nearly normal pMad gradient (E). Expression of Babo* altered P-Mad staining, obliterating the normal gradient in the pouch (F). Note the normal P-Mad staining outside of the pouch where vg-GAL4 is not expressed. Babo* and dSmad2 RNAi together generated ectopic P-Mad in the entire wing pouch (G). Providing Babo* with additional Punt also produced ectopic P-Mad (H). tkv RNAi prevented P-Mad accumulation in the middle of the wing pouch (I), and addition of Babo* did not counteract this P-Mad pattern (J). Additional knockdown of dSmad2 led to ectopic P-Mad (K), which paralleled the results without tkv RNAi. Data in panels C, D, F, and G were from the same experiment and were stained in parallel. Panel H is from a different experiment, but the pouch signals can be compared to the others because the endogenous P-Mad along the posterior margin has similar staining. Samples in panels I–K were stained and processed in parallel.

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In concordance with our cell culture studies, this result indicates direct Baboon to Mad cross-talk in vivo.

Dosage effects at endogenous levels: P-Mad levels increase in some tissues of dSmad2 protein-null animals

Given the observation that the presence of dSmad2 in the wing disc profoundly alters the activity of exogenous Babo*, we asked if a similar effect is observed with endogenous levels of TGF-β signaling components. To do this, we required a protein-null condition to preclude competition between Mad and dSmad2 binding to Baboon. The only available dSmad2 mutant allele, dSmad2MB388, harbors a mis-sense mutation near the beginning of the MH2 domain. This mutation does not prevent interaction with Baboon, as the corresponding E300K protein was robustly phosphorylated in S2 cells by Babo* (Figure 4B). To generate a protein null allele, we excised P(G0348), a white-marked P element located approximately 400 nt downstream of the dSmad2 transcriptional start, and screened for white lethal progeny (see Materials and Methods). Among several candidates, one allele, dSmad2M12, was a deletion of the entire coding sequence (Figure 5A).

We next surveyed P-Mad levels by IHC in various tissues derived from dSmad2 mutant larvae. We observed that many, but not all, tissues displayed greater P-Mad staining in the dSmad2 mutant compared to control animals reared under the same conditions. For example, nuclear P-Mad in the fat body was weakly detected in control animals, but markedly elevated in dSmad2 mutants (Figure 5B1 vs FB2). A similar increase was seen at various positions along the gut tract: P-Mad staining was elevated in the gastric caeca, the midgut/hindgut junction, and Malpighian tubules compared to equivalently stained control larvae (examples in Figure 5C and 5D, and summary in Figure S3).

We next tested the influence of the Baboon receptor on P-Mad levels in the fat body and gut. In contrast to dSmad2 mutants, baboon mutant animals had normal P-Mad staining (Figure 5B3, 5C4 and 5D4). The low level of nuclear P-Mad seen in wildtype and baboon mutant gut tissue is presumably due to canonical BMP-family signaling through Thickveins and Saxophone receptors. We next performed an epistasis test in which we assayed P-Mad levels in a dSmad2 mutant; babo double mutant. We reasoned that if the ectopic P-Mad in a dSmad2 protein-null mutant is due to enhanced phosphorylation of Mad by Baboon, then it should be suppressed in the double mutant. We observed this result in all tissues examined (Figure 5B4, 5C5 and 5D5, and Figure S3). Thus, Baboon function is required to produce the elevated P-Mad levels observed in dSmad2 deficient tissues. To explain this phenomenon we conclude simply that Baboon activity is directed towards Mad when dSmad2 is not present in vivo.

Baboon activation causes bulk degradation of dSmad2

During our experiments in S2 cells, we observed that over-expression of activated Baboon significantly lowered the level of co-over-expressed dSmad2 protein. In these experiments S2 cells were transfected with actin promoter expression constructs and were assayed several days later. Thus the dSmad2 protein level detected by Western blot represents the steady-state level, which reflects the production and degradation rates. Protein production rates should be constant in this system, so changes in the steady-state level of dSmad2 can be interpreted as changes in the degradation rate. By this measure, the degradation rate of dSmad2 increased about 5- to 10-fold when Babo* was present (Figure 6A).

We next tested this behavior in several in vivo situations to determine the generality of the bulk degradation of dSmad2 upon Baboon activation. Western blot detection of dSmad2 from embryo extracts showed that co-expression of Babo* lowered the dSmad2 level about 5-fold, compared to dSmad2 expression alone (Figure 6B). A similar reduction of exogenously expressed dSmad2 was seen in the wing blade upon expression of Babo* (compare Figure 6D to 6C). These results confirm that Baboon stimulation lowers dSmad2 stability in vivo. Below we consider how such degradation could alter the output of Baboon signaling in the context of cross-talk.

Discussion

Comparison with cross-pathway signaling in other systems

In this report we present experimental results showing that the Baboon receptor can directly phosphorylate Mad in cell culture and in vivo, and that this cross-talk activity is tightly controlled by the availability of dSmad2. These findings extend the initial report describing canonical signaling between Baboon and dSmad2 where dSmad2, but not Mad, was shown to be a substrate of Baboon in mammalian cells [15]. There are several possible reasons why Baboon-Mad activity is observed in Drosophila cells but was missed in the initial report. First, it is possible that Mad binding to Baboon may be too weak or transient to be detected by immunoprecipitation. Alternatively, endogenous Smad2/3 may have blocked the interaction in heterologous cell systems, or species-specific co-factors may facilitate Mad-Baboon binding.

The strong functional conservation of TGF-β superfamily proteins prompts a comparison of our results in Drosophila with studies describing cross-pathway signaling in mammalian systems. The limited number of pathway members and the efficacy of RNAi in Drosophila enabled us to distinguish between competing models presented for mammalian epithelial cells. With regard to the key mechanistic question of whether TGF-β Type I receptors can directly phosphorylate the BMP-R-Smads, our observation of Mad phosphorylation in the absence of BMP Type I receptors (Figure 1) is inconsistent with the model of heteromic TGF-β/BMP Type I receptor complexes proposed by Daly et al. [7], but is consistent with the model of direct phosphorylation of BMP R-Smads by TGF-β/Activin receptors proposed by Liu et al. [8]. It is possible that both types of mechanisms exist, but are differentially utilized depending on the ligands and receptors present in the
Figure 5. P-Mad elevation in dsmad2 null mutant tissues depends on baboon. The schematic depicts the location of the l(X)G0348 P element insertion in relation to the dsmad2 locus (A). The F4 excision product removed the entire coding region of dsmad2 and portions of the P-element. The genomic breakpoints are indicated above the dsmad2 mRNA; they were determined by sequencing PCR products, indicated by dotted lines. (B–D) P-Mad was detected by IHC of fixed larval tissues from several genotypes. For each image, a merged DAPI (blue) and P-Mad (red) panel is displayed above the isolated P-Mad channel. (B) Single confocal sections of P-Mad staining in the fat body. Under the staining conditions employed, endogenous nuclear P-Mad in a control fat body was barely detected (B1), but was increased in a dsmad2 null mutant animal (B2). Baboon single mutants and dsmad2; baboon double mutants had normal P-Mad staining (B3,4). (C, D) P-Mad staining at two representative positions along the digestive tract. Images are Maximal Intensity Projections of 3 micron interval confocal sections through the entire sample. The P-Mad primary antibody was omitted from “No Ab Control” samples to convey any background staining and auto-fluorescence in the red channel. (C) In the gastric caeca near the proventriculus, dsmad2 mutants showed elevated P-Mad (C3) compared to wildtype control males (C2). baboon single mutants and dsmad2; baboon double mutants showed wildtype levels (C4 and C5). Distal Malpighian tubule staining (lumpy tubes marked with asterisks) showed the same pattern, with the dsmad2 mutant displaying the strongest P-Mad staining (D3 compared to D2, D4 and D5).

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functionality is unknown [20]. A similar point can be made cell culture under over-expression conditions, but their Drosophila situations. Mixed receptor complexes have been detected in particular tissue being examined. Even in Drosophila, we note that direct action of Baboon on Mad does not preclude the possibility that mixed receptors form active signaling complexes in some situations. Mixed receptor complexes have been detected in Drosophila cell culture under over-expression conditions, but their functionality is unknown [20]. A similar point can be made regarding mixed Smad oligomers detected in mammalian epithelial cells by Daly et al. [7]. In our adult wing assay, we found that the Babo* phenotype depended primarily on Mad and that dSmad2 was not required. If the wing development defect was caused by the activity of a complex containing both P-Mad and P-dSmad2, then removal of either one should have blocked the Babo* phenotype. Again, this observation does not argue against the formation or activity of mixed R-Smads complexes in some contexts, but shows that productive signaling by cross-pathway phosphorylation can take place independently of such complexes.

One key observation in this report concerning the mechanism of cross-pathway signal regulation is that the degree to which it occurs, both in cell culture and in vivo, appears to be regulated by competition between the R-Smads, likely for receptor binding. Further work is required to determine how general this mechanism might be. Epithelial cell culture models showed that cross-talk is important for the TGF-β-induced migratory switch [8]. Our results in the larval wing disc and gut represent the first examples of cross-talk in vivo, and we expect that additional examples will be found in various animals and tissues. With regard to developmental studies, other systems should be evaluated to see if loss-of-function mutations of Smad2/3 orthologs lead to increased signaling through BMP R-Smads. Additionally, several human diseases have been attributed to mutations in TGF-β components [21]. A cautionary implication of our work is that mutations in the TGF-β branch may have unanticipated loss- or gain-of-function influences on the BMP branch.

Curiously, TGF-β/Actinin Type I receptors appear to have gained or retained cross-phosphorylation activity throughout evolution, but BMP Type I receptors do not appear to have reciprocal activity. We have never seen phosphorylation of dSmad2 by activated Drosophila Type I BMP receptors (Figure 6A and data not shown), or in response to various ligands. Likewise, no phosphorylation of Smad2/3 by BMP receptors has been reported in vertebrate cells. Why is there this distinction? The growing number of complete genome sequences has allowed phylogenetic reconstruction of the evolution of TGF-β signaling [5]. Apparently all metazoans, even the simple placozoan [22], have a complex TGF-β network containing both TGF-β/Actinin and BMP subfamilies. It is tempting to speculate that a single receptor with dual Smad targets could have played a transitional role in the expansion of the signaling network. The all-or-none nature of the TGF-β-superfamily network (both BMPs and TGF-β/Actins present, or none) in extant organisms, however, does not provide support for this idea. What it does support is the possibility that relationships between core pathway proteins stabilized hundreds of millions of years ago. If the dual-kinase activation of Baboon orthologs has been available to all metazoans during the radiation of animal forms, we would expect this activity to be deployed by different animals and different cell types in diverse ways. We are currently investigating several phenotypes that differ between baboon and dSmad2 mutants to determine which depend on Babo-Mad cross-talk and which might depend on other forms of Smad-independent signaling [23].

**Smad2 degradation as a signaling switch**

The different responses of adult wings and larval imaginal tissue upon Baboon activation (Figures 3 and 4) illustrate that TGF-β pathway wiring and output can vary with developmental context. Given the relationship between Babo and dSmad2, the cross-talk activity can be viewed two ways. From one perspective, the response to loss of dSmad2 depends on the level of Baboon signaling; only cells with Baboon activity can produce P-Mad by cross-talk. From the other perspective, the response of wildtype

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**Figure 6. Hyperactive Baboon signaling leads to dSmad2 destabilization.** The steady-state level of transfected dSmad2 in S2 cells was significantly reduced when Babo* was co-expressed (A). Quantified band intensities are displayed as a percentage of the control for each channel. Note that the P-dSmad2 level was greatly increased but the total dSmad2 level was reduced in the presence of Babo*, causing a greater than 1000-fold increase in the ratio of P-dSmad2 to total Flag-dSmad2. This effect was specific to Babo*, as inclusion of activated Saxophone (Sax*) neither stimulated P-dSmad2 nor significantly decreased the FLAG-dSmad2 signal (A, right lane). FLAG-dSmad2 was expressed in embryos using the da-GAL4 driver, either alone or with Babo* (B). Total exogenous dSmad2 and P-dSmad2 was detected by Western blot and the amount of FLAG-dSmad2 was normalized to endogenous alpha-tubulin. (C, D) vg-GAL4 was used to express Flag-dSmad2 alone (C) or with Babo* (D) in the wing disc. FLAG antibody IHC was used to gauge dSmad2 levels (red), and DAPI (blue) and aPKC IHC (green) are shown as counter-stains. Each image is a single confocal plane through the wing blade, and represents at least three discs stained per genotype. doi:10.1371/journal.pone.0036548.g006
cells to Baboon stimulation depends on dSmad2 levels: efficient cross-talk will only occur in the absence of dSmad2.

Given the dominant control that dSmad2 exerts on the ability of Baboon to phosphorylate Mad, the simplest model is that output of TGF-β ligand stimulus in a given cell depends on the expression level of dSmad2 (Figure 7A). Although dSmad2 is widely expressed, as are Smad2/3 proteins in other animals, different subsets of cells might express different ratios of R-Smads that could influence the signaling output.

Our observation that Baboon activity can lower the overall level of dSmad2 protein offers an additional regulatory possibility, where the TGF-β/Activin signal itself can influence its response. In the substrate-switch model, a cell exposed to a prolonged

Activin signal would eventually degrade enough of its dSmad2 pool to allow Baboon signaling through Mad (Figure 7B). It is not known which tissues, if any, require Baboon-to-Mad signaling in normal developmental contexts. We have observed that in some cases, dSmad2 over-expression leads to mad loss-of-function phenotypes, which could represent disruption of a sensor incorporating dSmad2 concentration as an input and Mad activity as an output (unpublished observations). Proteosomal degradation of activated Smad proteins is a well documented mechanism of signal attenuation [24], but the current observation of bulk degradation adds a new dimension because it can redirect receptor signaling. The mechanism of signal-dependent bulk dSmad2 degradation is unknown, and preliminary experiments do not

Figure 7. Models of signaling outputs generated by dual kinase activity of the Baboon receptor. Given the potential of Baboon to phosphorylate and activate dSmad2 and Mad, we present several configurations of Baboon, dSmad2 and Mad interaction that would generate different signaling outputs. In each schematic, only the most relevant pathway components are drawn. The first model represents a logic gate, where the ability of Baboon to phosphorylate Mad is strictly controlled by the expression of dSmad2 in that cell (A). Another model incorporates feed-back or feed-forward of Mad activity through dSmad2 regulation. If P-Mad positively regulated dSmad2 this would prevent the complete loss of dSmad2. Conversely, if P-Mad negatively regulated dSmad2 expression, this would enforce the switch from Baboon signaling through dSmad2 towards Mad.

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support a simple ubiquitylation-proteosomal pathway (unpublished observations). Regardless of the molecular details, the observed reduction of total dSmad2 available for receptor competition is the key parameter in our competition model. Since activated R-Smad proteins can also be dephosphorylated to rejoin the pool of would-be substrates, the relative rates of recycling and bulk degradation would be predicted to influence the substrate switch to Mad.

What could be gained by the substrate switch? A general answer is that multiple interactions permit diverse regulatory schemes. For example, cross-talk would permit Mad signaling in a cell that is not exposed to BMP ligands or is otherwise not competent to transduce such signals. Other modes of signal integration are intriguing, such as the conditional formation of mixed R-Smad complexes observed by Daly et al. [7]. Another type of regulatory link that could contribute to the evolutionary maintenance of an integrated dSmad2-Babo-Mad triad is depicted in Figure 7C. If dSmad2 transcription was a target for P-Mad, this would affect the substrate switch. In one case, Baboon signaling to Mad would be triggered by dSmad2 depletion and serve to up-regulate Smad2 to return balance to the system. On the other hand, if dSmad2 were down-regulated by P-Mad, this would stabilize the switch to the Baboon-Mad interaction. Additional studies are required to explore these intriguing possibilities.

**Materials and Methods**

**Signaling assays in S2 cells**

The S2 signaling assay has been described [12]. Briefly, conditioned media was collected from cells that expressed a construct encoding Dawdle. This media was mixed 1:1 with cells that had been transfected with a combination of Smads [11] and resuspended in their own media. Conditioned media from cells that were mock transfected was used as a negative control; recombinant Dpp (R&D Systems) was added to conditioned media at a final concentration of 20 nM for BMP stimulation. Mutated forms of dSmad2 (E300K and HD = R446H + T449D) were generated by site-directed mutagenesis and the changes were verified by sequencing. Constructs encoding different isoforms of Baboon are described by Jensen et al. [12]. Expression vectors for Babo* and Sax* are described by Gesualdi and Haerry [10]. Mammalian Alk4* and Alk7* [25,26] were cloned into the pAcPA vector for S2 cell expression.

RNAi treatment of S2 cells was as described [12], except that for Figure 4A dsRNA was added only once after transfection. Primers used to make PCR products for T7 transcription templates against babo, sax, and punt are described by Shimmi and O'Connor [27] and GFP dsRNA was used as a negative control [28]. Additional primers for RNAi are as follows:

\[
\begin{align*}
\text{dSmad2 forward} &- \text{TATAAGCCTACTATAGGGAGGACCAATGGTCAGAGAA} \\
\text{dSmad2 reverse} &- \text{TATAAGCCTACTATAGGGAGGACCCACATAGAGACCTC} \\
\text{babo forward} &- \text{TATAAGCCTACTATAGGGAGGACCTGCGTCGGGGTAATCTTCC} \\
\text{babo reverse} &- \text{TATAAGCCTACTATAGGGAGGACGGATAGCCACAAACTCC} \\
\text{dsARL4 forward} &- \text{TATAAGCCTACTATAGGGATGATAGCCACAAACTCC} \\
\text{dsARL4 reverse} &- \text{TATAAGCCTACTATAGGGATTTAGGGTTTGCTGCGCTGGG.}
\end{align*}
\]

Cells for microtubule disruption tests were exposed to a final concentration of 2 μg/mL colcemid in their own media for 2 hours prior to signaling assays. Control samples were treated similarly, but without drug pre-treatment. After the signaling assay, cells were pelleted, resuspended in 1 x sample buffer, and boiled. Samples were resolved on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and transferred to PVDF or nitrocellulose (Figure 4A) membrane after electrophoresis. Membranes were probed with primary antibodies against C-terminally phosphorylated Mad (1:1000, gift from E. Leof), phosphorylated dSmad2 (1:1000, Cell Signaling #3108), the FLAG epitope (1:2000, Sigma M2), or Saxophone (1:1000, Fabgrenix). Membranes were exposed to secondary antibodies (Rockland (IRDye)) and imaged on the Odyssey Infrared Imaging system. To obtain fold change of degradation rates of FLAG-dSmad2 under steady-state conditions, we quantified the bands and calculated the ratio of unstimulated to Babo*-stimulated FLAG-dSmad2. This simple formula applies if production is constant and degradation is assumed to be a first order reaction.

**Fly lines**

The vg-GAL4 driver (Bloomington stock #9229) was used to drive expression of several UAS constructs in the developing wing, and dv-GAL4 (Bloomington stock #5460) was used to drive expression in the embryo. UAS-Babo* lines have been reported previously [15], as has the UAS-FLAG-dSmad2 overexpression line [29]. dSmad2#15310 and babo#1242 are described in Zheng et al. [19]. UAS-punt is described in Marquès et al. [30].

New RNAi lines were made using the pUAST-R57 vector (National Institute of Genetics, Japan) and included gene-specific sequences spanning bases 1264–1700 of the mad transcript (U0328; insertion line 6C3), 1469–1928 of the dSmad2 transcript (AF101386; insertion line 25A3 or 3B3), and 926–1461 of the tkv transcript (NM_175975; recombinant line 38D2, 1A2). P-element-mediated insertions were made and mapped using standard techniques.

**Larval IHC and tissue handling**

Tissues from wandering third-instar larvae were fixed in 3.7% formaldehyde in PBS for 25 minutes, washed in PBS-Trition (0.1%), treated with primary antibody overnight at 4°C, exposed to secondary antibodies (Alexa Fluor 488 or 568; 1:200) at room temperature for two hours, and counterstained with DAPI. The P-Mad antibody (gift from E. Leof) was affinity purified against a peptide corresponding to the di-phosphorylated C-terminus of Mad. D2 monoclonal antibody (Sigma) was used to detect FLAG-tagged proteins. Anti-PKCGs (rabbit polyclonal, Santa Cruz) was used to visualize cell membranes for Figure 6. Confocal images were collected using a Zeiss Axiovert microscope with a CARV attachment, or with a Zeiss LSM710. The images in Figure 4C–H and 6C,D were collected using a 20×0.75NA objective (CARV). The images in Figure 5C,D were collected using a 10×0.45NA objective (CARV). The images in Figure 4I–K and Figure 5B were collected using a 20×0.80 NA objective (LSM). Where indicated in Figure Legends, Maximum Intensity Projections were generated using Axiovision (CARV images) or ImageJ (LSM images) software.

Wings from adult females were dissected in ethanol and mounted in 1:1 Canada balsam:wintergreen oil under a coverslip for light microscopy using a 4×0.16NA objective. All flies for wing and wing disc studies were reared on standard cornmeal medium at 25°C. Larvae for whole-animal P-Mad IHC were grown on yeast paste agar plates, and mutant animals were identified by the absence of GFP balances. Embryo extracts were identified by the absence of GFP balances. Embryo extracts were
made by boiling dechorionated 6–20 hour old embryos in 2× reducing sample buffer, and the soluble fraction was used for Western blot analysis.

Generation of a null dsmad2 mutant

A new dsmad2 allele was generated by imprecise excision of a P element from stock XG0348 (Flybase allele smox(G0348)), which is located in the ds mad2 5′ UTR. P-element males carrying a duplication (Tp1;2 sn+ 72d) to provide viability were outcrossed, and male progeny lacking the visible eye color marker were recovered and screened for lethality. Lethal chromosomes were analyzed by PCR and sequencing to map the extent of deletion. The d smad2P4 deletion removed a portion of the 5′ UTR (breakpoint at nucleotide 457 of sequence AF101386) and the entire coding region. The 3′ breakpoint was found to be 275 bp downstream of the d smad2 transcript and the deletion does not disrupt the neighboring gene. Several segments of the original P element were left behind (see Figure 5A). Hemizygous d smad2P4 males died as larvae or pupae. The d smad2P4F4 allele was fully rescued to viability and fertility by one copy of a small duplication covering d smad2 (either Dp[1;3]DC185 or Dp[1;3]DC186). An independent excision allele was generated in parallel from a yellow-marked P-element insertion (Flybase allele smox(G01120), using a slightly different strategy (details available upon request to LAR). The d smad2P11 allele from this screen was found to be an internal deletion of the transposable element. Like d smad2P4F4, hemizygous d smad2P11 males died as larvae or pupae and were fully rescued by duplications overlapping d smad2. We chose the F4 allele for our mutant analysis because it is an unambiguous protein null.

Supporting Information

Figure S1 Efficient knockdown of sax and tkv in S2 cells. (A) Cells were transfected with a Sax over-expression (O/E) construct with or without sax RNAi treatment. Western blotting for Sax showed that the RNAi treatment rendered Sax undetectable. (B) A similar test for Tkv showed approximately 98% reduction of overexpressed Tkv-FLAG upon RNAi as detected by FLAG Western blot. In both panels, the loading control is a FLAG cross-reactive band from the same gel lanes. Note that endogenous Sax and Tkv are not detected under these conditions.

Figure S2 Relative phosphorylation of d smad2 and Mad are unaffected by knockdown of SARA or disruption of microtubules. (A) Smad phosphorylation upon Dap treatment in S2 cells without or with dsMrna RNAi. As judged by the FLAG bands (red in merged image, and isolated in bottom slot), the RNAi samples had less Smad per lane, but the relative P-dSmad2 versus P-Mad ratios were similar between control and dsMrna RNAi samples. (B) Colcemid treatment did not affect the preferential activation of d smad2 by Baboon. In this particular experiment pMad stimulation by Dap was weak, but P-Mad was not increased in cells pre-treated with colcemid to depolymerize microtubules. This is in contrast to the increase in P-Mad caused by knockdown of d smad2. Together these suggest that d smad2 delivery to Baboon is not compromised upon microtubule disruption. In both panels, rDpp was included as a control for the ability of the cells to produce P-Mad.

References

1. Shi Y, Massagué J (2003) Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113: 685–700.
2. Shimzu O, Umeda D, Ohtner H, O’Connor MB (2005) Facilitated transport of a Dpp/Sev heterodimer by Sog/Tsg leads to robust patterning of the Drosophila blastoderm embryo. Cell 120: 873–886.
3. Sapkota G, Alcarón C, Spagnoli FM, Brivanlou AH, Massagué J (2007) Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol Cell 25: 441–454.
4. Zhang YE (2009) Non-Smad pathways in TGF-β signaling. Cell Res 19: 128–139.
5. Humíniček L, Goldovsky L, Fredich S, Moustakas A, Ouzounis C, et al. (2009) Emergence, development and diversification of the TGF-β signaling pathway within the animal kingdom. BMC Evol Biol 9: 28.
6. Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, et al. (2003) Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGF-β signaling. J Biol Chem 278: 9753–9763.
7. Wrighton KH, Lin X, Yu PB, Feng XH (2009) Transforming Growth Factor βCan Stimulate Smad1 Phosphorylation Independently of Bone Morphogenic Protein Receptors. J Biol Chem 284: 9763–9763.
8. Liu JM, Schilling SH, Knouse KA, Choy L, Derynck R, et al. (2009) TGF-β-stimulated Smad1/5 phosphorylation requires the ALK5 L45 loop and mediates the pro-migratory TGFβ switch. EMBO J 28: 88–96.
9. Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, et al. (2003) Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGF-β signaling. J Biol Chem 278: 9753–9763.
10. Wu H, Li Z, Sun L, Liang Y, Bao G, et al. (2007) Distinct signaling of Drosophila Activin/TGF-β family members. Fly (Austin) 1: 212–221.
11. Ross JJ, Shimzu O, Vilmos P, Pospykh A, Kim H, et al. (2001) Twisted gastrulation is a conserved extracellular BMP antagonist. Nature 410: 479–483.
12. Jensen PA, Zheng X, Lee T, O’Connor MB (2009) The Drosophila Activin-like ligand Dazdelle signals preferentially through one isoform of the Type-I receptor Baboon. Mech Dev 126: 950–957.
13. Feng XH, Derynck R (1997) A kinase subdomain of transforming growth factor-β (TGF-β) type I receptor determines the TGF-β intracellular signaling specificity. EMBO J 16: 3912–3923.
14. Padgett RW, Wozney JM, Gelbart WM (1993) Human BMP sequences can confer normal dorsal-ventral patterning in the Drosophila embryo. Proc Natl Acad Sci U S A 90: 2905–2909.
15. Brummel T, Abdollah S, Haerry TE, Shimell MJ, Merriam J, et al. (1999) The Drosophila activin receptor baboon signals through dSmad2 and controls cell proliferation but not patterning during larval development. Genes Dev 13: 98–111.

16. Tsukazaki T, Chiang TA, Davison AF, Artisano L, Wrana JL (1998) SARA, a FYVE domain protein that recruits Smad2 to the TGFβ receptor. Cell 95: 779–791.

17. Batut J, Howell M, Hill CS (2007) Kinesin-mediated transport of Smad2 is required for signaling in response to TGF-β ligands. Dev Cell 12: 261–274.

18. Chen YG, Massague J (1999) Smad1 recognition and activation by the ALK1 group of transforming growth factor-β family receptors. J Biol Chem 274: 3672–3677.

19. Zheng X, Wang J, Haerry TE, Wu AY, Martin J, et al. (2003) TGF-β signaling activates steroid hormone receptor expression during neuronal remodeling in the Drosophila brain. Cell 112: 303–315.

20. Haerry TE (2010) The interaction between two TGF-β type I receptors plays important roles in ligand binding, SMAD activation, and gradient formation. Mech Dev 127: 358–370.

21. Wu MY, Hill CS (2009) Tgf-β superfamily signaling in embryonic development and homeostasis. Dev Cell 16: 329–343.

22. Srivastava M, Begovic E, Chapman J, Putnam NH, Hellsten U, et al. (2008) The Trichoplax genome and the nature of placozoans. Nature 454: 953–960.

23. Ng J (2008) TGF-β signals regulate axonal development through distinct Smad-independent mechanisms. Development 135: 4025–4035.

24. Alarcón C, Zaromytidou AI, Xi Q, Gao S, Yu J, et al. (2009) Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-β pathways. Cell 139: 757–769.

25. Nakao A, Imamura T, Soucek W, Kawabata M, Ishiaki A, et al. (1997) TGF-β receptor-mediated signalling through Smad2, Smad3 and Smad1. EMBO J 16: 5353–5362.

26. Jernvall H, Blesijärvi A, ten Dijke P, Ibáñez CF (2001) The orphan receptor serine/threonine kinase ALK7 signals arrest of proliferation and morphological differentiation in a neuronal cell line. J Biol Chem 276: 5140–5146.

27. Shimizu O, O’Connor MB (2003) Physical properties of Tld, Sog, Tsg and Dpp protein interactions are predicted to help create a sharp boundary in Bmp signals during dorsoventral patterning of the Drosophila embryo. Development 130: 4673–4682.

28. Wang L, Brown JL, Cao R, Zhang Y, Kanis JA, et al. (2004) Hierarchical recruitment of polycomb group silencing complexes. Mol Cell 14: 637–646.

29. Ting CY, Herman T, Yonekura S, Gao S, Wang J, et al. (2007) Tiling of r7 axons in the Drosophila visual system is mediated both by transduction of an activin signal to the nucleus and by mutual repulsion. Neuron 56: 793–806.

30. Marques G, Rao H, Haerry TE, Shimell MJ, Duchek P, et al. (2002) The Drosophila BMP type II receptor Wishful Thinking regulates neuromuscular synaptic morphology and function. Neuron 33: 529–543.