

# Neuroendocrine regulation of *Drosophila* metamorphosis requires TGF $\beta$ /Activin signaling

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## SUMMARY

In insects, initiation of metamorphosis requires a surge in the production of the steroid hormone 20-hydroxyecdysone from the prothoracic gland, the primary endocrine organ of juvenile larvae. Here, we show that blocking TGF $\beta$ /Activin signaling, specifically in the *Drosophila* prothoracic gland, results in developmental arrest prior to metamorphosis. The terminal, giant third instar larval phenotype results from a failure to induce the large rise in ecdysteroid titer that triggers metamorphosis. We further demonstrate that activin signaling regulates competence of the prothoracic gland to receive PTTH and insulin signals, and that these two pathways act at the mRNA and post-transcriptional levels, respectively, to control ecdysone biosynthetic enzyme expression. This dual regulatory circuitry may provide a cross-check mechanism to ensure that both developmental and nutritional inputs are synchronized before initiating the final genetic program leading to reproductive adult development. As steroid hormone production in *C. elegans* and mammals is also influenced by TGF $\beta$ /Activin signaling, this family of secreted factors may play a general role in regulating developmental transitions across phyla.

**KEY WORDS:** TGF $\beta$ , Activin, Ecdysone, Insulin, PTTH, Prothoracic gland

## INTRODUCTION

In many multicellular organisms, developmental transitions are necessary for proper progression from the juvenile stage to adulthood. In humans, for example, puberty initiates a series of physical, morphological and physiological changes that culminate in an adult capable of reproduction. Likewise, insects, particularly those in the holometabolous group, undergo a process known as metamorphosis that transforms sexually immature individuals into breeding adults. Both puberty and insect metamorphosis rely on steroid hormones that systemically coordinate gene expression changes that change the physiology and morphology of the whole animal. (Mauras et al., 1996; Sisk and Foster, 2004; Gilbert et al., 1997; Gilbert et al., 2002).

During larval and pupal stages of insect development, the prothoracic gland (PG) is the major endocrine organ in which biosynthesis of the insect steroid hormone ecdysone (E) takes place. The production of E in the PG requires the action of the enzymes encoded by the Halloween gene family (Gilbert et al., 2002; Gilbert, 2004). With the exception of *neverland* (*nvd*) (Yoshiyama et al., 2006) and *shroud* (*sro*) (Niwa et al., 2010), all the Halloween genes identified so far including *spook* (*spo*), *spookier* (*spok*), *phantom* (*phm*), *disembodied* (*dib*) and *shadow* (*sad*) code for cytochrome P450 proteins that sequentially convert cholesterol to E (Warren et al., 2002; Warren et al., 2004). Once made and released from the PG, E is rapidly converted in peripheral tissues to 20-hydroxyecdysone (20E), the active derivative of E, by Shade, another P450 enzyme (Petryk et al., 2003). In *Drosophila* and other holometabolous insects, a large

increase in the titer of E/20E generated at the end of the third-instar stage triggers metamorphosis by inducing the expression of the downstream target genes (Andres and Thummel, 1992; Baehrecke, 1996).

Elucidating the mechanisms that modulate and time the production of the E/20E titer is crucial to understanding several aspects of developmental timing in insects. In the past few decades, a great deal of work has centered on understanding the function of various neuropeptides in modulating the timing and size of the E/20E pulses during development (for a review, see Marchal et al., 2010). Key among these is prothoracicotrophic hormone (PTTH), a brain-derived factor that can trigger a surge in the production and secretion of E from the PG (Gilbert et al., 1997; Gilbert et al., 2002). Recently, Torso, a receptor tyrosine kinase that activates the Ras/Raf/ERK pathway during embryonic terminal development in response to Trunk (Li, 2005; Perrimon et al., 1995), a distant relative of PTTH, has been identified as the PTTH receptor (Rewitz et al., 2009). Knockdown of Ras, Raf or ERK in the PG gives rise to a developmental delay phenotype similar to that caused by PTTH-neuron ablation, suggesting that this MAPK pathway is also used to transduce the PTTH signal in the PG (Rewitz et al., 2009). Consistent with this view, over-activation of Ras or Raf in the PG accelerates larval development caused by increased E production (Caldwell et al., 2005). One potential mechanism for how the PTTH pathway promotes E biosynthesis is by stimulating transcription of E biosynthetic enzymes, as PTTH neuron ablation leads to marked reduction in the steady-state mRNA levels of several E biosynthetic enzymes prior to metamorphosis (McBrayer et al., 2007).

In addition to PTTH signaling, the highly conserved insulin pathway has also been implicated in controlling E synthesis (Colombani et al., 2005; Mirth et al., 2005; Caldwell et al., 2005). In *Drosophila*, there are seven insulin-like peptides (DILPs) that activate a single insulin-like receptor (InR) (Mirth and Riddiford, 2007; Wu and Brown, 2006). The most intensively studied DILPs, DILP2, DILP3 and DILP5, are expressed in two clusters of seven

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neurosecretory cells in the brain known as insulin-producing cells (IPCs) (Ikeya et al., 2002). PG-specific expression of a dominant-negative Pi3K, a downstream effector of InR, or PTEN, a phosphatase that antagonizes Pi3K (Goberdhan et al., 1999), results in low ecdysteroid titers (Colombani et al., 2005; Mirth et al., 2005), slow development (Caldwell et al., 2005) and increased body size. By contrast, PG-specific expression of either wild-type or activated Pi3K, or hyperactivation of insulin signaling in IPCs reduces body size and advances the onset of metamorphosis, probably because of precocious ecdysone synthesis (Colombani et al., 2005; Caldwell et al., 2005; Walkiewicz and Stern, 2009). These observations suggest that insulin signaling influences both growth rate and metamorphic timing by acting on the PG to affect E synthesis.

The ability of nutrient signals to regulate developmental transitions is not restricted to insects. Pubertal onset in humans is intimately linked to nutritional status, with precocious puberty becoming more prevalent in well-fed western societies (Kaplowitz et al., 2001). Likewise, in *C. elegans* the formation of dauer, an arrested larval state that allows survival through times of stress, is induced by nutrient deprivation. Recently, daftachronic acids have been identified as the steroid hormones controlling dauer diapause (Motola et al., 2006). The production of daftachronic acids involves G protein-coupled receptors that act as environmental sensors to control the production of TGF $\beta$ -type and insulin peptides that, either directly or indirectly, regulate hormone biosynthesis (for a review, see Fielenbach and Antebi, 2008).

The involvement of TGF $\beta$ -type factors in regulating this important developmental transition in *C. elegans* and the conservation of the pathway in regulating many developmental and physiological processes suggest that TGF $\beta$  signals might be general regulators of steroid hormone production. In *Drosophila*, the Activin/TGF $\beta$  family comprises the ligands Act $\beta$ , Dawdle (Daw) and Myoglianin (Myo), while the BMP family ligands are represented by Decapentaplegic, Glass bottom boat and Screw. The family assignment of the ligand Maverick (Mav) is unclear based on either sequence or signaling properties (Nguyen et al., 2000). Both ligand families probably use a common set of type II receptors, coded for by *punt* and *wishful thinking*, whereas pathway specificity is mediated by the type I receptor encoded by *baboon* (*babo*), in the case of Activins and either *thickveins* or *saxophone*, in the case of BMPs. Downstream signal transduction is mediated by the R-Smads, dSmad2 (Smox – FlyBase) for Activin ligands or Mad for BMPs, whereas Medea serves as a common co-Smad that complexes with the dSmad2 or Mad to regulate target gene transcription.

Here, we demonstrate that the TGF $\beta$ /Activin pathway plays a pivotal role in regulating developmental transitions in *Drosophila*. We found that knockdown of various Activin pathway components in the PG causes developmental arrest at the last larval stage owing to the absence of the E/20E peak required for eliciting metamorphosis. The defect in producing the E/20E peak is caused, in part, by loss of PTH and insulin signal reception in the PG, which primarily affect transcription and translation of E biosynthetic enzymes, respectively. We conclude that Activin signaling is required in the PG to maintain competence of the endocrine gland to respond to the metamorphic stimulation by PTH and insulin signaling pathways, and that each of these pathways regulates E biosynthesis by distinct molecular mechanisms.

## MATERIALS AND METHODS

### *Drosophila* strains and husbandry

All *Drosophila* stocks and crosses were maintained on the standard cornmeal food at 25°C. The strains used include *daw-Gal4* (Zhu et al., 2008), *Act $\beta$ -Gal4* (Ting et al., 2007), *UAS-babo(CA)* (Brummel et al., 1999), *phm-Gal4* (Ono et al., 2006) and *UAS-Rheb<sup>EP50.084</sup>* (Chang and Neufeld, 2009). *UAS-dSmad2* RNAi (#14609 and #105687), *UAS-babo* RNAi (#3825 and #853), *UAS-punt* RNAi (#848 and #37279) and *UAS-medea* RNAi (#19688 and #19689) were obtained from the Vienna *Drosophila* RNAi center. Additional RNAi *babo*, *mad*, *punt* and *dSmad2* lines were generated for this study (details available upon request). *UAS-nuGFP* (#4775), *UAS-Akt* (#8191) and *UAS-InR* (#8262) were obtained from the Bloomington *Drosophila* Stock Center. *UAS-Daw* and *UAS-Act $\beta$*  were generated in a *y,w<sup>1118</sup>* background by using standard methods. *UAS-Ras<sup>V12</sup>* and *tub>PH-GFP* are kind gifts from Dr Neufeld (UMN, USA).

### Staging larvae

Females were allowed to lay eggs on apple juice agar plates for 6 hours. Larvae were synchronized at the L2-to-L3 transition. The newly molted third instar larvae were collected, transferred into vials containing standard cornmeal food, and allowed to develop to the desired time points. The larvae were reared inside an insulated moist chamber at 25°C under a 12 hour light/dark cycle.

### 20E feeding and whole-body ecdysteroid titer determination

The 20E feeding experiment was performed as previously described (McBrayer et al., 2007). For ecdysteroid determination, 50 larvae per genotype per time point were collected and frozen at –80°C. The procedures for ecdysteroid extraction and determination by radioimmunoassay can be found elsewhere (Warren et al., 2006).

### In situ hybridization

For examining endogenous gene expression, in situ hybridization was carried out as described by Chávez et al. (Chávez et al., 2000). cDNAs were used to generate both the antisense and sense probes according to standard methods.

### Immunofluorescence

Brain-ring gland complexes were dissected in pre-chilled PBS, fixed in 3.7% formaldehyde in PBS for 20 minutes at room temperature and then washed 3 $\times$  in PBS with 0.1% Triton X-100 (PBT). The primary antibodies used in this study include: mouse anti-Coracle, 1:200 (Hybridoma, C566.9); rabbit anti-Phm, 1:1000 (Parvy et al., 2005); rabbit anti-Dib, 1:1000 (Parvy et al., 2005); rabbit anti-Sad, 1:1000 (raised against the peptide sequence CIRVQEDQRRPHDEA); guinea pig anti-Spo/Spok, 1:100 (raised against peptide sequence CDWSQLQKRRNLARRH); mouse monoclonal anti-MAPK activated, 1:10,000 (Sigma, M8159); and rabbit anti-dFOXO, 1:1000 (Puig et al., 2003). Guinea pig anti-DIMM antibody (1:500) is a kind gift from P. Taghert (WUSTL, MO, USA). Fluorescent-conjugated secondary antibodies (ALEXA FLUOR) were purchased from Invitrogen and were used at a 1:500 dilution. DNA was stained by DAPI (1  $\mu$ g/ml, Sigma) in PBT for 5 minutes. All primary and secondary antibodies were diluted in PBT. Primary antibody incubation was carried out at 4°C overnight and secondary antibody incubation was performed at room temperature for 2 hours. DNA was stained by DAPI. Images were acquired using a Zeiss LSM710 laser scanning confocal microscope.

### qRT-PCR

For each sample, 10 ring gland-brain complexes were dissected and stored in TRIzol Reagent (Invitrogen). Total RNA was isolated by using RNeasy mini kit and RNase-Free DNase Set (QIAGEN). Reverse transcription was carried out by using ThermoScript RT PCR System (Invitrogen). qRT-PCR was performed on Roche LightCycler 480 System (Roche) using LightCycler DNA Master SYBR Green I (Roche). The *Drosophila* ribosomal protein L23 (*rpL23*) was used as a reference gene to normalize gene expression levels. Three independent biological samples for each

genotype at each specific time point were collected for statistical analysis. The qRT-PCR primers used are listed in Table S2 in the supplementary material.

### Western blotting

Western blotting was carried out according to standard protocols. The primary antibodies include mouse anti- $\alpha$ -tubulin, 1:5000 (Sigma, # T9026), rabbit anti-PHM, 1:1000 (Parvy et al., 2005), rabbit anti-DIB, 1:1000 (Parvy et al., 2005), rabbit anti-Sad, 1:1000 (raised against the peptide sequence CIRVQEDQRRPHDEA), guinea pig anti-Spok, 1:5,000 [raised against the peptide sequence that matches the Spok protein (FBgn0086917) from amino acid 356 to 485]. The secondary antibodies used are IRDye 700 and 800 (1:10,000), and blots were scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences).

### Statistical analysis

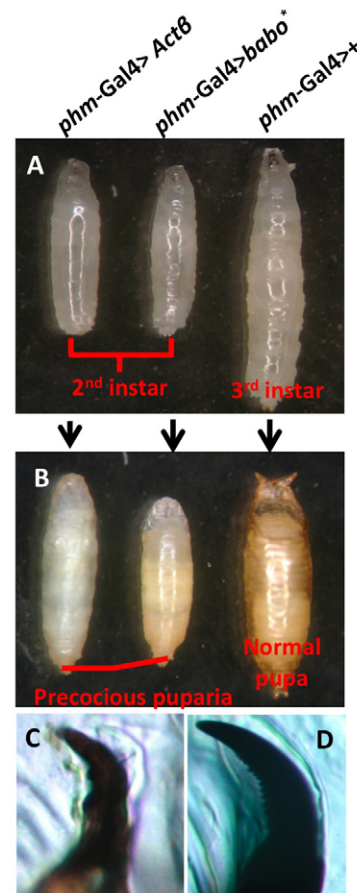
Student's *t*-test was used to calculate the statistical significance of the observed differences in the protein expression levels and gene expression levels. Error bars represent s.d.

## RESULTS

### Loss and gain of Activin signaling in PG causes developmental arrest

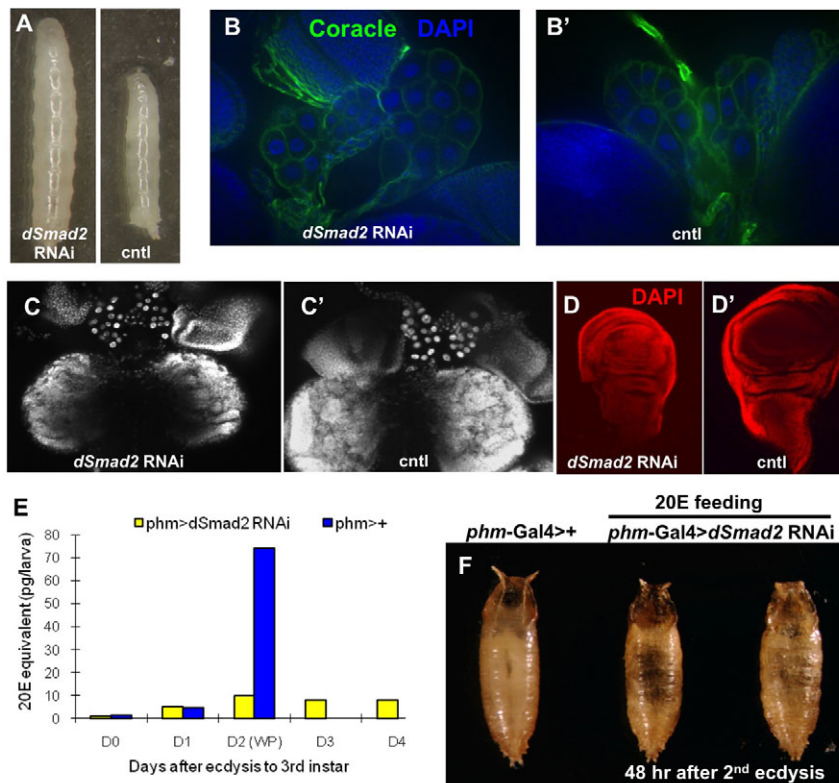
The finding that TGF $\beta$  signaling is involved in regulating dauer formation in *C. elegans* prompted us to ask whether this signaling pathway plays a similar regulatory role in controlling developmental transitions in *Drosophila*. Previously, we noted that *babo* mutant larvae are developmentally delayed and exhibit ~70% larval lethality (Brummel et al., 1999). This led us to examine whether perturbation of the Activin/Babo pathway specifically in the ring gland, a composite endocrine organ that secretes hormones essential for development (Jones and Jones, 2007), produces any developmental abnormalities. To this end, we used Akh (CC, corpus cardiacum), Phm (PG) and Aug21 (CA, corpus allatum) Gal4 drivers to specially overexpress or knockdown various Activin signaling components in different regions of the ring gland. Overexpression of a constitutively active form of Babo (Babo\*), the type I receptor for Activin-type ligands (Brummel et al., 1999) or knockdown of Smad2, the primary transcriptional transducer of *Drosophila* Activin signaling (Brummel et al., 1999) in either the CA or the CC produced no obvious developmental phenotypes (data not shown). By contrast, overexpression of Babo\* or the ligand Act $\beta$  in the PG resulted in larvae that wander at a relatively small size (Fig. 1A) with mouth hooks exhibiting second instar characteristics (Fig. 1C versus 1D), indicating that they have failed to progress to the third instar stage. After wandering for about 2 days, these second instar larvae formed stage precocious puparia that failed to survive to pharate adults (Fig. 1B), perhaps because they did not achieve minimal viable weight (see Fig. S1A in the supplementary material). Growth analysis shows that these second instar precocious larvae suffered from delayed development and took ~6.5 days after egg laying (AEL) to reach 50% pupariation, compared with the typical 5-day period in wild-type larvae (see Fig. S1B in the supplementary material).

Although these gain-of-function experiments demonstrate that excess Activin signaling in the PG can alter developmental progression through larval stages, we sought to determine whether Activin signaling is required in the PG for normal development. Accordingly, we employed RNAi to specifically knockdown *dSmad2* expression in the PG using a variety of Gal4 drivers. As *dSmad2* is the sole downstream mediator of canonical Activin signaling, its knockdown should remove contributions of all Activin-type ligands, including Act $\beta$ , Daw, Myo and perhaps Mav. Interestingly, larvae in which *dSmad2* is knocked down in the PG



**Fig. 1. Overactivation of Activin signaling in the PG leads to stage precocious pupariation.** (A) Ectopic expression of the ligand Act $\beta$  (left) or a constitutively active receptor Babo\* (middle) in the PG results in small wandering larvae compared with a 3rd instar control wandering larva (right). (B) The small wandering larvae in A (left and middle) formed stage precocious pupae. A wild-type pupa is shown on the right. (C,D) The mouth hook of the small wandering larvae from A exhibit 2nd instar characteristics (two or three large teeth) (C) compared with the mouth hooks of a typical 3rd instar control larva (D), which have many smaller serrated teeth.

(thereafter referred to as *dSmad2* RNAi larvae) failed to initiate metamorphosis and arrested at the third instar stage for more than 2 weeks, during which time they continued to feed and grow to a very large size (Fig. 2A). Knockdown of *dSmad2* was also able to suppress the stage precocious pupariation of activated Babo, indicating that the gain-of-function phenotype requires *dSmad2* (data not shown). To eliminate possible off-target effects of the RNAi technology, we used several UAS-*dSmad2* RNAi constructs that target different regions of the *dSmad2* gene. All of these constructs individually gave rise to the same non-pupariating phenotype (see Table S1 in the supplementary material). To show that the non-pupariating phenotype is specific to loss of Activin signaling rather than to other unrelated cellular functions of *dSmad2*, we knocked down the type I receptor, Babo, a type II receptor, Punt (Childs et al., 1993), and a transcriptional co-factor for *dSmad2*, Medea (Das et al., 1998). Again, using several RNAi lines for each gene, we observed the same non-pupariating phenotype (see Fig. S2A and Table S1 in the supplementary material). However, when the transcriptional transducer of the



**Fig. 2. Loss of Activin signaling in the PG leads to a failure to initiate metamorphosis.**

(A) Knockdown of *dSmad2* (left panel) results in larval developmental arrest and giant larvae compared with control (right panel). (B) Immunostaining for Coracle in both the control and *dSmad2* RNAi larvae. DAPI is in blue. (C,C') DAPI staining of RGCs of the control and *dSmad2* RNAi larvae. (D,D') DAPI staining of wing imaginal discs of the control and *dSmad2* RNAi larvae. (E) The whole larva ecdysteroid titer of the control and *dSmad2* RNAi larvae. Data are from a single determination using 50 larvae for each data point. (F) 20E feeding at 48 hours after the second ecdysis rescues the *dSmad2* RNAi larvae to the pupa stage.

BMP pathway, Mad, is knocked down in PG cells, no effect on developmental timing is observed, even though the same Mad RNAi lines were capable of generating strong BMP loss-of-function phenotypes when expressed in other tissues. In addition, simultaneous knockdown of both *dSmad2* and *Mad* in the PG produced no stronger phenotype than *dSmad2* knockdown alone (data not shown).

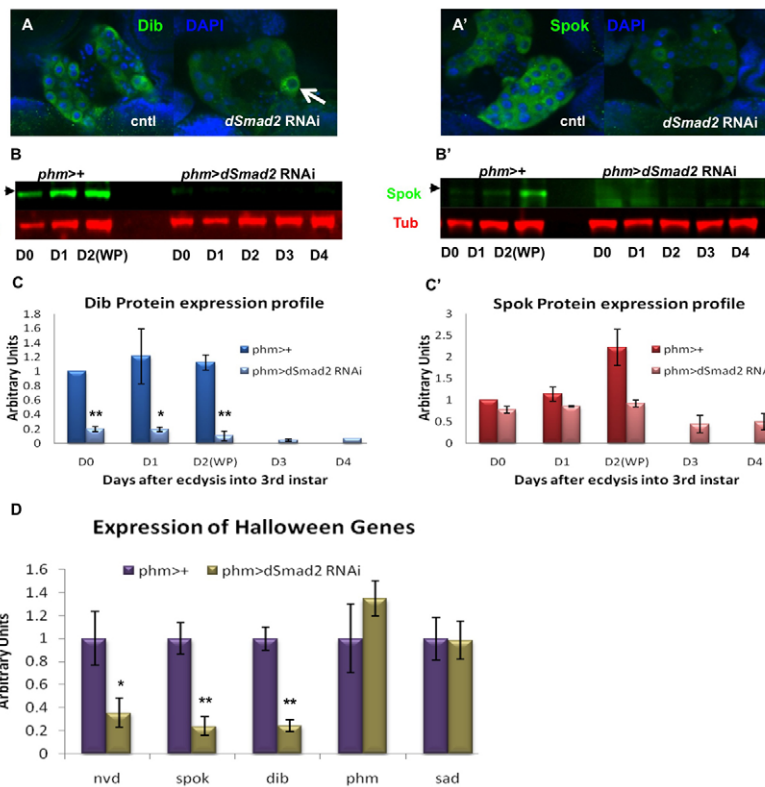
The above data suggest that Activin signaling mediated by *Babo*, *Punt*, *dSmad2* and *Medea* in the PG is essential for proper developmental timing and for the initiation of metamorphosis.

### Loss of Activin signaling leads to low 20E levels due to downregulation of E biosynthetic enzymes

Next, we explored the underlying mechanism of how TGF $\beta$ /Activin signaling affects metamorphosis. As TGF $\beta$  signaling has been shown to regulate cell fate during development (Larsson and Karlsson, 2005; Mondal et al., 2004; ten Dijke et al., 2003), we first examined whether Activin signaling is required for specifying PG cell fate. Both the PG morphology and cell number appear to be normal in the *dSmad2* RNAi larvae when compared with the control, at least when visualized by immunostaining for Coracle, a septate junction marker (Fehon et al., 1994) (Fig. 2B,B') and DAPI staining (Fig. 2C,C'). In addition, the fact that *Phm* and *Sad* are expressed at near normal levels in the *dSmad2* RNAi PG (see Fig. S3 in the supplementary material), and the fact that there is still detectable but low expression of several other ecdysone biosynthetic enzymes (see below), indicates that loss of TGF $\beta$  signaling does not generally interfere with the assignment of endocrine properties to the PG cells. Although we did not observe any major morphological defects in the PG, we noticed that *dSmad2* RNAi larvae have smaller brains and imaginal discs (Fig. 2C,C',D,D'), despite the fact that the larvae are abnormally large.

These phenotypes have been previously reported in *without children* mutants, which have low ecdysteroid levels (Wismar et al., 2000). As a high titer of 20E is essential for metamorphosis to occur, we examined whether *dSmad2* RNAi larvae exhibit a decreased 20E titer, and found that, indeed, they do not generate a 20E peak during the prolonged third instar stage. Although control larvae that normally undergo pupariation ~48 hours after ecdysis into the third instar stage have a sharp rise in total ecdysteroid content, *dSmad2* RNAi larvae failed to mount an ecdysone surge even after 4 days in the third instar stage (Fig. 2E). Feeding 20E to *dSmad2* RNAi larvae at 48 hours post second ecdysis, a stage where larvae mount an ecdysteroid titer under wild-type conditions, enables 45% of the larvae ( $n=54$ ) to undergo metamorphosis (Fig. 2F), but the pupae formed failed to progress to the pharate stage. The *dSmad2* RNAi third instar larvae that were aged for more than 2 weeks can also initiate pupariation in the presence of dietary 20E (see Fig. S2B in the supplementary material), although to a lesser extent (20%,  $n=10$ ). We note that many of the mitotic tissues, including brain, imaginal discs and parts of the gut show pronounced degeneration in these aged individuals likely accounting for the reduced pupation rate when feed 20E. These results confirm that 20E deficits underlie the failure to elicit metamorphosis. Based on these observations, we conclude that the Activin signaling pathway, mediated by *dSmad2*, modulates E metabolism in the PG.

One explanation for impaired 20E production in *dSmad2* RNAi larvae could be that the E biosynthetic enzymes themselves are not properly expressed in the PG. To test this idea, we used immunostaining to visualize expression of various E biosynthetic enzymes in the PGs of *dSmad2* RNAi larvae. Intriguingly, we found that staining for *Spok* and *Dib*, two P450 enzymes required in the PG for the synthesis of E, is reduced (Fig. 3A,A'). Curiously,



**Fig. 3. Activin signaling regulates expression of the Halloween genes in the PG.** (A,A') Immunostaining of DIB (A) and Spok (A') in the PG of wild-type and the *dSmad2* RNAi larvae. (B,B') Western blot analysis on the expression of DIB and Spok in the control and *dSmad2* RNAi larvae. A total of four RGBCs were used in each lane. Tubulin serves as a loading control. (C,C') Quantitative analysis of the Western blot results from B and B'. The DIB or Spok protein level is normalized to the tubulin level in the same sample. The normalized DIB or Spok level in the control larvae at D0 is set as 1. Three replicate blots were used for quantification. Data are mean±s.d. (D) qPCR analysis on the transcript levels of the Halloween genes in *dSmad2* RNAi larvae. Data are mean±s.d. \**P*<0.05, \*\**P*<0.005.

a rare individual PG cell (Fig. 3A, arrow) will exhibit significant staining, probably because of cell heterogeneity in *phm*>Gal4 expression.

To quantify this downregulation, we performed Western blot analysis on brain-ring gland complexes (BRGCs) to measure the levels of the DIB and Spok proteins (Fig. 3B,B'). Densitometry shows that BRGCs from *dSmad2* RNAi larvae have extremely low levels of DIB, ranging from a fivefold reduction at the beginning of the third instar to an 11-fold loss at the onset of metamorphosis when compared with the well-staged control larvae (Fig. 3C). Spok also shows significant reduction in *dSmad2* RNAi larvae (Fig. 3C') compared with wild type. Conversely, Phm and Sad protein levels are unaffected (see Fig. S3 in the supplementary material).

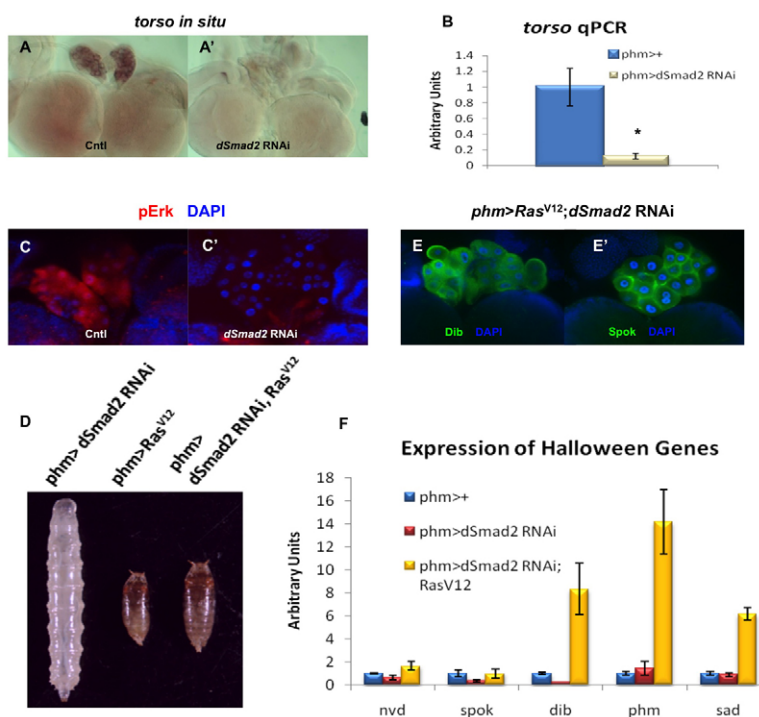
Since TGFβ signaling is known to regulate gene transcription, we examined whether downregulation of DIB and Spok at the protein level is a consequence of reduced transcription. Using qRT-PCR analysis of the RNA samples isolated from BRGCs in *dSmad2* RNAi and the control larvae, we found that the steady-state levels of *dib*, *spok* and *nvd* transcripts are significantly decreased (Fig. 3D), whereas, consistent with the protein expression data, the transcript levels of *phm* and *sad* are unaltered (Fig. 3D). These results identified the E biosynthetic genes *dib*, *spok* and *nvd* as being potential regulatory targets of Activin signaling in the PG, and suggest that the reduced expression of these genes probably contributes to the *dSmad2* RNAi phenotype.

**PTTH signaling is impaired by blocking Activin signaling**

In Lepidoptera and *Drosophila*, the rise of the E/20E titer at the onset of metamorphosis is thought to be triggered in part by the neuropeptide PTTH (Ishizaki and Suzuki, 1994; McBrayer et al., 2007; Smith and Gilbert, 1989). Therefore, we investigated the possibility that loss of Activin signaling might indirectly affect E

production by modifying PTTH signaling. First, we examined whether the ligand PTTH is expressed normally in *dSmad2* RNAi larvae and found that the *ptth* transcript is expressed in a pair of neurons in each brain hemisphere at a level comparable with wild type (see Fig. S4A,B in the supplementary material). Next, we investigated whether the downstream components of the PTTH pathway are affected. Recently, Torso, a receptor tyrosine kinase, has been shown to function as a PTTH receptor and signal through the Ras-Raf-Erk cascade (Rewitz et al., 2009). When we examined the expression of *torso* in *dSmad2* RNAi larvae, the level of *torso* mRNA was markedly reduced, as demonstrated by both in situ hybridization and qRT-PCR analysis (Fig. 4A,B). Furthermore, phosphorylated Erk (pErk), an indicator of PTTH signal activation, also shows strong downregulation (Fig. 4C'), consistent with a block of the PTTH signaling pathway at the receptor level.

If loss of PTTH signaling is responsible for the lack of E in *dSmad2* RNAi larvae, we rationalized that reactivating the PTTH pathway might partially restore E biosynthetic enzyme expression and rescue the pupariation defect. To this end, we expressed in the PG a constitutively active form of Ras (Ras<sup>V12</sup>), an essential component in PTTH signaling that acts downstream of Torso (Rewitz et al., 2009). Similar to a previous report (Caldwell et al., 2005), we found that expression of Ras<sup>V12</sup> alone in the PG accelerated larval development and gave rise to very small third instar pharate pupae (Fig. 4D) that failed to eclose. When Ras<sup>V12</sup> was expressed in the PG of *dSmad2* RNAi larvae (*phm*-Gal4>*dSmad2* RNAi; Ras<sup>V12</sup>), the larvae successfully initiated metamorphosis and died at the pharate stage, similar to the *phm*-Gal4> Ras<sup>V12</sup> animals (Fig. 4D). We reasoned that this rescue was probably a consequence of restored E biosynthetic enzyme expression. At the protein level, Spok and DIB, which are barely detectable in *dSmad2* RNAi larvae, are strongly expressed in the PG of *phm*-Gal4>*dSmad2* RNAi; Ras<sup>V12</sup> RNAi larvae (Fig. 4E).



**Fig. 4. Loss of Activin signaling reduces PTTH signal reception and Halloween gene mRNA levels.** (A,A') In situ hybridization of the *torso* transcript in a control and a *dSmad2* RNAi PG. (B) qPCR analysis of the *torso* transcript levels in control and *dSmad2* RNAi larvae. Data are mean $\pm$ s.d. \* $P$ <0.05. (C,C') Immunostaining of pErk (red) in the control and the *dSmad2* RNAi. DAPI is shown in blue. All the images were taken with the same exposure time. (D) Expression of Ras<sup>V12</sup> in the PG rescues the *dSmad2* RNAi larvae to the pharate stage. (E,E') Immunostaining of DIB and Spok in the PG of the *phm>dSmad2* RNAi; Ras<sup>V12</sup> larvae. (F) Comparison of the transcript levels of the Halloween genes in the control, *dSmad2* RNAi and *phm>dSmad2* RNAi; Ras<sup>V12</sup> larvae. Data are mean $\pm$ s.d.

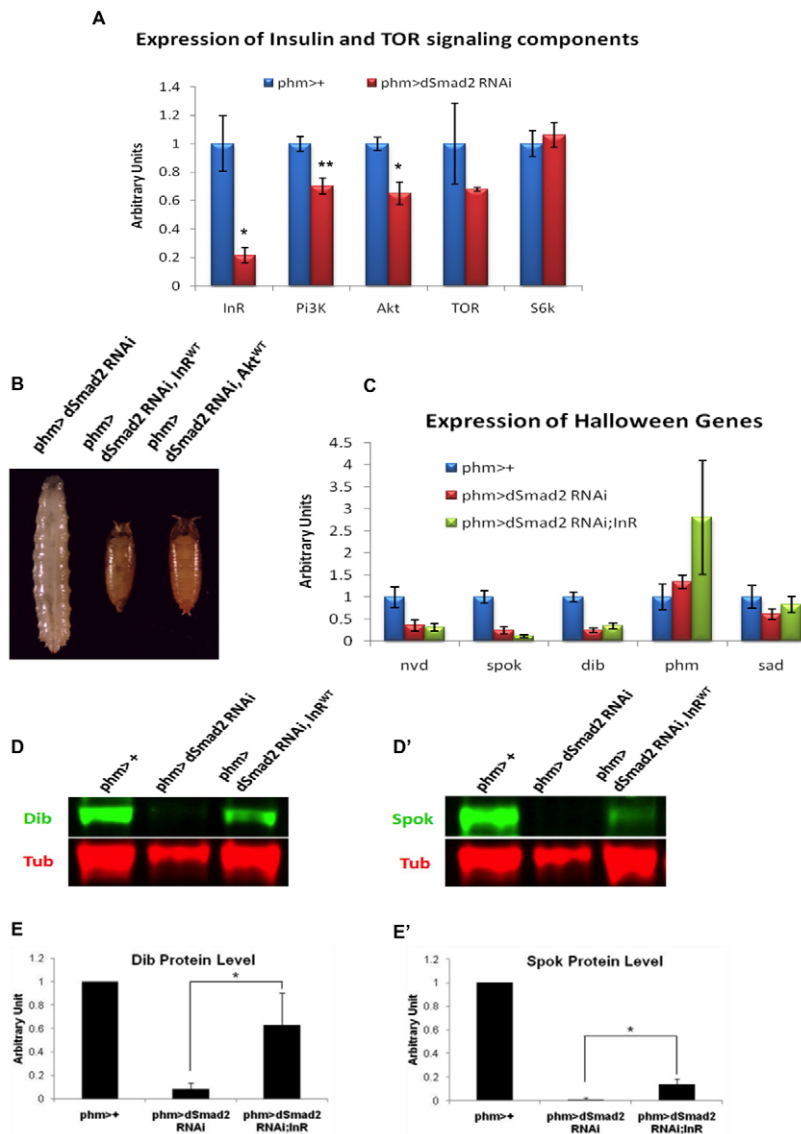
This increase at the protein level is probably a result of increased transcription because the amount of the *nvd*, *spok* and *dib* mRNA increased to almost normal or even more than the wild-type levels upon Ras<sup>V12</sup> expression (Fig. 4F). Surprisingly, transcription of *phm* and *sad*, which are not affected by *dSmad2* RNAi, also exhibit dramatic upregulation (Fig. 4F), confirming that PTTH signaling regulates the expression of *phm* and *sad*, as suggested previously (McBrayer et al., 2007). The expression of *molting defective* (*mld*), which encodes a transcription factor required for *spok* expression (Neubueser et al., 2005; Ono et al., 2006), is not influenced by Ras<sup>V12</sup> expression (data not shown), suggesting that the upregulation of mRNA is not seen for all PG-expressed genes when the PTTH signal pathway is hyperactivated. Overall, these results indicate that loss of Activin signaling in the PG impairs the expression of the PTTH receptor Torso and thus indirectly reduces E levels by rendering PG cells incompetent to respond to PTTH stimulation.

### Insulin signaling is disturbed by loss of Activin signaling

Although loss of *torso* expression in *dSmad2* RNAi larvae is consistent with the observed development timing defect, it is important to recognize that ablation of PTTH-producing neurons or knockdown of *torso* expression in the PGs only delays development during the third instar stage (McBrayer et al., 2007; Rewitz et al., 2009) and does not arrest it in the way that the knockdown of Activin signaling components does. We previously speculated that a second prothoracicotrophic signal exists that is responsible for producing an eventual rise in the E titer in larvae lacking PTTH signal that leads to metamorphosis (McBrayer et al., 2007). The more severe developmental arrest phenotype exhibited by *dSmad2* RNAi larvae could be accounted for by a loss of competence to respond to the hypothesized alternative prothoracicotrophic signal. We speculated that insulin-like factors might be good candidates as genetic manipulations of insulin

signaling in the PG have revealed that insulin signaling has an impact on 20E titers (Colombani et al., 2005; Mirth et al., 2005). We first sought to determine whether insulin signaling in the PG is disturbed by loss of Activin signaling. We used a pleckstrin homology domain-GFP (PH-GFP) fusion protein, which serves as an indicator of insulin signal activation (Britton et al., 2002). Although none of the control PG cells show cytoplasmic localization of the PH-GFP protein, we observed that this protein is diffusely distributed throughout the cytoplasm of many *dSmad2* RNAi PG cells (see Fig. S5A in the supplementary material), indicating that insulin signaling is probably downregulated when Activin signaling is blocked. Consistently, we also found that dFOXO is present in the nucleus of the *dSmad2* RNAi PGs, further suggesting reduced insulin signaling (Puig et al., 2003), whereas this nuclear localization pattern was not observed in the control PGs (see Fig. S5B in the supplementary material). Analysis of the mRNA levels of the insulin signaling components revealed that, whereas *Pi3K* and *Akt* show mild but significant reduction, *InR* exhibits a dramatic decrease in the BRGC of the *dSmad2* RNAi larvae (Fig. 5A). Although downregulation in both the PG and the brain could account for the observed transcriptional reduction of *InR*, expressing *InR* or its downstream mediator *Akt* specifically in the PG rescued the ability of the *dSmad2* RNAi larvae to undergo the larval-to-pupal transition and form viable adults (Fig. 5B). These results strongly imply that loss of *InR* in the PG contributes to the negative effect of *dSmad2* RNAi on 20E production.

To determine whether the rescue by upregulating insulin signaling was a result of enhanced expression of the E biosynthetic enzymes, we analyzed the mRNA levels of *nvd*, *dib* and *spok*, and the protein levels of DIB and Spok. Although upregulation of insulin signaling has no effect on the steady-state mRNA levels of these E enzymes (Fig. 5C), there is a considerable elevation of the amount of the DIB and Spok protein upon insulin signaling stimulation in *dSmad2* RNAi larvae (Fig. 5D,D'). Altogether, the



**Fig. 5. Loss of Activin signaling in the PG reduces insulin receptor expression and lowers Halloween gene protein levels.** (A) The transcript levels of *InR*, *Pi3K*, *Akt*, *TOR* and *S6k* in the BPGC of the control and the *dSmad2* RNAi larvae. Data are mean±s.d. (B) Expression of either a *UAS-InR* or a *UAS-Akt* transgene in the PG rescues the *dSmad2* RNAi larvae to the adult stage. (C) Comparison of the transcript levels of the Halloween genes in the control, *dSmad2* RNAi and *phm>dSmad2* RNAi; *InR* larvae. No statistical differences are found between *dSmad2* RNAi larvae and *phm>dSmad2* RNAi; *InR* larvae. Data are mean±s.d. (D,D') Western blot analysis on DIB and Spok proteins levels in the control, *dSmad2* RNAi and *phm>dSmad2* RNAi; *InR* larvae. (E,E') Quantitation. The levels of DIB and Spok are normalized to Tubulin and are relative to the *phm>+* animals. Data are mean±s.d. \**P*<0.05, \*\**P*<0.005.

above results suggest that insulin signaling is compromised in *dSmad2* RNAi larvae and that its primary effect is to regulate post-transcriptionally the levels of several E biosynthetic enzymes.

**DISCUSSION**

Developmental transitions are carefully timed processes that enable organisms to adapt and respond to various environmental and developmental cues so that reproduction occurs under conditions most advantageous for species survival. Previous work in a number of holometabolous insects, including *Drosophila*, has highlighted the importance of the PTTH and insulin signaling pathways in stimulating 20E production in the PGs to trigger metamorphosis (Colombani et al., 2005; Kawakami et al., 1990; McBrayer et al., 2007; Mirth et al., 2005; Sauman and Reppert, 1996; Walkiewicz and Stern, 2009). We demonstrate here that PG competence to respond to these two essential metamorphic stimuli in *Drosophila* is crucially dependant on TGFβ/Activin signaling, which controls production of the primary receptors for these two pathways. The ability of activins to act as either direct or indirect permissive signals in the PG is interesting as competence factors have long been thought to play a central role in modulating cellular responses

to hormonal signals (Broadus et al., 1999) and one generally recognized mechanism through which they function is by regulating the expression of receptors for a variety of signals (Shi et al., 1996). In the mammalian ovary, Activin has been shown to induce the expression of the receptor for follicle stimulating hormone (FSH) in rat granulosa cells (Hasegawa et al., 1988; Xiao et al., 1992), contributing to the stage-specific response of the developing follicles to FSH stimulation (Knight and Glister, 2001). It is also interesting to note in this regard that in *Bombyx*, the prothoracic gland, has been shown to be refractory to PTTH signals at certain stages (Ciancio et al., 1986) and that Torso levels fluctuate dramatically during the 5th instar stage (Rewitz et al., 2009), potentially accounting for the lack of PTTH response at certain times. At present we do not know whether Torso or InR levels in the PG fluctuate during normal *Drosophila* larval development, nor do we know whether activins play a role in regulating Torso and InR levels in response to some specific timing or nutritional cue. It is possible that activins simply impart constitutive expression of these receptors in the PG as part of a general developmental program responsible for endowing the PG cells with their steroidogenic capacity. As the general

morphological features of the PG cells are not perturbed by Activin signal knockdown, even when using the *phm*-Gal4 driver, which is activated in the early embryo, we do not suspect that activins are required for specification of the gland itself. However, this conclusion must be tempered by the fact that RNAi knockdown is probably not complete and the *phm*-Gal4 driver may not turn on until after gland formation is largely finished. To fully rule out a role of activins in gland specification, null germline clones for *babo* or *dSmad2* need to be analyzed, and at this time it is not clear that any such alleles exist for either gene (M.B.O., unpublished).

The lack of true null mutations in both *babo* and *dSmad2* may also account for the stronger phenotype produced by PG-specific knockdown of *babo* or *dSmad2* compared with the reported *babo* genetic loss-of-function phenotype. Our previous studies examining the phenotype of *babo* zygotic mutants revealed a 2-3 day developmental delay in puparium formation, but up to 30% of the mutant larvae do initiate metamorphosis (Brummel et al., 1999). By contrast, elimination of activin signal reception only in the PG produces a stronger phenotype where virtually 100% of the knockdown larvae arrest development without puparium formation. Although residual Babo function is a possible explanation for this difference, we point out that another explanation exists that highlights the difficulties associated with interpreting tissue-specific knockdown experiments. It is theoretically possible to produce a stronger whole animal phenotype by tissue-specific knockdown than by a genetic null mutation, owing to potential compensatory changes in other mutant tissues in the genetically null animal. This is especially true when the factor potentially regulates multiple systemic signals in different tissues. For example, the ligand Act $\beta$  is produced in IPCs of the brain (M.B.O., unpublished). If Act $\beta$  negatively regulates insulin production or secretion, then, in a *babo* genetic null background, upregulation of insulin signal upon loss of Act $\beta$  signaling in IPCs may compensate for reduced insulin reception capacity in the PGs. This compensatory mechanism may enable a percentage of the *babo* mutant larvae to undergo puparium formation. By contrast, knockdown of activin signal reception in the PG alone, as in the case of *phm*>*babo* RNAi animals, would probably not lead to this compensatory response and thus results in a stronger developmental arrest phenotype. Consistent with this view, we find that using a ubiquitous driver, such as *daughterless*-Gal4, to knockdown *dSmad2* in all tissues, including the PG, does not lead to developmental arrest, whereas using a PG specific driver does. These potential complications in evaluating phenotypic differences obtained using genetic mutations and tissue-specific knockdown methods should be borne in mind as they are likely to be observed more frequently with the increasing use of tissue-specific knockdown analyses in numerous model organisms.

The issue of which activin-like ligand(s) are responsible for providing the competence signal and whether they are regulated by particular developmental or nutritional cues is also important to answer but is problematic because of redundancy concerns. We suspect that Act $\beta$  probably plays a role as it is expressed in numerous neurosecretory cells including the IPCs (M.B.O., unpublished), which innervate the heart tube (Rulifson et al., 2002) and thereby probably provide systemic delivery of this ligand to many tissues. In addition, overexpression of this ligand in the PG causes stage precocious pupation, similar to that produced by expression of activated Babo. However, the one available Act $\beta$  loss-of-function mutation does not produce substantial developmental delay, and the majority of larvae (>90%) instead undergo slightly precocious pupation (A. Ghosh and M.B.O.,

unpublished) consistent with potential upregulation of insulin signaling, as suggested above. Likewise, mutations in *daw*, a second Activin-like ligand that is produced in the PG (M.B.O., unpublished), do not elicit major developmental delay when fed a yeast-enriched diet. However, when strong *daw* alleles are combined with the one available Act $\beta$  mutation, then only 25-35% of the larvae are able to initiate pupariation on rich food (Zhu et al., 2008) (M.B.O., unpublished). This observation suggests a functional redundancy between these ligands for regulating developmental timing, similar to their previously noted redundant roles in regulating neuroblast proliferation in the larval brain (Zhu et al., 2008). The residual pupation ability of the *daw*-Act $\beta$  double mutants may be accounted for by the compensation mechanism described above or further functional redundancy provided by the two other Activin-like ligands, Myo and Mav. At present, no mutations are available in these genes.

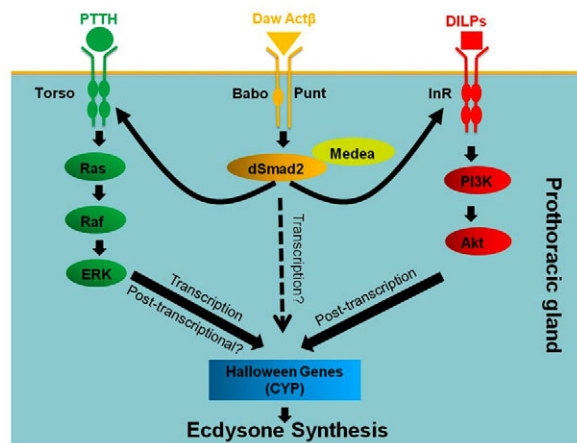
### Direct or indirect regulation of steroid production by Activin signaling?

Although our observations clearly show that loss of E biosynthetic enzyme expression underlies the *dSmad2* developmental arrest phenotype, we cannot say with certainty that this downturn is due solely to the loss of insulin and PTTH signaling or whether *dSmad2* might also participate directly in regulating biosynthetic enzyme gene expression. In addition, whether *dSmad2* binds directly to target sequences within Torso and InR regulatory sequences also remains uncertain as no *dSmad2* responsive elements have been identified for any gene in *Drosophila*. It is interesting to note, however, that one other molecular process, SUMOylation, has recently been implicated in regulating E biosynthetic enzyme expression and localization in the PG (Talamillo et al., 2008). Knockdown of *smt3*, the sole SUMO-encoding gene in *Drosophila*, in the PG results in third instar larval arrest phenotype that is rescuable by feeding the larvae 20E. In addition, these larvae show low levels of Dib protein accumulation in the PG (Talamillo et al., 2008). These phenotypes are strikingly similar to those seen in *dSmad2* RNAi larvae. Interestingly, previous studies have demonstrated that Medea can be SUMOylated in vitro, providing a potential link between SUMOylation and Activin signaling (Miles et al., 2008). However, we have been unable to detect any SUMOylated forms of Medea or *dSmad2* under various signaling conditions in vivo or in S2 cells. Additional studies will be required to determine whether SUMOylation and Activin signaling are linked in a common pathway or whether they act through independent means to control steroid production in the PG.

### A conserved role for TGF $\beta$ /Activin signaling in regulating developmental transitions

Despite the uncertainty in determining whether one or more ligands act as a PG competence factor, the observation that TGF $\beta$ /Activin signaling regulates metamorphosis may highlight an ancient and conserved role for these factors in regulating developmental transitions in many organisms. For example, the nematode worm *C. elegans* employs the TGF $\beta$  pathway to make a nutritionally dependent decision on whether to continue the normal development program into a mature adult or to enter a developmentally arrested stage known as dauer. The activation of the TGF $\beta$  pathway promotes normal development whereas its inactivation results in dauer formation (for a review, see Fielenbach and Antebi, 2008). Interestingly, a key target of the TGF $\beta$  pathway is *daf-9*, which encodes a cytochrome P450 protein. DAF-9 is involved in the





**Fig. 6. Model for TGF $\beta$ /Activin regulation of metamorphosis.** The onset of metamorphosis is regulated by the activities of multiple signaling pathways within the PG, including PTTH, insulin and TGF $\beta$ /Activin. PTTH signaling plays a primary role in upregulation of the E biosynthetic gene transcription, while insulin signaling regulates their translation and/or stability. Activin signaling ensures proper expression of Torso and InR in the PG. Without Activin signaling, the PG is unable to respond to either PTTH or to insulin signal, leading to a failure to express sufficient levels of the E biosynthetic enzymes that trigger metamorphosis.

synthesis of dafachronic acid, an ecdysone-like steroid hormone that prevents the developmental arrest as a dauer (Gerisch et al., 2001; Jia et al., 2002). Likewise, in mammals, Activin signaling probably affects pubertal timing by controlling sex steroid synthesis. Activin signaling can stimulate the production of the sex steroid hormone estradiol by enhancing the activity of CYP 450 aromatase (Hutchinson et al., 1987; Miro et al., 1991; Miro and Hillier, 1992). These observations from *C. elegans* and mammalian studies, together with our finding that *Drosophila* Activin signaling also regulates cytochrome P450 expression, although probably by indirect means, strongly indicate that TGF $\beta$ /Activin signaling is a common means by which developmental transitions are regulated across species.

### PTTH and insulin signaling impinge on steroidogenic regulation in distinct ways

Previously, we have found that complete loss of PTTH signaling delayed metamorphosis but did not completely block it (McBrayer et al., 2007). This observation led us to speculate that a second metamorphic signal, perhaps supplied by insulin, eventually facilitated pupation in the PTTH negative larvae. Our finding here that simultaneous knockdown of both pathways by eliminating dSmad2 in the PG leads to third instar developmental arrest and that resupplying activity in either pathway promotes metamorphosis is consistent with this idea. Even more intriguing is our finding that the two metamorphic signals provided by PTTH and insulin appear to regulate the steroid biosynthetic capacity of the gland in two distinct ways: PTTH at the level of biosynthetic enzyme mRNA accumulation and insulin at the biosynthetic enzyme protein level (Fig. 6). The observation that loss of PTTH signal reception in dSmad2 PG knockdown larvae reduces the steady-state mRNA levels of the E biosynthetic enzymes is consistent with our earlier PTTH-neuron ablation results with one exception. In our present studies, we only observed downregulation

of *dib*, *spok* and *nvd* in response to reduction in Torso expression, whereas in PTTH-neuron ablated larva, transcription of *phm* and *sad* are also reduced (McBrayer et al., 2007). One possible explanation for this discrepancy is that the five biosynthetic enzymes exhibit different sensitivities to the strength of the PTTH signal. In PTTH-neuron ablation experiments, all signaling is lost, whereas in the dSmad2 knockdown, it is likely that some Torso expression remains and provides enough signal to activate *phm* and *sad*. Consistent with this view, we find that in the rescued larvae, where we resupply PTTH signaling in the PG using activated Ras, the mRNA levels of the *phm* and *sad* genes are dramatically upregulated compared with the other three E biosynthetic enzymes (Fig. 4F), indicating they are very sensitive to the PTTH signal.

The effects of insulin signaling on protein levels is intriguing and has not been examined previously, although there are two reports to indicate that insulin signaling also affects transcription of at least two biosynthetic enzymes, *dib* and *phm* (Colombani et al., 2005; Walkiewicz and Stern, 2009). However, the reported effects were modest (between 30% to 2 fold) and it was not clear whether the determinations were normalized for differences in body size and/or ring gland size, which are crucial when considering the small reported differences. In our measurements, we used ring gland-brain complexes that were of similar size and staging, and did not detect any differences in transcription of biosynthetic enzymes between the dSmad2 knockdown animals and those in which InR expression was restored to the PG. Instead, we observe significant differences in biosynthetic enzyme protein levels. The known role of insulin in modulating translational capacity of cells is consistent with the idea that these changes in biosynthetic enzyme levels are the result of translational differences. Whether this effect is through Tor, which has been shown to be an important mediator of developmental timing in the PG (Layalle et al., 2008), and its modulation of S6 kinase, remains to be determined. In addition, we cannot exclude effects on protein stability, and further studies examining protein turnover will also be required to address this issue fully. Overall, this two-tiered regulatory control of biosynthetic enzyme expression may better enable the larva to fine-tune its ecdysone level depending on conditions, or perhaps may serve as a coincidence detector to ensure that both developmental and nutritional conditions are appropriate before triggering a terminal developmental program such as metamorphosis.

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### Competing interests statement

The authors declare no competing financial interests.

### Supplementary material

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**Table S1. The RNAi constructs that give rise to larval arrest phenotype when expressed in the PG**

<i>dSmad2</i> RNAi constructs	<i>punt</i> RNAi constructs	<i>babo</i> RNAi constructs
#14609 (Vienna Stock Center) Target sequence: cDNA (nucleotides 652-1049)	#848 and #37279 (Vienna Stock Center) Target sequence: cDNA (nucleotides 1076-1247)	#853 and #3825 (Vienna Stock Center) Target sequence: cDNA (nucleotides 1771-1942)
#105687 (Vienna Stock Center) Target sequence: 3' UTR	#26756 (Bloomington Stock Center) Target sequence: cDNA (nucleotides 785-1236)	#41B3 (The O'Connor Lab) Target sequence: cDNA (nucleotides 1123-1640)
#3A2 (The O'Connor Lab) Target sequence: cDNA (nucleotides 713-1136)		

**Table S2. The primers used for qPCR experiments**

Gene	Forward	Reverse
<i>nvd</i>	5'-GGAAGCGTTGCTGACGACTGTG-3'	5'-TAAAGCCGTCCACTTCCTGCGA-3'
<i>dib</i>	5'-TGCCCTCAATCCCTATCTGGTC-3'	5'-ACAGGGTCTTCACACCCATCTC-3'
<i>spok</i>	5'-GCCGAGCTAAATTTCTCCGCTT-3'	5'-TATCTCTTGGGCACACTCGTG-3'
<i>phm</i>	5'-GGATTTCTTTCGGCGCGATGTG-3'	5'-TGCCTCAGTATCGAAAAGCCGT-3'
<i>sad</i>	5'-CCGCATTACAGCAGTCAGTGG-3'	5'-ACCTGCCGTGTACAAGGAGAG-3'
<i>rpl23</i>	5'-GACAACACCGGAGCCAAGAACC-3'	5'-GTTTGCCTGCCGAATAACCAC-3'
<i>ptth</i>	5'-CCACGAGCTCATTTCGTAACCTTGC-3'	5'-CACTCCACATCCACAGAGATGGCGATGG-3'