THE ACTIVIN LIGAND DAWDLE LINKS DIET AND METABOLISM VIA RECEPTOR ISOFORM-SPECIFIC SIGNALING IN *DROSOPHILA*

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
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FOR THE DEGREE OF
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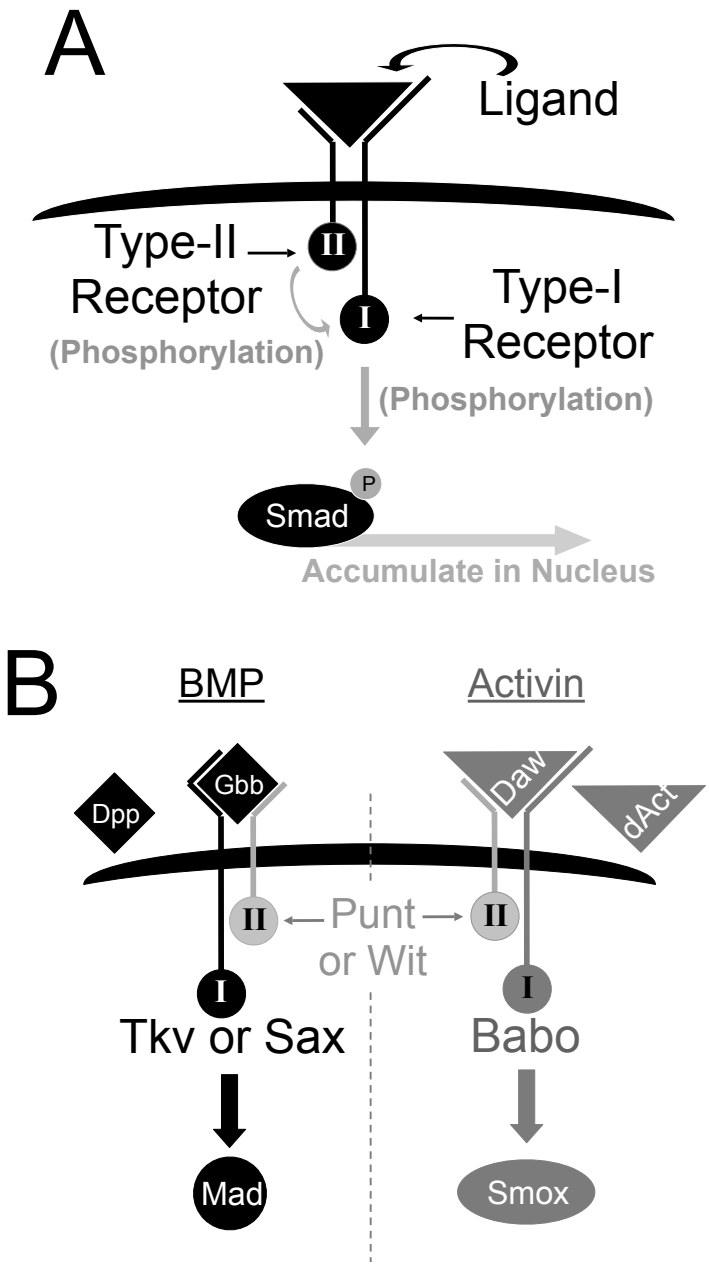
Chapter 1:
The *Drosophila* Activin-like ligand Dawdle signals preferentially through one isoform of the Type-I receptor Baboon.

The TGF-β superfamily of secreted signaling factors represents an ancient communication pathway that is conserved in all animals. TGF-β factors are dimeric ligands that bind to a complex of Type-II and Type-I receptors. After ligand binding, the Type-II receptor phosphorylates and activates the Type-I receptor, enabling it to recognize and phosphorylate an R- (Receptor-) Smad. Phosphorylated R-Smads bind a Co-Smad to form a trimeric complex that accumulates in the nucleus and regulates transcription in association with other cell type-specific factors (see Figure 1.1A and Shi and Massague, 2003).

The TGF-β signaling cascade is conserved throughout the animal kingdom and plays myriad roles within diverse tissues in both development and disease. In the fruit fly *Drosophila melanogaster*, the TGF-ß superfamily is divided into the Bone Morphogenic Protein (BMP) and Activin branches (for schematic of signaling pathways, see Figure 1.1B or Parker et al., 2004). Each branch is thought to have a unique set of ligands, Type-I receptors, and R-Smads, but the two Type-II receptors, Punt and Wishful thinking (Wit), function in both pathways (Zheng et al., 2003). The Type-I receptors Thickveins and Saxophone are BMP-specific receptors that phosphorylate the R-Smad Mad in response to the binding of one of the three BMP ligands: Decapentaplegic (Dpp), Screw (Scw), or Glass bottom boat (Gbb) (Bangi and Wharton, 2006; Shimmi and O'Connor, 2003). In contrast, the Activin pathway has only

Figure 1.1: *Overview of TGF-β Signaling and the BMP and Activin Pathways in* Drosophila. A) TGF-β signaling involves the formation of a complex that contains an extracellular ligand, a Type-II receptor, and a Type-I receptor. After ligand binding, the Type-II receptor phosphorylates the Type-I receptor, which then phosphorylates the intracellular R-Smad protein. Phosphorylated R-Smads then accumulate to the nucleus, where they function as transcription factors. B) The BMP ligands Dpp and Gbb (black diamonds) are capable of forming complexes with the BMP-specific Type-I receptors Tkv and Sax, leading to phosphorylation of Mad, the R-Smad of the BMP pathway. The Activin-family ligands dAct and Daw (gray triangles) can stimulate the Type-I receptor of the Activin pathway, Baboon, which subsequently phosphorylates Smox, the R-Smad in the Activin pathway. Note that the Type-II receptors Punt and Wit (light gray) can function in both the BMP and Activin pathways. The plasma membrane is depicted by a black crescent in both Figures A and B.

Figure 1.1:


A
Ligand
Type-II
Receptor
(Phosphorylation)
II
I
Type-I
Receptor
(Phosphorylation)
P
Smad
Accumulate in Nucleus
B
BMP
Activin
Dpp
Gbb
Daw
dAct
II
Punt
or Wit
II
I
I
Tkv or Sax
Babo
Mad
Smox

one Type-I receptor – Baboon (Babo) – for its four potential ligands: dActivin (dAct), Dawdle (Daw), Myoglianin (Myo), and Maverick (Mav) (Lo and Frasch, 1999; Nguyen et al., 2000; Parker et al., 2007; Serpe and O'Connor, 2006; Zhu et al., 2008). Several of these ligands have been shown to interact with Baboon and either Punt or Wit, leading to phosphorylation of Smox (dSmad2), the Activin R-Smad (Lee-Hoeflich et al., 2005; Parker et al., 2006; Serpe and O'Connor, 2006; Zhu et al., 2008).

Depending on the tissue, the *Drosophila* Activin ligands Dawdle and dActivin act either redundantly or independently to regulate proliferation, axon guidance and axon remodeling (Parker et al., 2006; Serpe and O'Connor, 2006; Zheng et al., 2003; Zhu et al., 2008). Both ligands are thought to signal via the Type-I receptor Baboon, yet the two ligands are not fully interchangeable. For example, overexpressing *daw* in the *Drosophila* wing disc does not phenocopy the large wings that result after similar overexpression of *dAct* (Gesualdi and Haerry, 2007) or an activated form of Baboon (Brummel et al., 1999). Why is the wing tissue sensitive to one Activin-like signal but not to another, even though both molecules apparently signal upstream of the same receptor in other contexts? How does the wing tissue discriminate between the signals? This issue is especially important in vertebrates, where many of the 33 different ligands circulate systemically via the blood but signal using only 7 Type-I receptors. How do different tissues discriminate between the many different ligands that they likely see during development?

One answer to these questions might involve tissue-specific expression of different receptor isoforms that vary in their extracellular ligand-binding domains.

Previously, we described two such isoforms (*babo*$_a$ and *babo*$_b$*)* for the Activin Type-I receptor Baboon (Brummel et al., 1999), although whether they signal with only one or a subset of ligands has not been established. In this study we report the discovery of a third isoform, *babo*$_c$. We demonstrate that in tissue culture Babo$_c$ is the only Type-I receptor capable of transducing a signal from the Activin-like ligand Dawdle, and does so only in the presence of a specific Type-II receptor, Punt. Furthermore, the three *babo* isoforms are differentially expressed in several tissues during larval development. In the wing, for example, expression of *babo*$_c$ is not detected, and wing tissues do not respond to overexpression of *daw* (Gesualdi and Haerry 2007). When we ectopically express *babo*$_c$ and *daw* together in the wing, however, we observe a phenotype that mimics high-level signaling produced by expression of a strong activated Babo receptor. To our knowledge these findings are the first example of Type-I isoform-specific activation by a TGF-β ligand. This signaling specificity, coupled with differential, tissue-specific expression of receptor isoforms, exemplifies a new level of spatial regulation of TGF-β signaling during animal development.

Results

Dawdle signals via the Type-II receptor Punt, but not via the Type-II receptor Wit.

Drosophila S2 cells respond to stimulation by TGF-β ligands in cell-based signaling assays as measured by increased phosphorylation of R-Smads (Ross et al., 2001; Serpe and O'Connor, 2006; Shimmi et al., 2005). Exposure to the Activin-like ligand Dawdle (Daw) results in increased levels of phosphorylated Smox (P-Smox), the

Activin R-Smad, while addition of the BMP 5/6/7 homologue Glass bottom boat (Gbb) results in increased levels of phosphorylated Mad (P-Mad), the BMP R-Smad (Figure 1.2A; McCabe et al., 2003; Serpe and O'Connor, 2006). We sought to elucidate which receptors transduce Daw's signal, starting with the Type-II receptors.

The *Drosophila* genome encodes two TGF-β Type-II receptors: Punt and Wishful thinking (Wit) (Childs et al., 1993; Marques et al., 2002). Punt is the only Type-II receptor expressed in S2 cells (McCabe *et al.*, 2003), and eliminating its expression by adding double-stranded RNA complementary to a portion of its transcript rendered cells incapable of responding to Gbb or Daw (Figure 1.2B). This indicates that Punt is sufficient for mediating Daw signaling. To determine if Daw can also signal via Wit, we again targeted the *punt* transcript by RNAi but also transfected cells with a construct coding for Wit. As shown previously, Gbb was able to signal via Wit (Figure 1.2C; McCabe *et al.*, 2003), but Daw was not able to signal when Wit was the only available Type-II receptor (Figure 1.2C). We conclude that in S2 cells, Punt is necessary and sufficient for Daw signaling, but Wit is neither necessary nor sufficient to transduce the same signal.

Isolation of $babo_c$.

The *Drosophila* Activin receptor Babo has two known isoforms, $Babo_a$ and $Babo_b$, which differ only in the extracellular domain that is critical for ligand binding (Brummel et al., 1999; Wrana et al., 1994). These isoforms appear to be products of differential splicing – each isoform's mRNA transcript contains a unique fourth exon.

Figure 1.2: *Dawdle signals in the presence of Punt, but not Wit.* A) S2 cells with endogenous levels of receptors respond to Gbb and Daw by phosphorylating Mad and Smox, respectively, to high levels. B) S2 cells do not respond to Gbb or Daw when the *punt* transcript is targeted by double-stranded RNA. C) S2 cells respond to Gbb, but not to Daw, when Punt is removed by RNAi and Wit is expressed ectopically. In Figures 1, 3, and 4, phospho-specific antibodies (rabbit) for both species of R-Smad are shown in the green channel, while FLAG antibody (mouse) bound to the Smads' N-terminal epitope tags is depicted in the red channel. Smad-FLAG levels demonstrate loading consistency; though FLAG-Smox (red) bands may be difficult to visualize in the color images, grayscale representations of the blots' red channels are shown in Figure 1.S1, which demonstrates the presence of Smox in all lanes at comparable levels.

Figure 1.2:

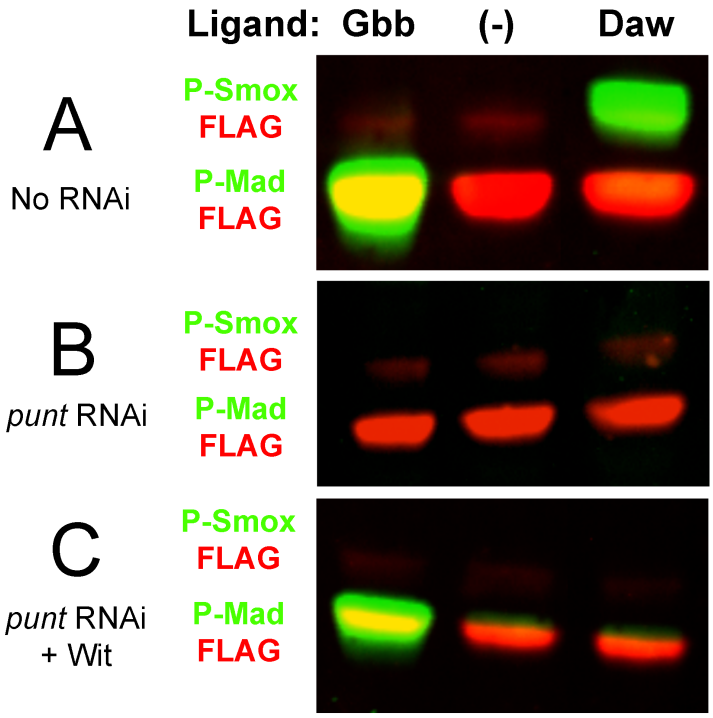
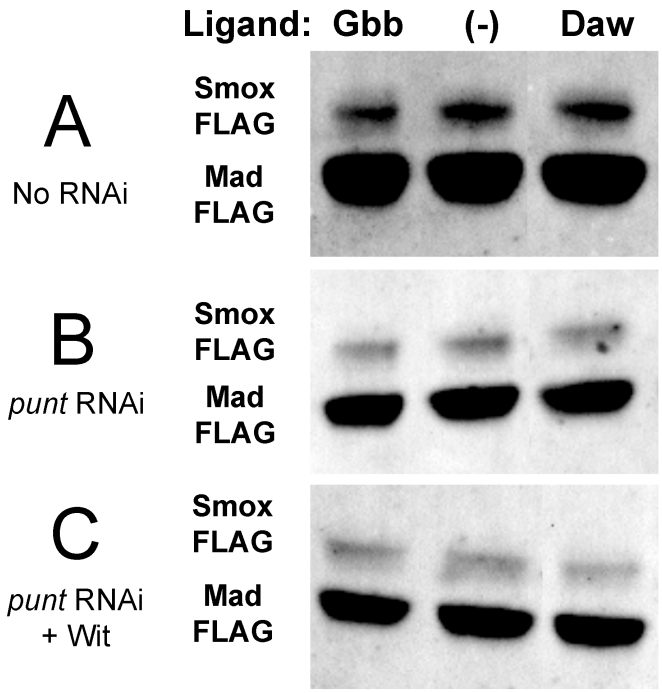

Ligand: Gbb (-) Daw
A
No RNAi
P-Smox
FLAG
P-Mad
FLAG
B
punt RNAi
P-Smox
FLAG
P-Mad
FLAG
C
punt RNAi
+ Wit
P-Smox
FLAG
P-Mad
FLAG

Figure 1.S1: *Grayscale representation of FLAG Western Blots in Figure 1.2.* Because the red SmoxFLAG bands may be difficult to visualize in the full-color images in Figure 1.2, the red channel alone is displayed in grayscale to demonstrate consistent loading between lanes.

Figure 1.S1:


Ligand: Gbb (-) Daw
A
No RNAi
Smox FLAG
Mad FLAG
B
punt RNAi
Smox FLAG
Mad FLAG
C
punt RNAi + Wit
Smox FLAG
Mad FLAG

We discovered another isoform of *baboon*, $babo_c$, with its own unique fourth exon that codes for a novel ligand-binding domain (Figure 1.3). Notably, all three of these isoform-specific exons encode an Asp-Phe-Cys-Asn motif that is typical of sequences found in the previously reported cysteine box region (Wrana *et al*., 1994) (Figure 1.3C). This motif is present in all Type-I receptors across the animal kingdom. Because this D-F-C-N motif is not found in any other reading frame between Exons 3 and 5 in the *baboon* genomic locus, it appears that there are only three *babo* isoforms.

$Baboon_c$ is a necessary Type-I receptor for Dawdle signaling.

We examined if different isoforms of Babo had different abilities to transduce the Daw signal. First, we measured expression of each isoform in S2 cells by RT-PCR. $babo_a$ was expressed at a low level compared to $babo_c$, and the $babo_b$ transcript was not detected (Figure 1.S2). To determine if Daw signaling utilizes a specific Babo isoform, we eliminated expression of individual isoforms by adding isoform (fourth exon) - specific double-stranded RNA to S2 cells. We then exposed the cells to Dawdle and measured phosphorylation of Smox. To make sure that a cell's inability to respond to Daw was due to the loss of the Babo isoform by RNA interference (RNAi) and was not due to the cells' general inability to respond to a TGF-β signal, an aliquot from each batch of cells was exposed to Gbb, which signals through separate BMP Type-I receptors. As shown in Figures 1.4A-H, all batches of cells were able to respond to Gbb as measured by an increase in the levels of P-Mad.

Figure 1.3: *Baboon$_c$ is an isoform with a novel ligand-binding domain.* A) Schematic representation showing which exons encode which portions of the Baboon protein. Isoforms of Babo are identical except for an extracellular region encoded by the fourth exon (gray), which contains the cysteine box domain that is critical for ligand-binding. The transmembrane and intracellular kinase domains of the protein are also shown. Drawing is to scale and represents the Babo$_a$ isoform. B) Schematic representation of the region surrounding the fourth exon(s) of the *baboon* genomic locus: *babo$_a$*, top; *babo$_b$*, middle; and *babo*$_c$, bottom. Alternative splicing leads to inclusion of one isoform-specific fourth exon in the mRNA transcript; unspliced, coding exons are black, and exons that are spliced out of the transcript are gray. C) Divergent amino-acid sequences encoded by the unique fourth exons of each *babo* splice variant. Because each fourth exon encodes a diverged sequence interspersed between four conserved cysteine residues that are involved in disulfide bonds necessary for ligand binding (Kitisin et al., 2007), each isoform likely exhibits a unique Activin ligand-binding spectrum. Cysteine residues are shown in gray, and the horizontal solid bar denotes the cysteine box motif. [Note: *baboon$_c$* was discovered by X. Zheng and T. Lee of the University of Massachusetts Medical School.]

Figure 1.3:

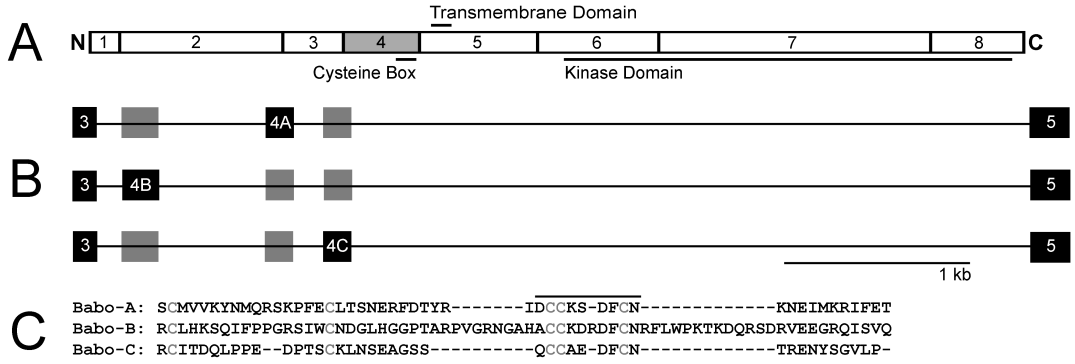

A
Transmembrane Domain
N
1
2
3
4
5
6
7
8
C
Cysteine Box
Kinase Domain
B
3
4A
5
3
4B
5
3
4C
5
1 kb
C
Babo-A: SCMVVKYNMQRSKPFECLTSNERFDTYR-------IDCCKS-DFCN------------KNEIMKRIFET
Babo-B: RCLHKSQIFPPGRSIWCNDGLHGGPTARPVGRNGAHACCKDRDFCNRFLWPKTKDQRSDRVEEGRQISVQ
Babo-C: RCITDQLPPE--DPTSCKLNSEAGSS----------QCCAE-DFCN------------TRENYSGVLP-

Figure 1.S2: *RT-PCR for transcripts of* babo *isoforms in S2 cells.* Amplified products for $babo_a$, $babo_b$, and $babo_c$ are 205, 268, and 187 bp, respectively. The three S2 PCR reactions were generated from the same master mix that contained cDNA template, providing an internal loading control. To demonstrate that the null result in the $babo_b$ S2 PCR reaction is not due to the primers' inability to amplify a product, all primer sets were used to generate products from appropriate plasmid templates.

Figure 1.S2:

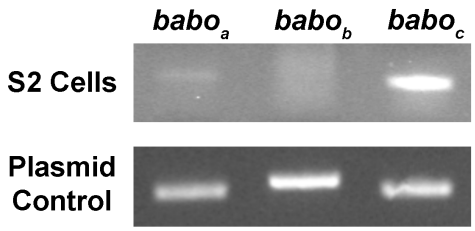
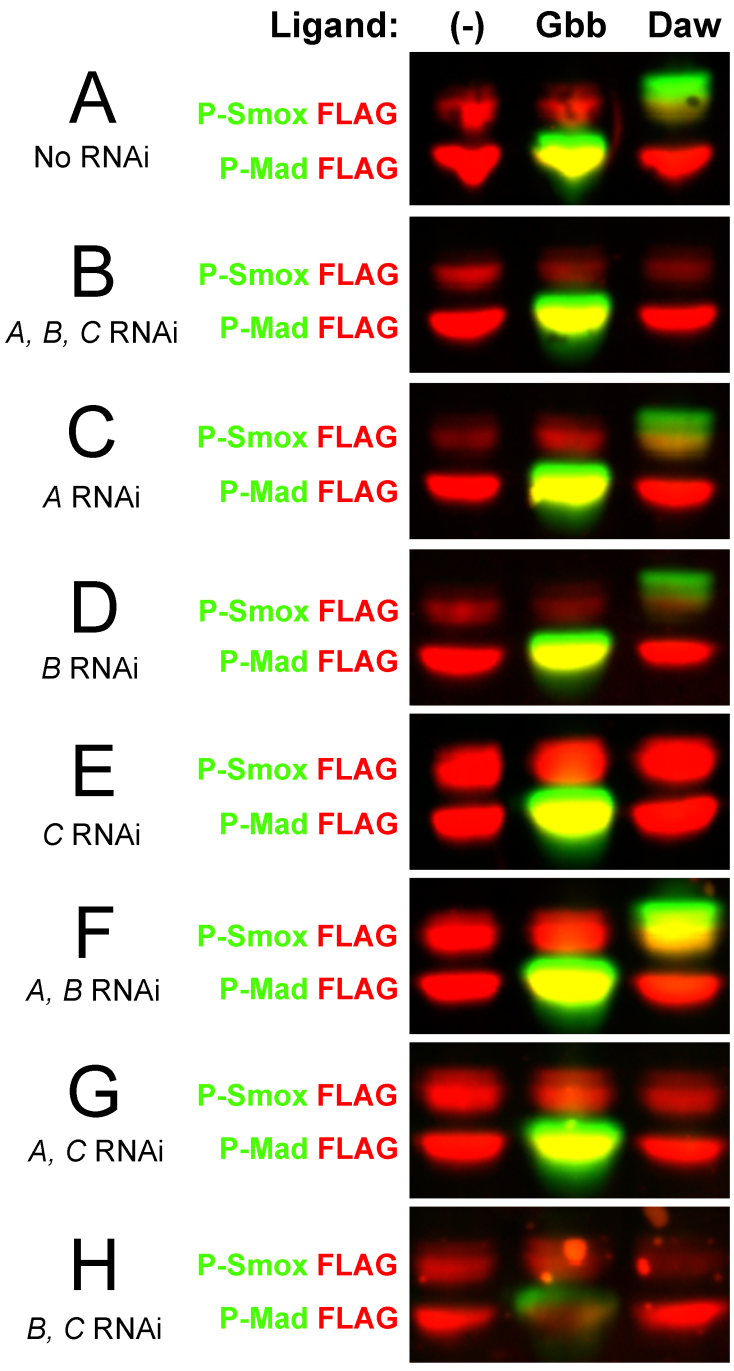


Figure 1.4: *Babo$_c$ is indispensible for Daw signaling.* A-H) Cells were treated with double-stranded RNA (dsRNA) that targeted different combinations of *babo* isoforms and were exposed to Gbb and Daw. Cells exposed to no dsRNA (A) or to dsRNA targeting isoforms *A* (C), *B* (D), or *A* and *B* (F) responded to Daw. In cells where *babo$_c$* was targeted by RNAi, either alone (E) or in combination with other isoforms (B, G, and H), cells could no longer respond to Daw stimulation. No combination of dsRNA eliminated a response to Gbb (A-H).

Figure 1.4:


Ligand: (-) Gbb Daw
A
No RNAi
P-Smox FLAG
P-Mad FLAG
B
A, B, C RNAi
P-Smox FLAG
P-Mad FLAG
C
A RNAi
P-Smox FLAG
P-Mad FLAG
D
B RNAi
P-Smox FLAG
P-Mad FLAG
E
C RNAi
P-Smox FLAG
P-Mad FLAG
F
A, B RNAi
P-Smox FLAG
P-Mad FLAG
G
A, C RNAi
P-Smox FLAG
P-Mad FLAG
H
B, C RNAi
P-Smox FLAG
P-Mad FLAG

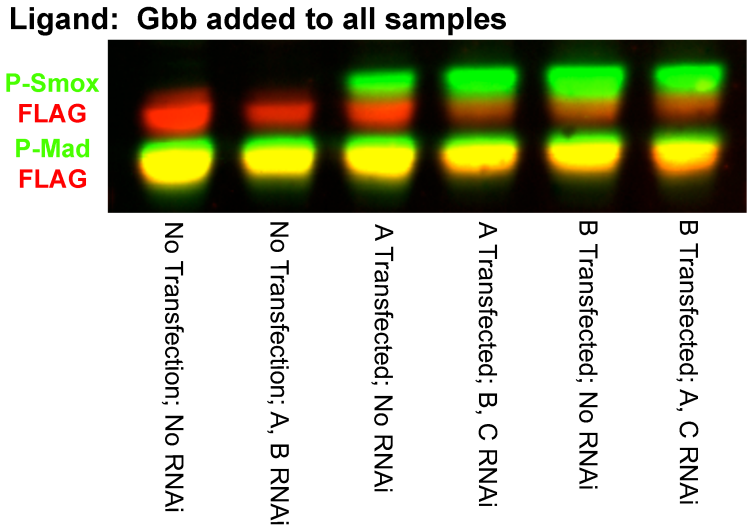
While cells with endogenous levels of receptors responded robustly to Daw (Figure 1.4A), simultaneous RNAi against all three *babo* isoforms eliminated Smox phosphorylation (Figure 1.4B). RNAi against $babo_a$ or $babo_b$ had no effect on the stimulation of cells by Daw (Figures 1.4C and 1.4D). Cells with $babo_a$ and $babo_b$ simultaneously targeted by RNAi, leaving only $Babo_c$, were also able to respond to Daw (Figure 1.4F). However, cells whose $babo_c$ was targeted by RNAi, regardless if other isoforms were similarly targeted, were never able to transduce Daw's signal (Figures 1.4E, 1.4G, and 1.4H). These data demonstrate that $Babo_c$ is a necessary Type-I receptor for Daw signaling in S2 cells.

Other isoforms of Baboon are neither necessary nor sufficient for Dawdle signaling.

$babo_a$ and $babo_b$ are expressed at lower levels than $babo_c$ in S2 cells (Figure 1.S2). This observation raises the possibility that $Babo_a$ and $Babo_b$ might indeed be able to transduce the Daw signal, but do not appear to do so in S2 cells because they are expressed at low levels. We increased the levels of $Babo_a$ and $Babo_b$ in S2 cells by transfection with constructs encoding each individual isoform. This expression was combined with the RNAi strategy utilized in Figure 1.4 to enable us to test each isoform individually for its ability to transduce the Daw signal. As before, Gbb was able to stimulate each batch of cells, suggesting that any loss of Daw signaling was due to the loss of Babo and not to the cells' general inability to respond to TGF-β ligands (Figure 1.S3).

Figure 1.S3: *Gbb stimulates phosphorylation of Mad (P-Mad) in all cell samples depicted in Figure 1.5*. An aliquot of cells from each batch used in Figure 1.5 was exposed to Gbb and tested for P-Mad. Compare P-Mad bands in each lane of this Figure with the lack of P-Mad in the appropriate (-) lanes in Figure 1.5. (Lanes 1 and 2: Figure 1.5A; Lanes 3 and 4: Figure 1.5B; Lanes 5 and 6: Figure 1.5C.)

Figure 1.S3:


Ligand: Gbb added to all samples
P-Smox
FLAG
P-Mad
FLAG
No Transfection; No RNAi
No Transfection; A, B RNAi
A Transfected; No RNAi
A Transfected; B, C RNAi
B Transfected; No RNAi
B Transfected; A, C RNAi

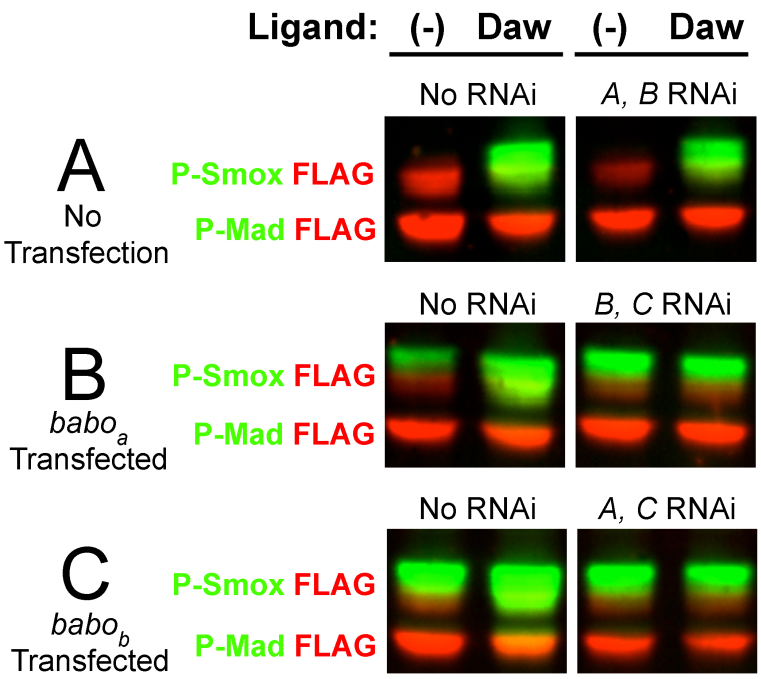
As previously shown in Figure 1.4, cells expressing endogenous receptors responded robustly to Daw, and RNAi against $Babo_a$ and $Babo_b$, leaving only $Babo_c$, had no effect on this response (Fig. 1.5A). Cells ectopically expressing $Babo_a$ had a baseline level of P-Smox even when exposed only to conditioned medium, indicating that the overexpressed receptor was able to signal to some extent in the absence of added ligand (Figure 1.5B). However, these cells still showed a significant increase in Smox phosphorylation in response to Daw when endogenous $Babo_c$ was not targeted by RNAi (Figure 1.5B). In contrast, when $Babo_b$ and $Babo_c$ were eliminated by RNAi, leaving only overexpressed $Babo_a$, cells could no longer respond to Daw (Fig. 1.5B). The same was true for $Babo_b$: cells overexpressing $Babo_b$ could respond to Daw at levels above background stimulation, but only if endogenous $Babo_c$ was not targeted by RNAi (Figure 1.5C). These results suggest that neither $Babo_a$ nor $Babo_b$ is capable on its own of responding to Daw, even under overexpressed conditions.

Isoforms of baboon *are expressed in different tissues during development.*

We sought to determine whether individual *babo* isoforms are differentially expressed in various tissues during larval development. Previous *in situ* hybridization experiments have shown that all larval tissues express *baboon* mRNA (Brummel *et al*., 1999), but the probe that was used in these studies was complementary to a nucleotide sequence shared by all three *babo* isoforms. To determine if the isoforms of *baboon* have distinct spatial expression patterns during larval development, we isolated RNA

Figure 1.5: *Daw cannot signal using other isoforms of Babo*. Daw is able to signal in the presence of endogenous receptors or when $babo_a$ and $babo_b$ are targeted by RNAi, leaving only $Babo_c$ (A). Expression of $babo_a$ did not change cells' ability to respond to Daw, but addition of dsRNA targeting $babo_b$ and $babo_c$, leaving only $Babo_a$, left cells unable to respond to Daw stimulation (B). Similarly, cells expressing $Babo_b$ could respond to Daw in the presence of endogenous $Babo_c$, but could not respond if $babo_a$ and $babo_c$ were targeted by RNAi (C).

Figure 1.5:


Ligand: (-) Daw (-) Daw
No RNAi
A, B RNAi
A
No Transfection
P-Smox FLAG
P-Mad FLAG
No RNAi
B, C RNAi
B
babo$_a$ Transfected
P-Smox FLAG
P-Mad FLAG
No RNAi
A, C RNAi
C
babo$_b$ Transfected
P-Smox FLAG
P-Mad FLAG

from tissues that were dissected from third-instar larvae and performed semi-quantitative RT-PCR analysis using isoform-specific primers.

Consistent with the previous study of *baboon* expression, all larval tissues express at least one *baboon* isoform (Figure 1.6). The third-instar brain expresses predominantly $babo_a$, while the wing disc expresses both $babo_a$ and $babo_b$ but not high levels of $babo_c$. In contrast $babo_c$ and not $babo_a$ or $babo_b$ is expressed principally in the gut and fat body. Punt, the necessary and sufficient Type-II receptor for Daw signaling in S2 cells (Figure 1.2), is expressed ubiquitously during development (Childs *et al.*, 1993).

Dawdle induces wing patterning defects only in the presence of ectopically-expressed $babo_c$.

Since we found that signaling by Daw in tissue culture requires $Babo_c$, the inability of Daw to produce a phenotype when ectopically expressed in the wing might be attributable to the low endogenous level of $Babo_c$ in the wing. To examine this possibility, we ectopically expressed Daw in the wing either with or without coexpression of $Babo_c$.

Strongly activating the Activin pathway in the *Drosophila* wing disc results in significant vein-patterning defects, as shown by overexpression of a constitutively active form of Babo (Babo*) (Figures 1.7A and 1.7B). A milder phenotype – one seen using weaker Babo* transgenes (Brummel *et al*., 1999) – is seen upon overexpression of the ligand dActivin (Figure 1.7C). Ectopic expression of neither Daw nor $Babo_c$

Figure 1.6: *Expression of isoforms of* babo *in third-instar larval tissues*. Primers for *actin* (A) and each isoform of *babo* (B-D) amplified PCR products from cDNA collected from tissues that were dissected from third-instar larvae. *actin* transcript was present in all tissues tested (A). $babo_a$ is expressed in the brain and the wing (B), and $babo_b$ was detected primarily in the wing disc (C). $babo_c$ is expressed in the gut and the fat body, but not in the brain or the wing disc at measurable levels (D).

Figure 1.6:

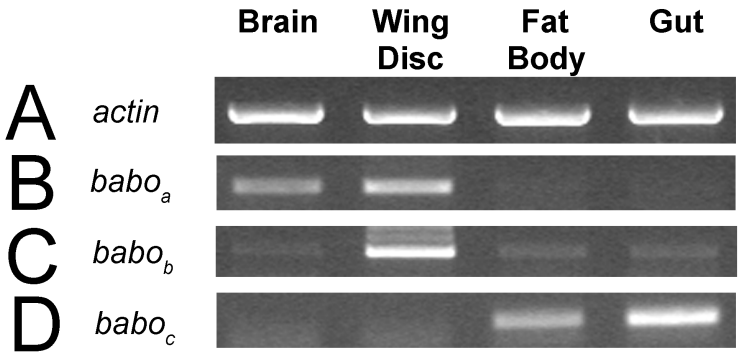
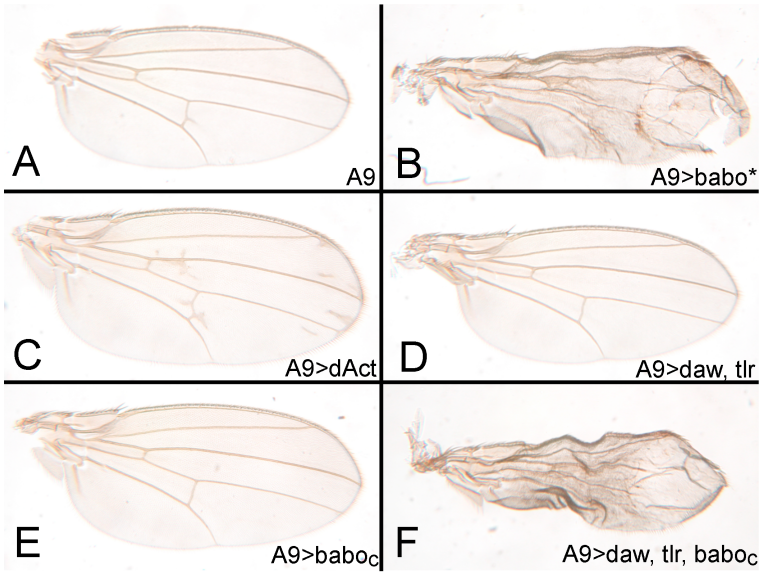


Figure 1.7: *Dawdle overexpression induces wing patterning defects only in the presence of $Babo_c$.* Patterning defects result from ectopic expression of a constitutively active form of Baboon (Babo*) using the A9 Gal4 driver (A and B). Overexpression of the ligand dActivin produces a moderate vein-patterning defect (C), but A9-driven Dawdle does not induce either of these phenotypes, even when it is co-overexpressed with its activating protease Tlr (Serpe and O'Connor, 2006) (D). However, patterning defects occur when Daw and Tlr are co-overexpressed with $Babo_c$ (F), even though $Babo_c$ has no effect when it is expressed alone (E). [Note: the UAS *$baboon_c$* transgenic fly line was created by X. Zheng and T. Lee of the University of Massachusetts Medical School.]

Figure 1.7:



induces a patterning phenotype (Figures 1.7D and 1.7E), but when Daw is co-overexpressed with $Babo_c$ we observe a striking defect in wing patterning that is identical to that seen upon overexpression of a strong activated Babo receptor line (Figure 1.7F). Taken together, these data suggest that $Babo_c$ is necessary *in vivo* to transduce a high level of Daw signal. Importantly, these data also demonstrate that tissue-specific isoform expression can dictate which tissues respond to different Activin-family signal(s) during development.

Discussion

The data presented in this study demonstrate that the gene for the *Drosophila* Activin receptor, Baboon, encodes three isoforms that differ only in their extracellular ligand-binding domains and that one of these isoforms, $Babo_c$, is uniquely required for signaling by the Activin-like ligand Dawdle. The ability to express ligand-specific receptor isoforms is likely to have important implications for cells during development. For example, different profiles of receptor expression would allow neighboring cells to receive different levels of Activin signaling, even if they are exposed to the same suite of Activin ligands. Restricted receptor expression patterns also offer a means to enable systemically delivered ligands such as Daw to still exhibit tissue-specific effects. One example of this regulation may occur at the *Drosophila* neuromuscular junction, where multiple Activin-like ligands are expressed either pre- or post-synaptically (Zhu *et al.*, 2008; Serpe and O'Connor, 2006; Parker *et al*., 2006; M.B.O., unpublished).

Differential expression of Baboon isoforms may be an important way for the muscle and neuron to distinguish between different Activin inputs.

Because Daw appears to signal though a specific Type-I receptor isoform that is not necessary for dAct signaling (Figure 1.7C), it is curious that the two ligands appear to function redundantly in one case, the regulation of neuroblast proliferation (Zhu *et al.*, 2008). This is especially surprising since $babo_c$ is not highly expressed in the brain, at least as measured by low-cycle RT-PCR (Figure 1.6D). One possible explanation for this discrepancy is that $babo_c$ may be expressed only in a small subset of cell types in the brain, like neuroblasts, and our whole-brain RT-PCR using a pan-*babo* 5' primer was biased towards the more prevalent $babo_a$ transcript. However, our attempts to examine the tissue distribution of individual isoforms by *in situ* hybridization using isoform-specific mRNA probes has not been successful. Ultimately, isoform-specific antibodies may be necessary to elucidate higher-resolution spatial expression patterns of the three isoforms. Such studies, together with a more careful analysis of each ligand's expression pattern and the generation of isoform-specific loss-of-function mutants, will help elucidate the extent of potential functional redundancies between ligands and if specific receptor isoforms regulate unique biological processes.

Baboon is not the only *Drosophila* Type-I receptor with multiple isoforms: the *Drosophila* BMP receptors Sax and Tkv also have several isoforms that differ in their extracellular regions (Brummel *et al.*, 1994). Similarly, recent work has uncovered Type-I receptor isoforms that are divergent in their extracellular domains in many mammalian species (Konrad et al., 2007). In both of these cases, however, the divergent

isoforms do not affect the cysteine box as seen in the Babo isoforms. For this reason it is not clear if, or to what degree, these more subtle changes might affect affinity of ligand-binding. Genes encoding mammalian Type-II receptors also produce multiple isoforms that differ in their extracellular domains (Suzuki *et al*., 1994; Hirai and Fujita, 1996), and some of these isoforms can bind the same ligand with different affinities (Attisano et al., 1992) or bind different ligands with different affinities (Parker et al., 2007; Rotzer et al., 2001). Some of these isoforms are also expressed tissue-specifically (Rotzer *et al.*, 2001). When coupled with these findings, our study uncovers an evolutionarily conserved mechanism by which cells can regulate their response to TGF-β ligands via the type of receptor isoforms that they express. The many potential combinations of Type-I and Type-II isoforms likely enable a cell to fine tune its response when presented with numerous TGF-β family members, especially in mammals, where 33 ligands appear to signal via a limited set of 5 Type-II and 7 Type-I receptors (Kitisin et al., 2007).

In *Drosophila*, only one isoform of each Type-II receptor has been found, but because flies express so few ligands, many combinations of Type-I-Type-II signaling complexes may not be needed. For example, the *Drosophila* genome encodes two Type-II receptors and three isoforms of Babo, giving six combinations of homomeric Type-I/Type-II receptor complexes. The fly genome also encodes four Activin-style ligands (*e.g.*, with nine cysteines versus the seven found in BMPs): dActivin, Dawdle, Myoglianin and Maverick. It is possible, therefore, that each ligand could have a specific combination of receptors for signaling. We have examined this possibility using

S2 signaling assays, but we have been unable to see reproducible signaling *in vitro* from dActivin, Myoglianin, and Maverick, even in the presence of every combination of receptors (data not shown). Others have noted similar difficulties (Gesualdi and Haerry, 2007; Lee-Hoeflich, 2005). Perhaps, in addition to expressing different isoforms of Baboon, cells also control other Activin-like signals by regulating expression of a necessary co-receptor not found in S2 cells. Indeed, a co-receptor is required for signaling by the related signaling molecule Nodal in mammals (Yeo and Whitman, 2001).

In summary, our demonstration of Type-I receptor isoform-specific signaling reveals an additional mechanism by which signal specificity can be achieved within the TGF-ß pathway. Our findings suggest that, by regulating which complement of receptor isoforms they express, distinct cell types within a tissue may be able to discriminate between Activin-family signals during development. This specificity may allow a cell type to maintain a unique level of Activin signaling – one that is different from a neighboring tissue's – despite its exposure to several systemically expressed ligands that all can activate the intracellular Activin signaling cascade.

Experimental Procedures

S2 Signaling Assay

S2 cell culture and transfection have been described previously (Serpe and O'Connor, 2006). Briefly, conditioned medium was collected from cells that were transfected with an expression construct coding for a *Drosophila* TGF-β ligand.

Conditioned medium from mock-transfected cells was used as a negative control. Cells used in the signaling assay were all transfected with constructs coding for N-terminally FLAG-tagged Mad and Smox, and, as noted, were also transfected with constructs coding for Wishful thinking or various isoforms of Baboon. Double-stranded RNA was prepared as described previously (Shimmi and O'Connor, 2003), and 10 μg RNA was added to cultured cells at the time of transfection and then subsequently at 24 hours and 48 hours post-transfection. Separate batches of cells were transfected with ligand or with Smox and receptors. After 72 hours of incubation, 250μL of supernatant from ligand-expressing cells were added to 250μL of Smad/receptor-transfected cells suspended in their own culture media and incubated at room temperature with rolling for 90 minutes. Cells were then pelleted, resuspended in 1X SDS sample buffer, and boiled.

Western Blotting and Antibodies

Samples were loaded onto NuPAGE© 4-12% Bis-Tris Gels (Invitrogen) and transferred after electrophoresis to PVDF membrane (Bio-Rad). Membranes were probed with primary antibodies against phosphorylated Mad (gift from E. Leof, 1:1000), phosphorylated Smox (Cell Signaling, 1:1000), and the FLAG epitope (M2 from Sigma, 1:2000). IRDye secondary antibodies (Rockland) were used 1:2000, and blots were imaged on a Li-Cor Odyssey infrared imaging system.

Isolation of baboon_c

The *babo$_c$* isoform was identified by sequencing *baboon* PCR products amplified from a cDNA library. As illustrated in Figure 1.S2, the *babo$_c$* isoform is also expressed by the embryonically-derived S2 cell line. The *babo$_c$* cDNA sequence is available from FlyBase (www.flybase.org; transcript ID: FBtr0300599).

RNA Isolation and Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

RNA was isolated from either S2 cells or from tissues that were dissected from third-instar *Drosophila* larvae using Trizol® (Invitrogen); 1 μg total RNA was reverse transcribed using the Thermoscript® RT-PCR kit (Invitrogen) and an Oligo dT primer. All products were used per suppliers' instructions. 1 μL of the RT reaction was amplified by PCR for 25 cycles (S2 cDNA) or 30 cycles (fly-tissue cDNA). Sequences of primers used in PCR reactions are listed in Table 1.

Drosophila *stocks and husbandry*

The A9 Gal4 driver was used to induce overexpression of transgenes in the wing disc (Brummel et al. 1999). A9 females were crossed to males carrying the following transgenes or combinations thereof, as noted: *UAS babo*1A2*, *UAS dAct 2X*, *UAS babo$_c$*, or the *UAS daw 9D2 UAS tlr2* recombinant. Flies were reared at 25 degrees C on standard food, and wings from adult females were mounted in standard mounting medium.

Table 1: List of Primers for RT-PCR and RNAi

Primer Name	Sequence
punt T7 RNAi 5’	TAATACGACTCACTATAGGGAGAGACAACGGGCATCCTGCGC
punt T7 RNAi 3’	TAATACGACTCACTATAGGGAGCCGCAGGGCTTGCCTGGCTG
babo RT-PCR 5’ (common to all isoforms)	GGCTTTTGTTTCACGTCCGTGGA
babo-A RT-PCR 3’	CTGTTTCAAATATCCTCTTCATAATCTCAT
babo-B RT-PCR 3’	CTTGGACGGAGATTTGCCGCCC
babo-C RT-PCR 3’	CTGGTAGGACACCACTGTAATTCTC
babo-A T7 RNAi 5’	TAATACGACTCACTATAGGGTTGCATGGTTGTCAAGTACAATATGC
babo-A T7 RNAi 5’	TAATACGACTCACTATAGGGCTGTTTCAAATATCCTCTTCATAATCTCAT
babo-B T7 RNAi 5’	TAATACGACTCACTATAGGGATGTCTGCACAAGTCTCAAATCTTTCCGC
babo-B T7 RNAi 3’	TAATACGACTCACTATAGGGCTTGGACGGAGATTTGCCGCCC
babo-C T7 RNAi 5’	TAATACGACTCACTATAGGGATGCATAACAGACCAGTTGCCACC
babo-C T7 RNAi 3’	TAATACGACTCACTATAGGGCTGGTAGGACACCACTGTAATTCTC

Chapter 2:
The Activin ligand Dawdle links diet and metabolism via regulation of a *Drosophila* insulin-like signal, Dilp6.

Coordinated growth of cells and organs is essential for proper animal development. A developing animal simultaneously regulates many cell biological processes – including proliferation, growth, and differentiation – and utilizes many intercellular signal transduction pathways to dovetail these events. However, just as cells must work in concert with other cells during development, they must also respond to environmental cues, such as nutrition. Cellular growth and metabolism must have the ability to match available nutrients to ensure that proper development can occur across a range of environments, a versatility that is critical for any species' survival. The fruit fly *Drosophila melanogaster* demonstrates rapid growth during development (a one-hundred-fold increase in body mass in a four-day period), and does so reproducibly even when grown on a variety of food sources. This developmental trait of balancing nutrition and metabolism makes the fly model an attractive system for studying the interface of developmental biology and environment.

Many animals across the animal kingdom use insulin signals to control cellular growth and metabolism (reviewed in Broughton and Partridge, 2009). These small peptides initiate a strongly conserved intracellular signaling cascade within cells that can govern many traits, including body size, developmental timing, and life-span in many animals (Kimura *et al*., 1997; Brogiolo *et al*., 2001; Walkiewicz and Stern, 2009). Furthermore, aberrant insulin signaling in humans is famously connected to the onset of the prevalent metabolic disorder diabetes mellitus. Tight regulation of insulin signaling,

therefore, is critical for both proper development and normal physiology in species across the animal kingdom.

Another evolutionarily conserved mechanism of cell-to-cell communication is the Transforming Growth Factor-β (TGF-β) signaling pathway. Like insulins, TGF-β family members catalyze well-studied intracellular signaling pathways; these cascades ultimately regulate myriad cell biological processes by affecting transcription of target genes. In *Drosophila*, one branch of the TGF-β pathway, the Activin pathway, is known to regulate cell proliferation, neuronal patterning, and axon guidance (Zheng *et al*., 2003; Parker *et al*., 2006; Serpe *et al*., 2006; Zhu *et al*., 2008). In this report we implicate Activin signaling in metabolism during *Drosophila* development; we demonstrate that Activin signaling regulates expression of an insulin-family signal.

Our results demonstrate that Dawdle (Daw), a member of the Activin family, is necessary for normal development across a range of dietary conditions. Diet influences the expression level of Dawdle, which negatively controls transcription of the gene that encodes Dilp6, one of several *Drosophila* insulin-like peptides. To show that this lack of negative regulation of insulin is responsible for the phenotypes of *daw* mutants, we demonstrate a suppression of the *daw* phenotypes by concurrent mutation of *dilp6*. This simple circuit that links diet to Dawdle to Dilp6 provides a mechanism by which a *Drosophila* larva can dovetail its nutrition and cellular metabolism using two ancient intercellular signaling pathways.

Results

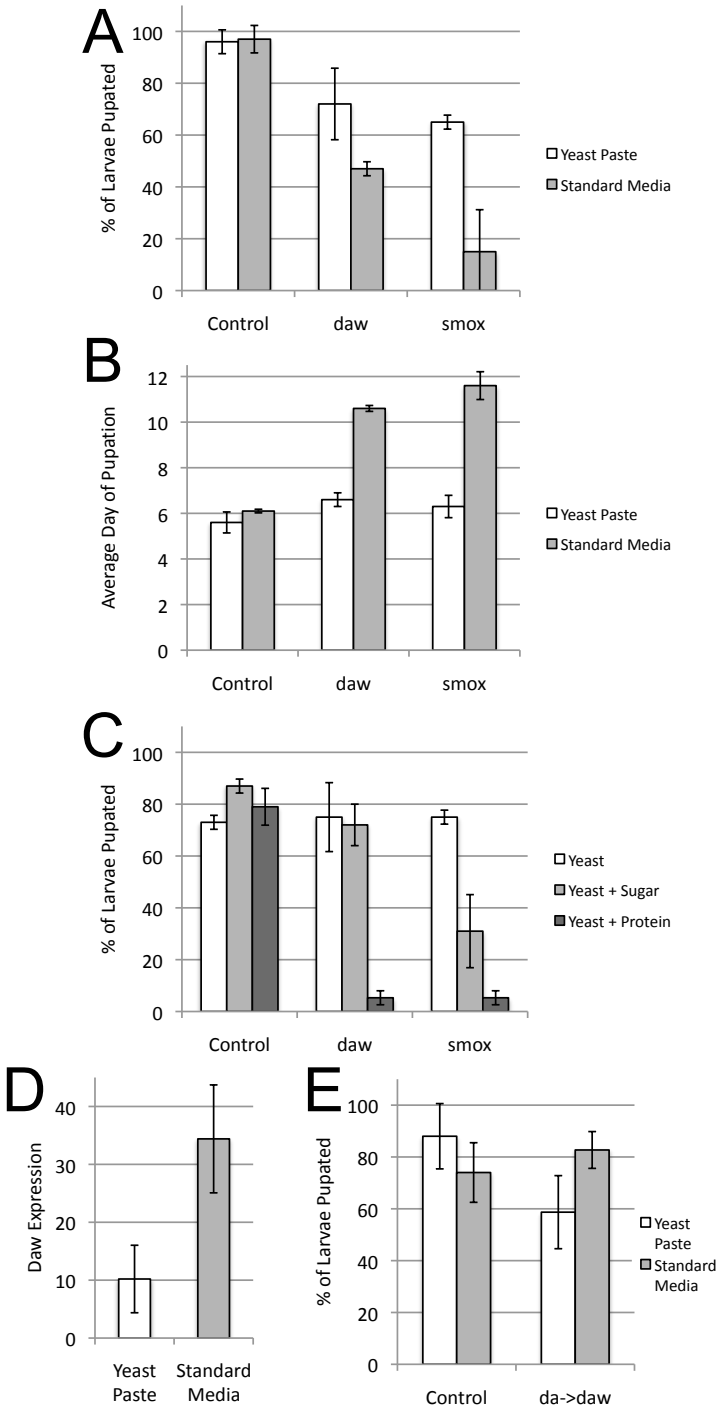
daw *mutants display diet-specific developmental arrest and delay.*

Drosophila larvae develop and pupate normally when raised on either yeast paste or standard *Drosophila* media (Figures 2.1A and 2.1B). When *daw* mutants are raised on yeast paste, they pupate at a frequency that is similar to that of controls, but the mutants often die as larvae when they develop on standard fly food ($p < 0.001$; Figure 2.1A). Furthermore, *daw* mutants pupate one day after controls when they are grown on yeast paste ($p = 0.02$), but they pupate over four days after controls when they are raised on standard food ($p < 0.001$) (Figure 2.1B). These results demonstrate that *daw* mutants display increased larval lethality and developmental delay when they are reared on standard media compared to yeast paste. Similar diet-dependent phenotypes are seen in animals that are mutant for Smox, the Smad in the Activin pathway (Figure 2.1B).

We next sought to characterize which component of the standard *Drosophila* media was poisoning our Activin-pathway mutants during development. Because the media consists largely of sugar and protein, we measured pupation rates of larvae that were grown on a yeast food that included either glucose or BSA. On a basic yeast food, *yw* control and *daw* mutant larvae formed puparia with indistinguishable frequency (Figure 2.1C). The addition of glucose to the media did not induce significant lethality in *daw* larvae compared to controls, but only 5% of *daw* mutants pupated when BSA was added to the food, compared to 79% of control animals (Figure 2.1C). *smox* mutant larvae also fared poorly on protein-rich food compared to the yeast-only diet; however, the glucose-rich diet also induced lethality in *smox* mutants (Figure 2.1C).

Figure 2.1: *Activin signaling is necessary for appropriate response to varied dietary conditions in* Drosophila*.* A) On yeast paste *daw* mutants pupate at a frequency similar to control larvae, but on standard food a significant percentage of *daw* mutants die as larvae. *smox* mutants die on both yeast and standard diets, but the lethality is more severe on standard food. B) Both *daw* and *smox* mutant larvae are severely developmentally delayed when raised on a standard diet; on yeast paste *daw* mutants are delayed one day. C) Addition of 10% glucose to a yeast diet has little effect on the development of *daw* larvae, but addition of 10% BSA results in significant larval death. *smox* mutants do not develop on either sugar- or protein-rich food. D) Transcription of *daw* is significantly increased in *yw* animals when they are raised on standard food compared to yeast paste (arbitrary units). E) Ectopic expression of *daw* results in larval lethality only on a diet of yeast paste, a condition in which *daw* is not normally expressed at high levels. On standard media, a diet on which animals already have elevated *daw* levels, ectopic expression does not affect development.

Figure 2.1:


A
% of Larvae Pupated
100
80
60
40
20
0
Control
daw
smox
Yeast Paste
Standard Media
B
Average Day of Pupation
12
10
8
6
4
2
0
Control
daw
smox
Yeast Paste
Standard Media
C
% of Larvae Pupated
100
80
60
40
20
0
Control
daw
smox
Yeast
Yeast + Sugar
Yeast + Protein
D
Daw Expression
40
30
20
10
0
Yeast Paste
Standard Media
E
% of Larvae Pupated
100
80
60
40
20
0
Control
da->daw
Yeast Paste
Standard Media

Taken together, these results suggest that Dawdle and the Activin signaling pathway are important for proper development, but that this importance varies with diet. Unlike control animals, who can pupate with indistinguishable frequency on diets of yeast, yeast + sugar, and yeast + protein, *daw* mutants die as larvae in the presence of high protein levels. High levels of sugar and protein are toxic to *smox* mutants. These findings indicate that Activin signaling plays a role in interpreting dietary cues during *Drosophila* development.

To investigate why Dawdle signaling is important for development on standard media but is dispensable for proper development on yeast paste, we compared levels of *daw* expression in animals that were grown on each food source. Transcription of *daw* was significantly upregulated in third-instar larvae that were grown on standard food compared to yeast paste (Figure 2.1D). This result is consistent with the loss-of-function experiment described above: mutation of *daw* is of little consequence when larvae are grown on yeast paste, which is when the animal expresses only low levels of the gene. Larvae that are grown on standard food, however, typically express higher levels of *daw* and therefore develop abnormally in its absence.

These results demonstrate that the transcription level of *dawdle* varies with diet, and that disrupting this diet-matched expression has deleterious effects on development. To further investigate the relationship between *daw* transcription and nutrition, we sought to misregulate expression levels of *daw* using a gain-of-function approach. Because *daw* loss-of-function was lethal only under conditions where *daw* is normally highly expressed, we hypothesized that overexpression of *daw* should disrupt

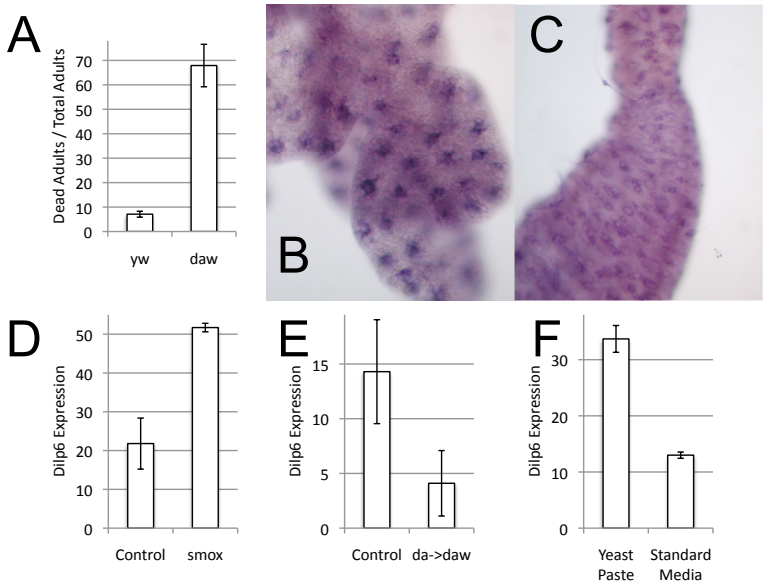
development most severely when the gene is typically transcribed at low levels. To test this hypothesis we induced ectopic expression of *dawdle* using the ubiquitous *daughterless* (*da*) promoter and the Gal4/UAS system. As shown in Figure 2.1E, overexpression of *daw* had little effect on development when the larvae were raised on standard food, a condition that on its own robustly induces *daw* transcription. However, ectopic expression of *daw* resulted in significant larval lethality when larvae were grown on yeast paste, a diet that does not stimulate high levels of *daw*. Taken together, the experiments described in Figure 2.1 demonstrate that diet-dependent regulation of *dawdle* expression is critical for proper development in *Drosophila*.

Activin signaling regulates expression of dilp6*, a gene encoding an insulin-like peptide.*

Because the severity of *daw* mutant phenotypes varies with diet, we sought to find a transcriptional target of Activin signaling that is implicated in growth and metabolism. Our search for such targets was aided by another observation involving *daw* mutant adults. When grown on standard food, *daw* mutant adults have shorter life spans than controls; many of them die within days of eclosion (Figure 2.2A). This loss-of-function phenotype involving life-span, coupled with the aforementioned role in metabolism, suggested that *daw* may be regulating the insulin signaling pathway. Insulin signaling affects growth, metabolism, and lifespan in organisms across the animal kingdom (Kimura *et al*., 1997; Brogiolo *et al*., 2001; Saltiel and Kahn, 2001), and it functionally interacts with TGF-β signaling pathways in animals as diverse as nematodes and mammals (Shaw *et al*., 2007; Bertolino *et al*., 2008). For these reasons

Figure 2.2: *Activin signaling regulates expression of* dilp6*, a member of the insulin family.* A) Compared to *yw* controls, a significantly greater percentage of *daw* mutant adults died within 16 days of egg deposition. B and C) *daw* is expressed at high levels in third-instar fat body and gut (first gut turn shown). D) Transcription of *dilp6* is significantly increased in early *smox* third-instar larvae compared to *yw* controls (units in Figures 2D-2F are arbitrary units). E) *dilp6* transcription in early third-instar larvae is reduced upon overexpression of *daw* compared to *yw* controls. F) *yw* early third-instar larvae grown on standard media express lower levels of *dilp6* than larvae grown on yeast paste.

Figure 2.2:


A
Dead Adults / Total Adults
70
60
50
40
30
20
10
0
yw
daw
B
C
D
Dilp6 Expression
50
40
30
20
10
0
Control
smox
E
Dilp6 Expression
15
10
5
0
Control
da->daw
F
Dilp6 Expression
30
20
10
0
Yeast Paste
Standard Media

we chose to examine whether a link to insulin signaling might explain the phenotypes we observed in *daw* and *smox* mutants in *Drosophila*.

daw is expressed in many tissues during larval development, but it has especially high levels of expression in the fat body and gut (Figures 2.2B and 2.2C; Parker *et al*., 2006; Serpe *et al*., 2006). Similarly, unlike other *Drosophila* insulin-like peptides ("Dilps") whose expression patterns are restricted to specific neurons (Brogiolo *et al*., 2001; Miguel-Aliaga *et al*., 2008), the insulin gene *dilp6* is expressed in the fat body and the gut (Brogiolo *et al*., 2001; Okamoto *et al*., in preparation). The common expression patterns of *daw* and *dilp6* suggested that they may be involved in similar processes during development, and we sought to determine if the phenotypes of the Activin-pathway mutants were due to a misregulation of *dilp6*.

Third-instar larvae that are mutant for *smox* express significantly higher levels of *dilp6* mRNA than control animals (Figure 2.2D), suggesting that Activin signaling is a negative regulator of insulin expression. To further test a model of negative regulation, we overexpressed *daw* using the ubiquitous *daughterless* Gal4 driver and found subsequently reduced levels of *dilp6* transcript in early third-instar larvae compared to controls (Figure 2.2E). Taken together, these loss-of-function and gain-of-function experiments indicate that Activin signaling antagonizes expression of the insulin gene *dilp6* during *Drosophila* development.

Our findings involving *dawdle*'s diet-induced expression and its repression of *dilp6* suggested that wild-type larvae that are raised on standard food (and that express higher levels of *daw*) will transcribe lower levels of *dilp6* than wild-type larvae that

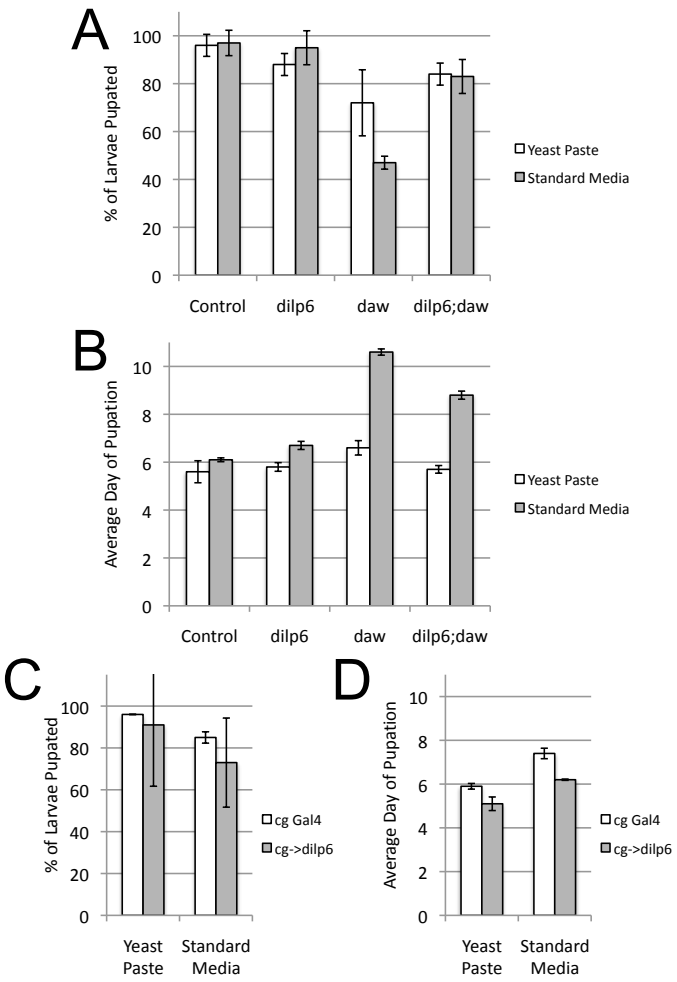
develop on yeast food. An RT-PCR experiment that measured RNA that was extracted from early third-instar larvae supports this hypothesis: larvae that grew on standard food expressed lower levels of *dilp6* compared to yeast-fed larvae (Figure 2.2F). This result is consistent with a diet-induced, Activin-dependent suppression of insulin expression during development. Furthermore, a negative regulation of insulin by Activin signaling is consistent with our previous observation involving life span: because inhibiting the insulin pathway results in longer lives in several organisms (Friedman and Johnson, 1988; Clancy *et al*., 2001; Holzenberger *et al*., 2003), an increased level of insulin signaling may indeed be responsible for the short adult lives of *daw* mutants.

Our findings demonstrate an antagonism of insulin expression by the Activin pathway, but we sought to determine whether misregulation of *dilp6* is responsible for the phenotypes of developmental delay and larval lethality in Activin-pathway mutants. If these diet-dependent phenotypes are due to increased expression of *dilp6*, they should be suppressed in a *dilp6*-mutant background. For this analysis we utilized a mutation in the *dilp6* gene that was generated by imprecise excision of a *P* element in the gene's promoter region (Okamoto *et al*., in preparation). The resulting deletion removed a portion of the gene's upstream sequence and the first exon of the putative mRNA transcript; this mutation significantly disrupts expression of the *dilp6* ORF and results in body-mass phenotypes that are characteristic of insulin pathway mutants (Okamoto *et al*., in preparation).

Mutation of *dilp6* influences neither developmental time nor larval viability when animals are grown on yeast food (Figure 2.3A). On standard food, *dilp6* mutants

Figure 2.3: *Mutation of* dilp6 *can suppress* daw *mutant phenotypes.* A) *dilp6* mutants do not die as larvae on either yeast paste or standard media. Mutation of *dilp6* suppresses the larval lethality of *daw* mutants grown on standard media. (In Figures 2.3A and 2.3B, the data regarding *daw* single mutants are identical to the corresponding data in Figure 2.1). B) *dilp6* mutants are slightly delayed on a standard diet, but they are not delayed when raised on yeast paste. The slight developmental delay of yeast-fed *daw* mutants is suppressed in a *dilp6*-mutant background, and *dilp6* mutation partially rescues the severe delay of *daw* mutants when they are raised on a standard diet. C and D) Overexpression of *dilp6* using a fat body-specific driver does not induce larval lethality, and it results in precocious pupation regardless of diet.

Figure 2.3:


A
% of Larvae Pupated
100
80
60
40
20
0
Control
dilp6
daw
dilp6;daw
Yeast Paste
Standard Media
B
Average Day of Pupation
10
8
6
4
2
0
Control
dilp6
daw
dilp6;daw
Yeast Paste
Standard Media
C
% of Larvae Pupated
100
80
60
40
20
0
Yeast Paste
Standard Media
cg Gal4
cg->dilp6
D
Average Day of Pupation
10
8
6
4
2
0
Yeast Paste
Standard Media
cg Gal4
cg->dilp6

do not die as larvae, but they do have a slight but significant developmental delay of 0.6 days (Figure 2.3B), a typical phenotype for other insulin pathway mutants (Clancy *et al*., 2001; Rulifson *et al*., 2002). These results demonstrate that mutation of *dilp6* does not induce larval lethality during development, but it does appear to induce slight developmental delay on standard food. Perhaps interestingly, mutation of either *daw* or *dilp6* appears to increase time until pupation under the same dietary conditions, albeit to different degrees.

To test if increased *dilp6* levels are responsible for our diet-induced phenotypes in Activin-pathway mutants, we measured pupation of *dilp6; daw* double mutants. Double mutants and *dilp6* single mutants pupated at indistinguishable frequencies regardless of diet, demonstrating that mutation of *daw* does not induce larval lethality in a *dilp6*-mutant background (Figure 2.3A). Furthermore, concurrent mutation of *dilp6* completely rescued the slight delay shown by *daw* mutants that develop on yeast food; the *dilp6*-mutant background partially rescued the four-day delay shown by *daw* mutants on standard media (Figure 2.3B). Taken together, these data demonstrate that mutating *dilp6* completely rescues the diet-induced larval-lethality phenotype of *daw* mutants, and it partially rescues the nutrition-dependent phenotype of developmental delay. That is, the *daw* mutant phenotypes are largely suppressed in a *dilp6* mutant background. These results are consistent with our model of negative regulation of *dilp6* expression by diet-induced Dawdle signaling.

Discussion

Here we show that *daw* mutants express increased levels of an insulin-like gene, *dilp6*, and that an intact *dilp6* locus is at least partly responsible for the phenotype of delayed onset of metamorphosis. Interestingly, a recent report demonstrates that an increased level of insulin signaling results in precocious pupation in *Drosophila* (Walkiewicz and Stern, 2009). To investigate the discrepancy between these findings, we mimicked *daw* mutation by overexpressing *dilp6* in the fat body using the *cgGal4* driver. Consistent with previous reports, overexpression of *dilp6* induced precocious pupation, and it did not result in larval lethality, regardless of diet (Figures 2.3C and 2.3D). Taken together, these results suggest that, while a high level of *dilp6* expression is necessary for diet-induced lethality and delay in *daw* mutants, it alone is not sufficient for manifestation of these phenotypes. One explanation for the severe *daw* phenotype might be that Activin signaling controls expression of another gene (or genes), the misregulation of which would make the animal unable to respond normally to high levels of *dilp6*.

The search for such a gene might be aided by a finding from a recent study of Dawdle signaling, which shows that Dawdle can stimulate only one of three isoforms of the Activin receptor Baboon (See Chapter 1). This isoform, which differs from others at its ligand-binding domain, is strongly expressed in the fat body and gut (Chapter 1). This finding prompts our speculation that the other putative target of Activin signaling is expressed in the fat body or gut. Such an idea is plausible when one considers that such a gene would be implicated in coordination of diet and metabolism during development, a task in which these organs play profound roles.

The discovery of receptor isoform-specific signaling is also important regarding the dynamic levels of *daw* expression in response to diet. Daw is present in the hemolymph and therefore comes into contact with larval tissues that are patterned by other Activin ligands during development (Parker *et al*., 2006; Zhu *et al*., 2008). Because these tissues express other receptor isoforms, and not the one required for Dawdle (see Chapter 1), many developmental processes that are dependent on other Activin ligands can proceed unchanged, regardless of the levels of Dawdle. Only organs that must respond to dietary cues, such as the fat body and gut, express the receptor isoform necessary for Dawdle signaling and can therefore interpret diet-dependent increases in signal strength. By using this mechanism of differential expression of receptor isoforms, the tissues in the larva can distinguish between which Activin signals they receive, making them blind to other ligands whose levels may vary based on unrelated external cues.

Interactions between TGF-β-like signals and insulin-family members apparently date to early in animal evolution – both signaling families are implicated in the general cellular processes of metabolism and growth in organisms as diverse as worms and humans (Ren *et al*., 1996; da Graca *et al*., 2004; Andersson *et al*., 2008). In conjunction with previous work (Shaw *et al*., 2007), our findings demonstrate the evolutionarily conserved linkage between these two signaling pathways during development. Interestingly, work in the nematode system has also shown that a TGF-β signal links diet and metabolism (Greer *et al*., 2008), and work in mice demonstrates a negative regulation of insulin production by an Activin signal (Bertolino *et al*., 2008). Our work

spans these studies in a single organism to show that TGF-β has linked diet and metabolism via control of the insulin pathway throughout animal evolution.

Experimental Procedures

Drosophila *stocks and husbandry*.

The daw^{32} allele has been described previously (Serpe *et al*., 2006); the $smox^{f4}$ allele is a deletion mutant (Rachel Herder, O'Connor lab, personal communication; courtesy of L. Raftery). The Gal4 expression lines used in this report have been reported previously: *da* Gal4 (Bloominton stock 5460), *cg* Gal4 (Asha *et al*., 2003); we used the UAS *daw* line #9A3. Unless noted otherwise, in pupation assays "control" animals are a stock whose original *P* element in the *dilp6* locus has been precisely excised (Okamoto *et al*., in preparation).

"Yeast food" consists of a smear of 60% (w/v) brewers' yeast in distilled water on a plate of apple-juice agar. The nutritive value of the agar plate itself is not sufficient to support *Drosophila* development. "Standard *Drosophila* media" is a typical recipe of a cornmeal- and corn syrup-based food that also includes glucose, malt, and de-fatted yeast.

Embryos that were deposited on yeast paste were transferred to appropriate media as early first-instar larvae. Mutants were distinguished by their lack of fluorescently-labeled balancer chromosomes. Each experiment included three biological replicates per sample; pupation assays included 25 animals per replicate. All flies were reared at 25°C.

RNA extraction and RT-PCR.

RNA was extracted from early third-instar larvae using the Trizol ® reagent (Invitrogen); cDNA synthesis utilized the SuperScript III ® kit (Invitrogen). All reagents were used per the manufacturers' instructions. Low (24)-cycle semi-quantitative PCR was used to analyze levels of gene expression; all experiments were conducted using biological triplicates that each represented ten animals. PCR products were run on agarose gels and quantified using ImageJ software (http://rsbweb.nih.gov/ij/). An Actin57B PCR product was used as a loading control for each sample. In every case where a difference in gene expression is reported, all replicates of the sample with higher expression showed greater band intensities than all of the replicates of the sample with lower expression. Primers for *dilp6*: 5': CACTCTTAGTCATTCGTCATCGAGG; 3': TACTCTAGGGGACACTGCTGCTTG. Primers for *daw*: 5': ACAAACAGAACCGCACCGACAC; 3': TCATTCCGCTGGAGCAGTTG. Primers for Actin 57B: 5': GAAGTTGCTGCTCTGGTCGTTG; 3': GATGCCGCAGGATTCCATTC.

Chapter 3:
Characterization of a molecular interaction between the Activin and BMP pathways in *Drosophila melanogaster*.

Transforming Growth Factor Beta (TGF-β) signaling comprises a family of evolutionarily conserved growth and differentiation signals that are utilized by all animals. The molecular mechanism of TGF-β signal transduction is similar across the animal kingdom. TGF-β ligands, which are secreted as covalently linked dimers, bind transmembrane receptor kinases to form a signaling complex at the cell surface. This complex consists of the ligand pair and four receptors: two Type-I and two Type-II. Upon formation of this tetrameric receptor complex, the Type-II receptors phosphorylate the Type-I receptors, which then phosphorylate intracellular transcription factors called R-Smads. Phosphorylated R-Smads bind a related protein called a Co-Smad, which shuttles them to the nucleus where they accumulate to regulate diverse transcriptional events.

The TGF-β family includes two closely related signaling branches: the Bone Morphogenic Protein (BMP) branch and the TGF-β/Activin branch. Each ligand, Type-I receptor, and R-Smad in the family have been assigned to one of these signaling branches based on sequence and structure, and these assignments have been supported by countless functional data. However, the strong similarity between the two sub-families is apparent: they often share Type-II receptors and the Co-Smad in order to function. This close structural relationship raises questions about the possibility of signaling events that span the conventional branches of the family.

Several reports in recent years have described, in varying levels of detail, such a crosstalk signaling event. In humans, for instance, two reports demonstrate that ligands from the TGF-β/Activin sub-family ultimately stimulate BMP signaling, though the accounts differ in where exactly within the cascade the signal crosses from one branch to the other (Daly *et al*., 2008 and Liu *et al*., 2009). Another report in *Drosophila* describes a similar crosstalk event, but how the two pathways interact molecularly has yet to be addressed (Gesualdi and Haerry, 2007).

In this report we also describe an Activin-to-BMP crosstalk event in *Drosophila*, and we present several lines of evidence that address the molecular mechanism by which these related sub-families interact. We conclude that the *Drosophila* Activin Type-I receptor, Baboon, is capable of phosphorylating both the R-Smad of the Activin family (Smox) as well as the BMP R-Smad (Mad); our data suggest that these two proteins compete for activation by Baboon. This finding is consistent with the model presented by Liu *et al*. (2009) in the human system and demonstrates for the first time an evolutionarily conserved mechanism for trans-branch crosstalk in TGF-β signaling.

Results

Stimulating the Activin pathway phenocopies BMP overexpression in vivo *and induces phosphorylation of Mad* in vitro.

Drosophila third-instar larvae that are mutant for the Activin receptor *baboon* (*babo*) have small imaginal discs, including the wing disc (Brummel *et al*., 1999). However, larvae that are mutant for the R-Smad in the Activin pathway – Smox – do

not display a small-disc phenotype like *babo* mutants. Instead, they have overgrown wing discs, a phenotype that is strikingly similar to one that results from increased stimulation of the related BMP signaling pathway in the wing (Figures 3.1A and 3.1B; Burke and Basler, 1996). Therefore, the developing *Drosophila* wing provides an example where genetically altering the levels of two related signaling pathways can produce similar phenotypes, suggesting an interaction between these two pathways. We sought to determine the molecular nature of an interface between the Activin and BMP pathways in *Drosophila*.

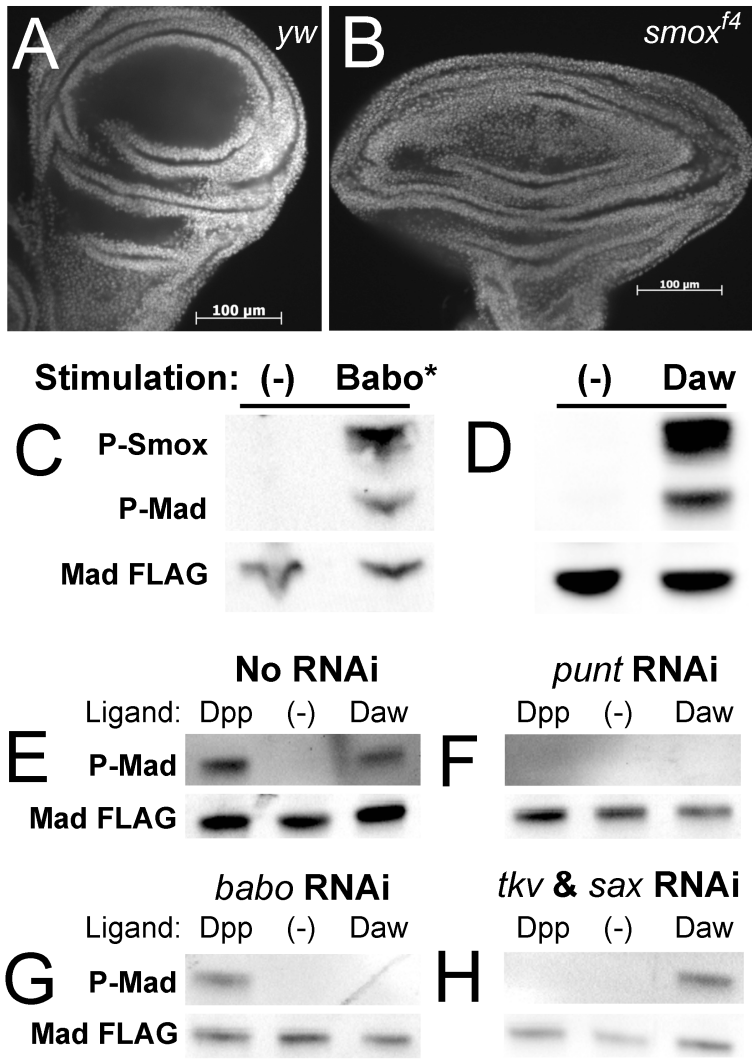
One clue about this interaction came from a cell culture-based signaling assay. In *Drosophila* S2 cells, expression of a constitutively active form of Baboon (Babo*) results in phosphorylation of both the Activin and BMP R-Smads, Smox and Mad (Figure 3.1C). To ensure that this phosphorylation of Mad is a property of the Baboon receptor itself and is not due to an artifact of the activating mutation in Babo*, we also exposed S2 cells to the Activin-like ligand Dawdle (Daw). These cells, which express only wild-type Baboon, also responded to Daw by phosphorylating both Smox and Mad (Figure 3.1D). These results suggest that an Activin ligand can stimulate the BMP pathway in *Drosophila* S2 cells.

BMP receptors, but not Baboon, are dispensable for Activin-induced BMP signaling.

Previous work has described an Activin-to-BMP crosstalk event in *Drosophila*, but the receptor responsible for the Activin-dependent phosphorylation of Mad remains unknown (Gesualdi and Haerry, 2007). For example, a previous report of a similar

Figure 3.1: *Stimulating the Activin pathway phenocopies increased BMP signaling both* in vivo *and in a cell-based signaling assay.* Third-instar *smox* mutant larvae have overgrown wing discs that strongly resemble discs with increased levels of BMP signaling (A and B). Both activated Baboon (Babo*) and the Activin ligand Dawdle can induce increased levels of P-Mad in S2 cells (C and D). Dawdle's ability to induce P-Mad depends on Baboon but not BMP Type-I receptors. Both the BMP ligand Dpp and Dawdle stimulate P-Mad in the presence of endogenous receptors in S2 cells (E), but neither has an effect on cells whose *punt* transcripts are targeted by RNAi (F). Depleting Babo levels has no effect on Dpp signaling, though Daw cannot induce phosphorylation of Mad in Babo's absence (G); lowered levels of the BMP receptors Tkv and Sax abolish Dpp signaling but have no effect on Daw stimulation (H). [Panels A and B are courtesy of Aidan Peterson.]

Figure 3.1:


A
yw
100 µm
B
smox^f4
100 µm
Stimulation: (-) Babo*
C
P-Smox
P-Mad
Mad FLAG
(-) Daw
D
No RNAi
Ligand: Dpp (-) Daw
E
P-Mad
Mad FLAG
punt RNAi
Dpp (-) Daw
F
babo RNAi
Ligand: Dpp (-) Daw
G
P-Mad
Mad FLAG
tkv & sax RNAi
Dpp (-) Daw
H

crosstalk event in human TGF-β signaling suggests that the phosphorylation of the BMP R-Smad is actually accomplished by a BMP receptor (Daly *et al*., 2008). In that model, the receptor signaling complex 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, another recent *in vitro* study demonstrates that a human TGF-β/Activin receptor is capable of phosphorylating BMP R-Smads directly, eliminating any role for a BMP receptor (Liu *et al*., 2009). These conflicting models have not been tested in the *Drosophila* system.

We sought to determine which of these mechanisms is responsible for the crosstalk that we observed in *Drosophila* S2 cells. To do this we combined a technique involving RNA interference (RNAi) with our cell-based signaling assay. We first treated cells with double-stranded (ds) RNA whose sequence matched a portion of the transcript of a TGF-β receptor, significantly reducing its expression. We then exposed these treated cells to Dawdle and measured levels of P-Mad. This RNAi-based assay allows determination of which receptors are necessary for Activin-induced phosphorylation of Mad, permitting elucidation of which of the above mechanisms is responsible for Activin-BMP crosstalk in *Drosophila*.

Similar to data shown in Figure 3.1D, cells whose receptors were not targeted by RNAi responded to both the BMP ligand Dpp and the Activin ligand Daw by phosphorylating Mad (Figure 3.1E). Reducing expression of the Type-II receptor Punt – the only Type-II receptor in S2 cells – by RNAi eliminated response to both ligands

(Figure 3.1F). Cells whose Baboon was depleted by RNAi phosphorylated Mad in response to Dpp but not in response to Daw (Figure 3.1G). In contrast, cells that were treated with dsRNA that targeted BMP receptors Saxophone and Thickveins still phosphorylated Mad upon exposure to Daw, even though they had no response to Dpp (Figure 3.1H). (Knockdown of these BMP receptors was confirmed by Western blot, as shown in Figure 3.S1.)

These results suggest 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 crosstalk. These findings are inconsistent with the model of heteromeric Type-I receptor complexes proposed by Daly *et al*. (2008). Our data suggest that crosstalk in *Drosophila* is more consistent with the model of direct phosphorylation of BMP Smads by TGF-β/Activin receptors in humans that has been proposed by Liu *et al*. (2009).

Drosophila *Smads compete for phosphorylation by Baboon.*

Because it is necessary and sufficient for Dawdle-stimulated phosphorylation of both Smox and Mad (Figure 3.1 and Brummel *et al*., 1999), it is curious that Baboon has been labeled as a member of the Activin pathway only. Perhaps Baboon's affinity for Mad has gone unnoticed because of a phenomenon highlighted in Figure 3.2A. In this time-course experiment, cells were exposed to Daw and harvested after incubation for 1.5 and 4 hours. A Western blot of these samples shows that levels of P-Smox increased rapidly after stimulation, while levels of P-Mad did not accumulate until the

Figure 3.S1: *RNAi in S2 cells effectively reduces expression of overexpressed receptors.* Endogenous levels of Sax are barely detectable by Western blot using an α-Sax antibody, but overexpression (O/E) of *sax* produces a robust band at the expected molecular weight (A). RNAi against *sax* reduces levels of overexpressed protein to below the threshold of detection (O/E + RNAi). Similar results are obtained using a construct that expresses FLAG epitope-tagged Thickveins (B). Again, RNAi against the receptor reduces levels of overexpressed protein to near the threshold of detection. In both cases (A and B), RNAi reduced levels of overexpressed protein by over 98% (quantification not shown). Background FLAG staining is used as a loading control.

Figure 3.S1:

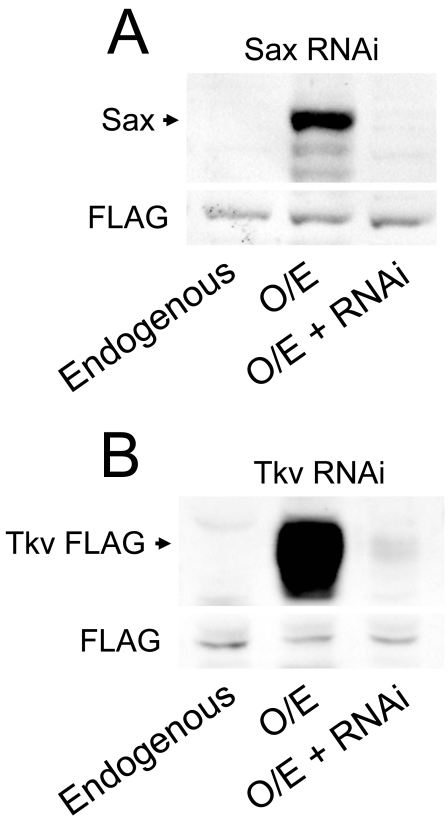
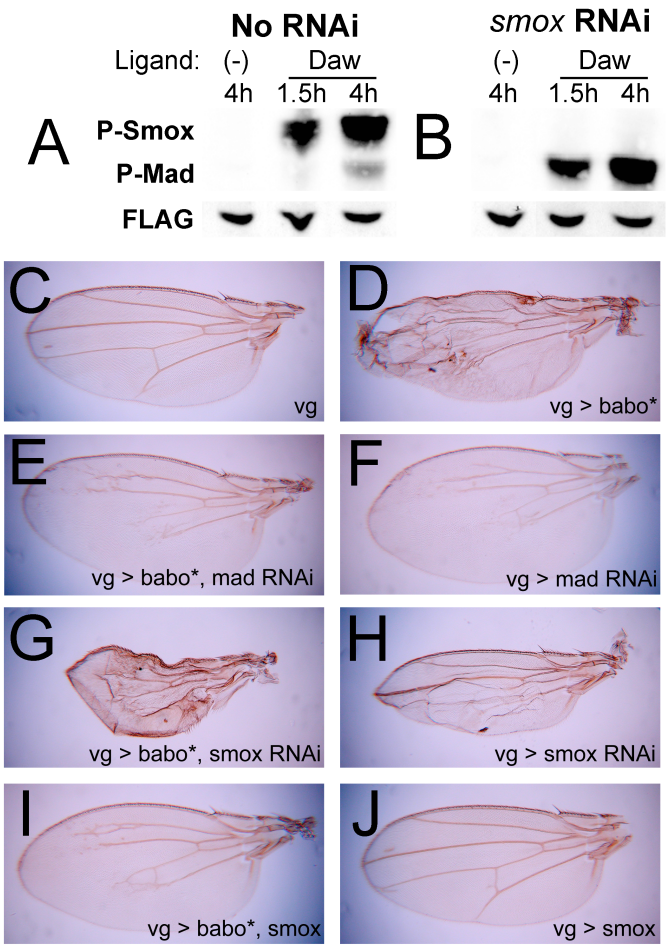

A
Sax RNAi
Sax
FLAG
Endogenous
O/E
O/E + RNAi
B
Tkv RNAi
Tkv FLAG
FLAG
Endogenous
O/E
O/E + RNAi

Figure 3.2: *Smox levels affect Baboon's ability to induce phosphorylation of Mad.* In S2 cells that overexpress both Smox and Mad, levels of P-Mad increase slowly after stimulation by Dawdle (A). When Smox is targeted by RNAi, however, P-Mad levels increase rapidly after identical stimulation (B). In the *Drosophila* wing, overexpressing a constitutively activated form of Baboon results in a crumpled-wing phenotype typical of BMP overexpression (C). Targeting expression of Mad by RNAi suppresses this phenotype (E) and gives a wing that is indistinguishable from one that expresses Mad RNAi alone (F). Concurrent expression of Babo* and Smox RNAi strengthens the BMP-dependent crumpling phenotype (G) to a level of severity greater than expressing Babo* or Smox RNAi alone (D and H). Overexpressing Smox, like targeting Mad expression by RNAi, suppresses the Babo* phenotype and mimics Mad loss of function (I), even though Smox overexpression alone has no effect on wing patterning (J). Panels C-J are courtesy of Aidan Peterson (O'Connor Lab). The loading control in panels A-B depicts background bands stained by the α-FLAG antibody.

Figure 3.2:


No RNAi
smox RNAi
Ligand:
(-)
Daw
4h
1.5h
4h
A
P-Smox
P-Mad
FLAG
B
C
vg
D
vg > babo*
E
vg > babo*, mad RNAi
F
vg > mad RNAi
G
vg > babo*, smox RNAi
H
vg > smox RNAi
I
vg > babo*, smox
J
vg > smox

later time point. These data hint that Baboon has a higher affinity for Smox than it has for Mad. Such a difference might explain why Baboon's role in BMP signaling was not initially appreciated.

To test a model of competitive binding between R-Smads, we manipulated levels of Smox – Baboon's hypothetically preferred substrate – in the cells used in the signaling assay. As mentioned, in S2 cells that were transfected with both Smads, P-Smox levels rapidly increased after Daw stimulation; P-Mad accumulation was delayed (Figure 3.2A). However, in cells whose Smox was targeted by RNAi, levels of P-Mad increased more rapidly after identical stimulation (Figure 3.2B). These results, which suggest that Smox inhibits an interaction between Baboon and Mad, are consistent with a model involving competitive binding of Smads.

To further test this competition model *in vivo*, we employed a similar RNAi-based approach using the *Drosophila* wing. Overexpression of a constitutively active form of Baboon (Babo*) results in a crumpled wing that is reminiscent of increased BMP signaling (Figures 3.2C and 3.2D; Bangi and Wharton, 2006). Indeed, concurrently reducing Mad expression by RNAi and expressing Babo* suppresses the wrinkling seen with Babo* alone; the resulting wing is indistinguishable from a wing expressing Mad shRNA only, suggesting that increased BMP signaling is the cause of the crumpling phenotype (Figures 3.2E and 3.2F). Consistent with a model of Smads' competition for phosphorylation by Baboon, simultaneous decrease of Smox levels by RNAi and expression of Babo* exacerbates the phenotype, leading to a more severely crumpled wing than either Smox RNAi or Babo* produces alone (Figures 3.2G and

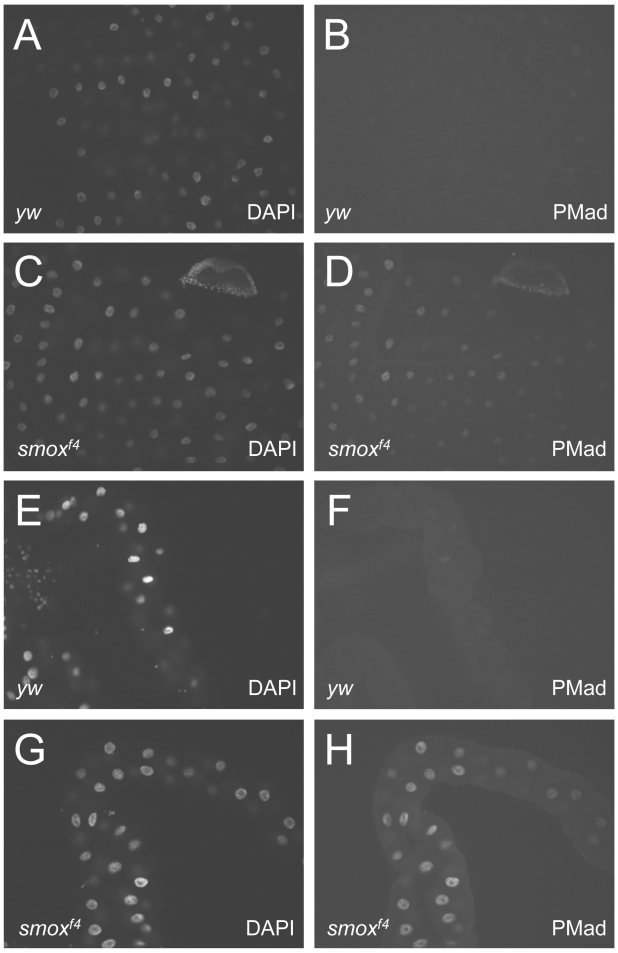
3.2H). Furthermore, overexpressing Smox suppresses BMP- and Babo*-dependent crumpling of the wing. This wing gives a loss-of-veins phenotype that is strikingly similar to that of Mad RNAi (Figure 3.2I). Smox overexpression alone does not have a noticeable effect on wing patterning (Figure 3.2J).

Taken together, these experiments in the adult wing demonstrate that altering the level of Smox affects Baboon's propensity to phosphorylate Mad. Namely, we see a dose-dependent effect of Smox's ability to out-compete Mad for phosphorylation by Baboon, even to the extent where Smox overexpression mimics Mad loss of function. These results further support a model of direct phosphorylation of a BMP R-Smad by an Activin receptor, with competitive binding governing relative rates of phosphorylation of the two species of R-Smad.

Crosstalk can also be observed in the presence of endogenous levels of Activin signaling in the developing *Drosophila* larva. Wild-type levels of P-Mad antibody staining in the fat body and gastric ceca of the third-instar larva are shown in Figure 3.3B and 3.3D, respectively. In animals mutant for *smox*, however, P-Mad levels in those tissues are increased (Figures 3.3F and 3.3H), which is consistent with a model involving Baboon's increased phosphorylation of Mad in the absence of its preferred substrate, Smox. This finding suggests that Baboon is capable of robustly phosphorylating Mad when stimulated by Activin ligands at endogenous levels *in vivo*, at least in the absence of Smox.

Figure 3.3: *Levels of P-Mad increase in various tissues of the third-instar larva.* Fat bodies from *yw* and *smoxf4* mutants were co-stained with DAPI and PMad antibody (A-D). In *yw* controls, little PMad is visible in nuclei of fat body cells (B), but *smox* mutants have high levels of PMad (D). *yw* and $smox^{f4}$-mutant gastric cecae were stained with DAPI and PMad antibody (E-H). PMad levels are increased (H) compared to *yw* controls (F). To ensure equivalent staining across samples, all tissues were exposed to aliquots of a singular DAPI/antibody mix and were imaged within minutes of one another at identical camera settings. [Images courtesy of Michael O'Connor.]

Figure 3.3:


A
yw
DAPI
B
yw
PMad
C
smox^f4
DAPI
D
smox^f4
PMad
E
yw
DAPI
F
yw
PMad
G
smox^f4
DAPI
H
smox^f4
PMad

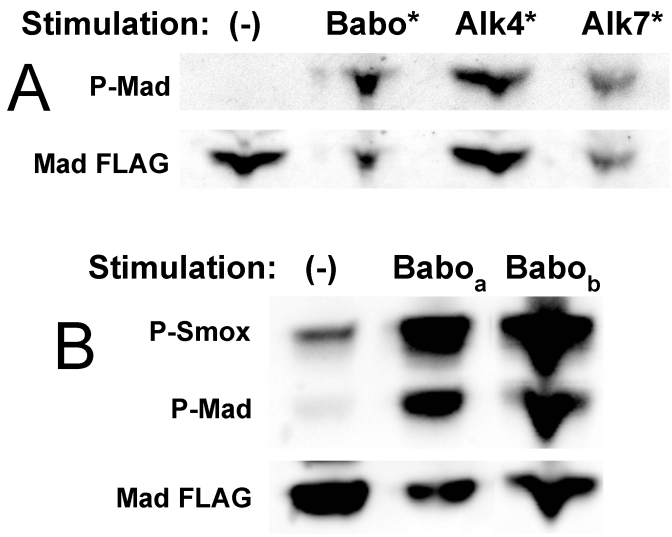
Activin/TGF-β receptors from humans and all isoforms of Baboon can phosphorylate Smox and Mad.

Many reports have demonstrated that members of the same TGF-β-family pathway are interchangeable across species. For example, expression of a human BMP ligand can rescue mutation of a *Drosophila* BMP ligand *in vivo* (Padgett *et al.*, 1993). We were curious if trans-species crosstalk was also possible. We tested whether activated TGF-β/Activin Type-I receptors from humans could phosphorylate Mad, the *Drosophila* BMP R-Smad. When transfected into S2 cells, the constitutively active forms of human Activin receptors Alk4 and Alk7, like Babo*, could induce phosphorylation of both Smox and Mad (Figure 3.4A). This interaction highlights the strong evolutionary conservation of the described crosstalk phenomenon.

Recent work has uncovered functional differences between isoforms of the Activin receptor in *Drosophila* (see Chapter 1). These isoforms have divergent extracellular domains and identical kinase domains. We wondered if the ability to phosphorylate Mad might be affected by these differences, perhaps via steric interactions outside the cell. Only one isoform, $Baboon_c$, is activated by the Activin ligand Dawdle in S2 cells (see Chapter 1). We sought to determine whether the other isoforms of Baboon, $Babo_a$ and $Babo_b$, were also able to stimulate crosstalk. When overexpressed in S2 cells, both of these isoforms are activated ligand-independently, and they are capable of phosphorylating both Smox and Mad (Figure 3.4B). This result is consistent with a previous report suggesting that the kinase domain's L45 loop – a

Figure 3.4: *Human Activin receptors and all isoforms of Baboon can initiate crosstalk*. Constitutively active versions of human Activin receptors Alk4 and Alk7 (Alk4* and Alk7*), like Babo*, induce phosphorylation of both *Drosophila* Smox and Mad in S2 cells (A). Overexpressing wild-type $Baboon_a$ or $Baboon_b$ in S2 cells leads to ligand-independent activation and results in phosphorylation of both Smox and Mad above background levels (B).

Figure 3.4:


Stimulation: (-) Babo* Alk4* Alk7*
A
P-Mad
Mad FLAG
Stimulation: (-) Babo$_a$ Babo$_b$
B
P-Smox
P-Mad
Mad FLAG

portion of the protein unchanged between Baboon isoforms – is primarily responsible for substrate recognition (Feng *et al*., 1997 and Liu *et al*., 2009). Extracellular differences between isoforms seem to be unimportant with regard to crosstalk.

Susceptibility of Activin-to-BMP crosstalk to the drug Dorsomorphin, a BMP inhibitor.

The drug Dorsomorphin is an inhibitor of BMP signaling in several animal systems (Yu *et al*., 2008). We sought to determine whether Baboon also would be unable to phosphorylate Mad after treatment with Dorsomorphin, or whether the drug was specific to interactions between BMP receptors and R-Smads.

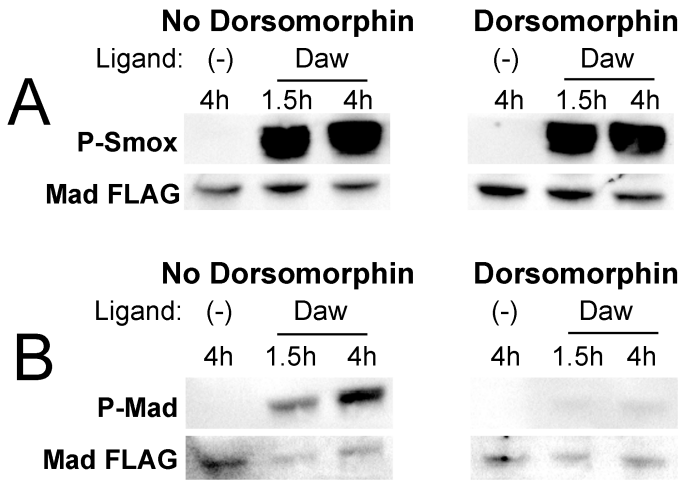
Regardless if they were treated with Dorsomorphin, cells were able to respond to Daw by phosphorylating Smox (Figure 3.5A), which is consistent with the drug's established role as only a BMP inhibitor. However, while cells that were not incubated with Dorsomorphin phosphorylated Mad after exposure to Dawdle, cells that were treated with the drug did not accumulate P-Mad after identical stimulation (Figure 3.5B). These results indicate that the BMP inhibitor Dorsomorphin affects Activin-to-BMP crosstalk in *Drosophila*. Similar results have been found in the human system (Liu *et al*., 2009).

Discussion

The experiments described in this report demonstrate that activation of the *Drosophila* Activin receptor, Baboon, can induce phosphorylation of the R-Smad in the BMP pathway, Mad. While such a link has previously been observed in flies (Gesualdi

Figure 3.5: *Dorsomorphin, a small molecule that inhibits BMP signaling, disrupts Baboon's phosphorylation of Mad but not Smox in S2 cells*. Exposing cells to Dawdle for either 1.5 or 4 hours resulted in increased levels of P-Smox regardless of Dorsomorphin treatment (A). Exposure to 10μM Dorsomorphin inhibited cells' phosphorylation of Mad in response to Daw stimulation (B).

Figure 3.5:


No Dorsomorphin
Dorsomorphin
Ligand: (-) Daw (-) Daw
4h 1.5h 4h 4h 1.5h 4h
A
P-Smox
Mad FLAG
No Dorsomorphin
Dorsomorphin
Ligand: (-) Daw (-) Daw
4h 1.5h 4h 4h 1.5h 4h
B
P-Mad
Mad FLAG

and Haerry, 2007), our study is the first to explore the molecular nature of this interaction in *Drosophila*. Our data suggest a mechanism of direct interaction between the Activin and BMP signaling pathways that appears to be conserved from flies to humans. The support for this direct-phosphorylation mechanism is manifold.

First, Baboon is the only necessary and sufficient Type-I receptor for this signaling event, and BMP receptors are neither necessary nor sufficient for the process. These two findings allow us to reject a model involving heteromeric receptor complexes and roles for BMP receptors in crosstalk. Second, we have presented both *in vitro* and *in vivo* data that show an interplay between Smox and Mad; the nature of this interaction suggests competition for an interaction with Baboon. Indeed, under the alternative model based on heteromeric receptor complexes, altering levels of one receptor's substrate would not affect the other receptor's ability to phosphorylate its target. Finally, a model of direct crosstalk is consistent with the mutant and phenotypes originally observed in the developing wing disc (Figure 3.1A and B), which match the phenotype of BMP overexpression (Burke and Basler, 1996): *smox* mutants may have overgrown discs because of Baboon's propensity to activate the BMP pathway in the absence of its preferred substrate, Smox; this phenotype would mimic overexpression of BMP. Ultimately, these results describe a previously unappreciated molecular interaction between the Activin and BMP signaling pathways in *Drosophila*, and highlight a mechanism that is evolutionarily conserved from flies to humans.

Interestingly, under no circumstance have we observed a BMP ligand or activated BMP receptor stimulating phosphorylation of Smox (Figures 3.1, 3.2, and data

not shown). This finding is consistent with published data in flies suggesting that crosstalk is unidirectional: an Activin-to-BMP interaction is robust, but no BMP-to-Activin link exists at the molecular level (Gesualdi and Haerry, 2007). This observation may give insight into the evolutionary origins of the separate Activin and BMP pathways in eumetazoans: is one pathway a duplication of the other? Even relatively simple animals like cnidarians have TGF-β signaling, but they appear to have only one branch of the family, and the sequences of the pathway members suggest that they compose an ancestral BMP branch. Proteins resembling R-Smads of the Activin family appear only later in animal evolution (Newfeld and Wisotzkey, 2006). One could envision a scenario early in animal evolution where a newly duplicated receptor – the founding Activin receptor, perhaps – would both retain its ability to phosphorylate the original BMP R-Smad and develop an affinity for a divergent Activin R-Smad. There might not have been a selection pressure for the original BMP receptor to similarly adopt a new molecular function. However, without experiments involving receptors from these ancestral animals, at this point such an evolutionary relationship is merely open to conjecture.

Our results show that Activin-to-BMP crosstalk in *Drosophila* is inhibited by the drug Dorsomorphin, a known BMP inhibitor that also blocks similar crosstalk in the human system (Liu *et al*., 2009). These results are perhaps inconsistent with the accepted model of Dorsomorphin's mechanism of action: that of a kinase inhibitor. As shown in Figure 3.5A, the drug does not compromise Baboon's ability to phosphorylate Smox. If Dorsomorphin were indeed a kinase inhibitor, one would not expect it to

inhibit Baboon substrate-specifically. Perhaps the drug gleans its specificity for BMP signaling because of a role disrupting kinase-substrate binding instead of disrupting enzymatic activity itself. Further work may be necessary to reconcile our and others' observations with Dorsomorphin's currently hypothesized mechanism of inhibition.

To date, all studies of TGF-β crosstalk have yet to uncover an *in vivo* process that is regulated by an Activin-induced BMP signal. In a genetic system like *Drosophila*, such a process would be revealed by a common phenotype of mutants from each of the two signaling pathways (*e.g.*, matching phenotypes from an Activin ligand and a BMP R-Smad). Such a finding would be important to establish for the first time biological relevance for these crosstalk events. To that end, the discovery of inter-pathway signaling may have important implications for the field of TGF-β research. For example, a phenotype displayed by an animal mutant for an R-Smad may have been associated with that R-Smad's signaling family by default, when actually the signal might have come from another family's ligand and receptor. Such an instance could be conceivable in a biological system, like early vertebrate development, where both the BMP and Activin branches appear to be working in concert to regulate related processes. Similarly, both branches of the TGF-β family have been implicated in the progression of human disease – perhaps these pathways are not actually working in parallel but are acting convergently. Such reevaluation of data may bring to light previously unappreciated connections between the two TGF-β signaling branches in multiple organisms.

Experimental Procedures

Immunohistochemistry and Antibodies

Tissues from wandering third-instar larvae were fixed in 3.7% formaldehyde in PBS for 20 minutes, washed in PBS-Triton (0.1%) and treated with P-Mad antibody (gift from E. Leof; 1:200) overnight at 4°C, and then exposed to secondary antibodies (Alexa Fluor 488 or 555; 1:200) at room temperature for two hours.

Cell-based Signaling Assays and Western Blots

The S2 signaling assay has been described in Chapter 1. Briefly, conditioned media was collected from cells that expressed a construct encoding Dawdle. This media was then mixed 1:1 with cells that had been transfected with a combination of Smads and that were 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 for BMP stimulation. Constructs encoding different isoforms of Baboon have been described in Chapter 1.

RNAi treatment of S2 cells has also been described in Chapter 1. Primers that were used to make PCR products for T7 transcription templates against *tkv*, *sax*, and *punt* have been reported previously (Shimmi and O'Connor, 2003). Primers used to make T7 templates for *smox* RNAi have the following sequences: 5': TAATACGACTCACTATAGGGAACTGTGTGTGTGCTCCTTGCG; 3': TAATACGACTCACTATAGGGGTCCTCGTTGCCCTTTTTGAG. Templates for *babo* RNAi were made using the following primers: 5':

TAATACGACTCACTATAGGGACCTGCGCTCCGGCTAATCTTCC; 3': TAATACGACTCACTATAGGGACGGATGATAGCCACAAACTCCC.

Cells that were treated with Dorsomorphin were exposed to a concentration of 10 μM with rolling in their own media for 1 hour before they were used in the signaling assay. Control samples were rolled similarly without drugs prior to stimulation.

After the signaling assay, cells were pelleted, resuspended in 1X sample buffer, and boiled. Samples were loaded into NuPAGE© 4-12% Bis-Tris Gels (Invitrogen) and transferred to PVDF membrane (Bio-Rad) after electrophoresis. Membranes were probed with primary antibodies against phosphorylated Mad (1:1000, gift from E. Leof), phosphorylated Smox (1:1000, Cell Signaling), the FLAG epitope (1:2000, Sigma (M2)), or Saxophone (1:1000, Fabgennix). Membranes were exposed to secondary antibodies (1:2000, Rockland (IRDye)) and imaged on the Odyssey Infrared Imaging system.

Signaling Assays in the Drosophila *wing*

The *vestigial* (*vg*) GAL4 driver (Bloomington stock #8229) induced expression of several UAS expression constructs in the developing wing. *babo** constructs have been reported previously (Brummel *et al*., 1999), as has the *smox* overexpression line (Ting *et al*., 2007). RNAi lines were made using the pUAST-R57 vector (http://www.shigen.nig.ac.jp/fly/nigfly/about/aboutRnai.jsp) and included gene-specific sequences spanning bases 1264:1700 of the *mad* transcript (U10328; insertion line 6C3) and 1469-1928 of the Smox (AF101386; insertion line 25A3 "sunspot") message. P-element-mediated insertions were made using standard techniques.

Wings from adult females were dissected in ethanol and mounted in Canada balsam:wintergreen oil under a coverslip for light microscopy. All flies were reared on standard medium at 25° C.

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