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Integration of an abdominal Hox complex with Pax2 yields cell-specific EGF secretion from *Drosophila* sensory precursor cells

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SUMMARY

Cis-regulatory modules (CRMs) ensure specific developmental outcomes by mediating both proper spatiotemporal gene expression patterns and appropriate transcriptional levels. In *Drosophila*, the precise transcriptional control of the serine protease *rhomboid* regulates EGF signaling to specify distinct cell types. Recently, we identified a CRM that activates *rhomboid* expression and thereby EGF secretion from a subset of abdominal sensory organ precursor cells (SOPs) to induce an appropriate number of lipid-processing cells called oenocytes. Here, we use scanning mutagenesis coupled with reporter assays, biochemistry and genetics to dissect the transcriptional mechanisms regulating SOP-specific *rhomboid* activation. Our results show that proper spatial activity of the *rhomboid* CRM is dependent upon direct integration of the abdomen-specific Hox factor Abdominal-A and the SOP-restricted Pax2 factor. In addition, we show that the Extradenticle and Homothorax Hox co-factors are differentially integrated on the *rhomboid* CRM by abdominal versus thoracic Hox proteins in the presence of Pax2. Last, we show that Abdominal-A uses both Pax2-dependent and Pax2-independent mechanisms to stimulate *rhomboid* CRM activity to induce proper oenocyte numbers. Thus, these data demonstrate how a CRM integrates Hox and neural transcriptional inputs to regulate the appropriate spatial pattern and levels of EGF secretion to specify an essential cell fate.

KEY WORDS: Cis-regulatory modules, Hox factors, EGF, *Drosophila*

INTRODUCTION

The differential control of gene expression is crucial for the specification of distinct cell types during development. At the transcriptional level, gene expression is controlled via the binding of transcription factors to DNA modules known as cis-regulatory modules (CRMs). A general framework has been established for how this process occurs: a cell activates or represses a gene based on the sum of active transcription factors within a cell and the composition of transcription factor-binding sites within each CRM (Levine, 2010). Once established, gene regulatory networks direct developmental programs that solidify cell-type differences and contribute to cell specification (Davidson and Levine, 2008; Levine and Davidson, 2005). Given the number of cell types and complexity of gene expression patterns that arise during development, understanding how distinct cell-types regulate genes in space and time, and indeed how similar cells differentially regulate target genes between body regions, is a complex and important problem in developmental biology.

One group of transcription factors that directs differential gene regulation along the anterior-posterior (AP) body axis is the highly conserved Homeotic Complex (Hox) genes (Krumlauf, 1994; Lewis, 1978; Pearson et al., 2005). Hox genes are expressed in broad regions along the AP axis and were discovered based on their ability to transform the fate of entire body regions. Subsequent studies revealed their importance in patterning the *Drosophila* embryo, and this function is conserved throughout metazoans (Carroll et al., 2001). Molecularly, Hox genes encode homeodomain proteins that bind AT-rich DNA sequences to regulate target genes within multiple cell and tissue types (Ekker et al., 1994; Krumlauf, 1994; McGinnis and Krumlauf, 1992; Noyes et al., 2008). It is this ability of Hox factors to differentially regulate target genes that ultimately specifies distinct cell types along the AP axis.

An interesting problem in Hox biology is that all family members bind similar DNA sequences in vitro yet regulate distinct sets of target genes in vivo (Berger et al., 2008; Ekker et al., 1994; McGinnis and Krumlauf, 1992; Noyes et al., 2008; Pearson et al., 2005). Studies have revealed that Hox target specificity is achieved, in part, by the formation of complexes with other transcription factors on DNA (Mann et al., 2009). The best-characterized Hox co-factors are the Extradenticle (Exd) and Homothorax (Hth) homeodomain proteins. Both Exd and Hth directly bind DNA and physically interact with each other and Hox factors to form transcription factor complexes on DNA (Mann et al., 2009). Hence, Exd and Hth enhance both Hox-binding selectivity and affinity for CRMs. However, like the Hox factors, Exd and Hth are broadly expressed during development, making their interactions insufficient to explain cell-type specificity (Aspland and White, 1997; Kurant et al., 2001; Rauskolb et al., 1993).

In this study, we dissect how a CRM of the protease *rhomboid* (*rho*) integrates Hox and neural transcription factors to yield cell-specific gene expression. In *Drosophila*, the precise expression of *rho* dictates the spatial secretion of the *spitz* epidermal growth factor (EGF) to pattern the embryo (Shilo, 2005). For example, a subset of abdominal sensory organ precursor cells (SOPs) activates *rho* to promote EGF secretion and induce an essential abdominal cell type (oenocytes) (Elstob et al., 2001; Rusten et al., 2001). Previous findings identified a *rho* CRM (RhoBAD) sufficient to

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recapitulate abdominal SOP expression and oenocyte induction (Li-Kroeger et al., 2008). These studies revealed RhoBAD contains a conserved element (RhoA) that binds a complex composed of the Abdominal-A (Abd-A) Hox factor, Exd and Hth, which stimulates enhancer activity by limiting the binding of the repressor Senseless (Sens). In segments that lack *abd-A* expression, however, Sens binds RhoBAD to repress *rho* (Li-Kroeger et al., 2008). However, additional transcriptional inputs are required for RhoBAD activation, as many Abd-A-expressing cells, including other SOPs, fail to activate this CRM. Here, we use a scanning mutagenesis approach coupled with reporter assays, genetics and DNA-binding assays to identify an additional input that activates RhoBAD in specific SOPs, and we dissect the transcriptional mechanisms underlying *rho*-dependent EGF signaling from these cells.

MATERIALS AND METHODS

Plasmid and transgenic fly generation

The wild type, Sens, Hth, Hox and Sens/Hox mutant *RhoBAD-lacZ* and wild-type and Hox mutant *RhoAAA-lacZ* P-elements have been described previously (Li-Kroeger et al., 2008; Witt et al., 2010). All other RhoBAD and RhoAAA mutations were generated by PCR and confirmed by sequencing (supplementary material Fig. S1 for mutations). *RhoAAA-luciferase* was generated by subcloning RhoAAA fragments into a luciferase vector containing a hsp70 minimal promoter. The following pAC5.1A expression constructs were generated: DPax2 (amino acids 1-844, GenBank #AAF59385) with a C-terminal V5 tag; Antp (1-374, GenBank #AAS65109) with a N-terminal Flag tag; Abd-A (1-330, GenBank #AAF55359) with a N-terminal Flag tag; Hth (1-458, GenBank #ACZ94879) with a N-terminal HA tag; and HthΔhd (1-320, corresponding to Arg321>stop from the *hth*$^{100.1}$ allele) (Kurant et al., 2001) with a N-terminal HA tag. Transgenic fly lines were established using P-element transformation or the ϕC31 recombinase system at locus 51C as indicated (Rainbow Transgenic Flies).

Fly stocks and embryo staining

Fly lines used include: *abd-A*^{m1}, *hth*^{p2}, *UAS-MycAbdA* and *UAS-MycAntp* (from Richard Mann, Columbia University, NY, USA); *hth*$^{100.1}$ (Kurant et al., 2001); *Def(4)G*, *yw*^{1118}, *PrdG4* and *TM6B,K>GFP* (Bloomington Stock Center); *UAS-Spa* (*UAS-DPax2* from Marcus Noll, University of Zurich, Switzerland); *UAS-DPax2-RNAi* (Charlton-Perkins et al., 2011); *UAS-Sv-RNAi* (Vienna *Drosophila* Resource Center); *eya*$^{P7b-1}$ (from Nancy Bonini, University of Pennsylvania, Philadelphia, PA, USA); *RhoBAD-LacZ*, *RhoBAD*$_{SensM}$*-LacZ*, *RhoBAD*$_{HoxM}$*-LacZ* and *RhoBAD*$_{HoxM/SensM}$*-LacZ* (Li-Kroeger et al., 2008); and *RhoAAA-LacZ*, *RhoAAA*$_{SensM}$*-LacZ* and *RhoAAA*$_{HoxM}$*-LacZ* (Witt et al., 2010). Embryos were harvested, fixed and immunostained using standard protocols. Expression of *lacZ* (anti-β-gal, Abcam, 1:1000), Abd-A (GP4, 1:500, Li-Kroeger et al., 2008), dPax2 (rabbit, 1:50, from Marcus Noll; or guinea pig, 1:500) (Charlton-Perkins et al., 2011), the Myc-epitope (mouse, 1:1000, Sigma), Eya (mouse, 1:50, DSHB), HNF4 (rat, 1:1000) (Gutzwiller et al., 2010) and Sens (rat, 1:125, Xie et al., 2007) were detected by fluorescent staining. Comparative β-gal levels were determined using age-matched embryos imaged as optical sections under identical settings on a Zeiss apotome microscope. Pixel intensity of a standardized area around each SOP was measured using ImageJ software and the brightest optical slice selected as the plane of focus. Mean pixel intensity was corrected for background. Oenocyte quantifications were performed on a minimum of 10 embryos per condition. All results were analyzed by ANOVA using Excel.

Co-immunoprecipitaion and luciferase assays

Drosophila S2 cells were cultured in HyClone serum free media (Fisher Scientific). For transfections, 0.6×10^6 cells were cultured in 12-well plates 24 hours prior to transfection. Each well was transfected with a total of 0.5 μg of DNA (12.5 ng luciferase reporter, 100 ng *pAc5.1-lacZ* and the indicated amount of expression construct) using 1.5 μl of Fugene (Roche). Empty pAc5.1 was added to bring the total DNA to 0.5 μg per well. Cells were harvested 48 hours post-transfection, lysates isolated and luciferase activity determined as described previously (Xie et al., 2007). Transfection efficiencies were normalized to β-gal using standard ONPG methods. Each experiment was performed three times in triplicate with similar results. For co-immunoprecipitation assays, 6×10^7 cells were cultured in 150 mm Petri dishes 24 hours prior to transfection. Each dish was transfected with a total of 5 μg of DNA using 15 μls of Fugene (Roche). Forty-eight hours post-transfection, cells were harvested, lyzed in RIPA buffer and incubated with 0.5 μg mouse V5 antibody (Invitrogen) in 500 υl of lysate. Complexes were purified using protein A/G beads (Santa Cruz Biotech) and western blot analysis was performed using standard protocols.

Protein purification and EMSAs

A DPax2 protein containing the paired domain (amino acids 169-309 with N-terminal His and C-terminal V5 tags) was purified from BL21 bacteria using Ni-chromatography (Gebelein et al., 2002). The His-tagged Antp, Abd-A, Exd, Hth and Sens proteins were purified as described previously (Gebelein et al., 2002; Gebelein et al., 2004; Li-Kroeger et al., 2008). Protein concentrations were measured using the Bradford assay and confirmed by SDS PAGE and Coomassie Blue analysis. EMSAs were performed using native PAGE (Gebelein and Urrutia, 2001). Supershifts were performed by incubating proteins and probes for 10 minutes, then incubating with the appropriate antibodies for 10 minutes prior to EMSA analysis. Competition assays were performed as described previously (Uhl et al., 2010). Dried gels were exposed to phosphor-screens and densitometry was performed using ImageQuant 5.1.

RESULTS

Abd-A inputs activate the RhoA CRM

Both *rho* and the RhoBAD CRM are active in a subset of abdominal SOPs to stimulate EGF secretion and thereby induce oenocytes (Brodu et al., 2002; Elstob et al., 2001; Li-Kroeger et al., 2008; Rusten et al., 2001). Our previous findings revealed that a Hox complex composed of Abd-A, Exd and Hth stimulates RhoBAD activity by restricting the binding of the Sens repressor to the conserved RhoA region of this CRM. These results help explain the abdominal specificity of RhoBAD, but fail to explain how RhoBAD is activated in only a subset of SOPs. Here, we use a simplified *rho* reporter containing three copies of RhoA (*RhoAAA-lacZ*) to identify potential activators of RhoBAD (Witt et al., 2010). Like *RhoBAD-lacZ*, *RhoAAA-lacZ* is sufficient to yield strong abdominal expression (Fig. 1A,B); mutations in the Hox-binding site (all three RhoA copies are mutated) result in a loss of reporter activity (Fig. 1C,D); and mutations in the Sens-binding site result in de-repression in thoracic SOPs (arrows in Fig. 1E,F) (supplementary material Fig. S1B for mutations). By contrast, SensM/HoxM double mutations result in differing effects on the activity of RhoBAD compared with RhoAAA. In the context of RhoBAD, the SensM/HoxM mutations result in gene expression in both abdominal and thoracic SOPs, whereas in RhoAAA they result in a loss of activity in abdominal SOPs (Fig. 1G,H). These results have three implications: first, RhoBAD must contain additional binding sites outside the RhoA element and the Hox-binding site that contribute to SOP gene activation. Second, the simplified RhoAAA CRM requires functional Hox-binding sites for activating abdominal SOP gene expression. Third, as Abd-A, Exd and Hth are broadly expressed throughout the abdomen, they cannot account for the restricted activity of RhoAAA in specific SOPs. Here, we focus on identifying additional transcriptional inputs that regulate the RhoA element in abdominal SOPs.

DPax2 directly activates the RhoA CRM

Sequence comparisons between *Drosophilid* species reveal RhoA has conservation extending past the Sens-, Exd-, Hth- and Hox-binding sites (supplementary material Fig. S1A). To determine

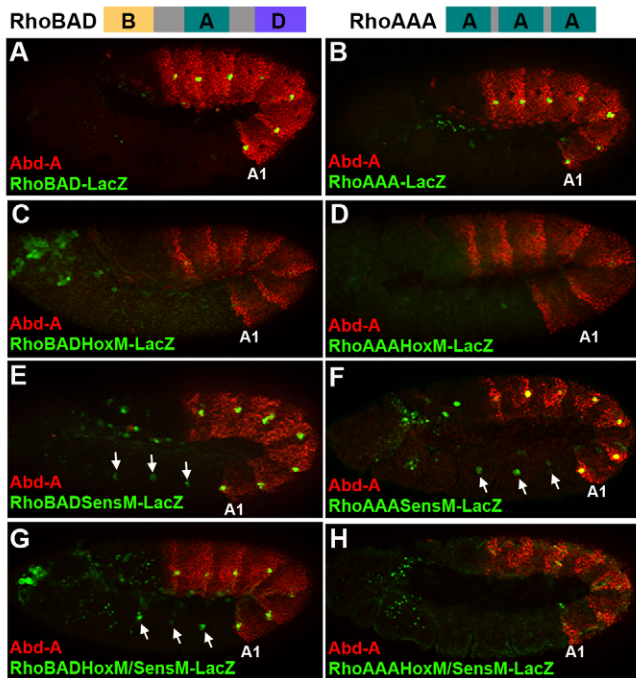

Fig. 1. The RhoA Hox-binding site is required for abdominal SOP activity of *rho* CRMs. Schematics of the RhoBAD and RhoAAA CRMs are shown at the top. (**A-H**) Expression analysis of *RhoBAD-lacZ* and *RhoAAA-lacZ* in stage 11 *Drosophila* embryos carrying either wild-type (A,B), HoxM (C,D), SensM (E,F), or HoxM/SensM (G,H) RhoA-binding sites. Reporter activity is observed using immunostaining for β-gal (green) and Abd-A (red). The first abdominal segment (A1) of each embryo is labeled and, when present, de-repression into the thorax is indicated by arrows.

whether these sequences are required for abdominal SOP activity, we performed a mutagenesis screen by sequentially altering three to four nucleotides across the uncharacterized RhoA sequences (Fig. 2A). Each mutation (*RhoAAA*$_{X1\ through\ X8}$*-lacZ*) was tested for reporter activity in embryos from three independently derived transgenic fly lines (supplementary material Fig. S2). These studies uncovered a series of mutations (X1-X3) that reduced or eliminated reporter activity compared with wild-type RhoAAA lines (Fig. 2B,C). Importantly, these mutations did not affect the binding of Sens, Exd/Hth or Abd-A to RhoA (supplementary material Fig. S3A,B). Thus, these findings indicate RhoA contains a binding site for one or more additional transcription factors required for proper *rho* expression.

To identify transcription factors that regulate the X1-X3 region of RhoA, we used a bioinformatics approach to uncover a potential binding site for Pax factors (Fig. 2A). Based on previous expression studies as well as our own analysis of Pax factors (Fu et al., 1998), we identified the *Drosophila* Pax2 homolog (DPax2) as a candidate to regulate RhoAAA. First, we found that DPax2 is expressed within the embryonic peripheral nervous system (PNS), including the RhoAAA-positive SOPs (Fig. 2D,D′; supplementary material Fig. S4). Second, we used loss-of-function approaches to show that DPax2 is required for RhoAAA activity in abdominal SOPs. As shown in Fig. 2E, a genomic deficiency [*Df(4)G*] that removes the entire DPax2 locus severely reduces *RhoAAA-LacZ* activity. Importantly, the loss of reporter activity is not due to gross defects in PNS development as SOP cells form relatively normally in these mutants (supplementary material Fig. S4). However, because *Df(4)G* removes a number of other genes, we also used *UAS-RNAi* lines against regions of DPax2 that are not highly conserved with other Pax factors. These *UAS-DPax2RNAi* lines were expressed in every other embryonic segment using *Paired-Gal4* (*PrdG4*), which resulted in a significant reduction in both DPax2 protein and *RhoAAA-LacZ* activity in *PrdG4*-expressing segments (Fig. 2F). Similar results were obtained using two additional RNAi constructs (not shown). Hence, DPax2 is expressed within abdominal SOPs that activate *RhoAAA-lacZ*, and two loss-of-function assays demonstrate DPax2 is required for RhoAAA activity within these cells.

We next assessed whether DPax2 directly binds RhoA using purified proteins containing the DPax2 DNA-binding domain in electromobility shift assays (EMSAs). As shown in Fig. 2I, DPax2 binds the wild-type RhoA probe, but not probes containing the X1, X2 and X3 mutations that disrupt in vivo reporter activity. Importantly, DPax2 binding to RhoA is not affected by mutations in the Hox-, Exd-, Hth- and Sens-binding sites (supplementary material Fig. S3C). Thus, RhoA contains a distinct binding site for DPax2 and mutations that compromise DPax2 binding correlate with a loss of in vivo RhoAAA reporter activity.

To determine whether DPax2 is sufficient to stimulate RhoAAA activity in a binding site-dependent manner, we used a *RhoAAA-luciferase* assay in *Drosophila* S2 cells. As shown in Fig. 2J, co-transfection of *RhoAAA-luciferase* with a DPax2 expression plasmid (pAc-DPax2) stimulated reporter activity 12-fold over controls. Moreover, DPax2 fails to stimulate a *RhoAAA*$_{X3}$*-luciferase* reporter containing a mutation that disrupts DPax2 binding (Fig. 2I,J). Similar results were obtained in *PrdG4* embryos, as ectopic DPax2 significantly expands the number of abdominal cells that activate *RhoAAA-lacZ* compared with non-DPax2 expressing segments (Fig. 2G) while *RhoAAA*$_{X3}$*-lacZ* embryos do not show ectopic activity (Fig. 2H). It is interesting, however, that DPax2 is not sufficient to stimulate gene expression in all cells, as it fails to stimulate *RhoAAA-lacZ* in thoracic segments (arrow in Fig. 2G) and only activates gene expression in a subset of abdominal cells. Nevertheless, the DPax2 expression pattern, loss- and gain-of-function genetics, cell culture and biochemical data show that DPax2 is a DNA binding-dependent activator of abdominal RhoAAA activity.

Direct DNA binding by Abd-A, but not Hth, is required for abdominal SOP gene expression

The finding that DPax2 stimulates *RhoAAA-lacZ* in the abdomen but not the thorax suggests an abdomen-specific Hox input is required for this activity. Consistent with this hypothesis, mutations in the Abd-A-binding site (*RhoAAA*$_{HoxM}$*-LacZ*, Fig. 1D) and the *abd-A* gene result in a loss of RhoAAA activity in the abdomen (Fig. 3A,B). In addition, ectopic expression of Abd-A (*PrdG4;UAS-MycAbdA*) is sufficient to stimulate *RhoAAA-lacZ* activity in the thorax (Fig. 3C). By contrast, comparative reporter assays using the thoracic Antennapedia (Antp) Hox factor (*PrdG4;UAS-MycAntp*) failed to significantly enhance *RhoAAA-lacZ* activity (Fig. 3D). Hence, the activation of RhoAAA in abdominal SOPs requires both Abd-A and DPax2 inputs, whereas a thoracic Hox factor fails to stimulate *RhoAAA-lacZ* with DPax2.

We next investigated the roles of the Hth and Exd Hox co-factors in mediating *RhoAAA-lacZ* activity in abdominal SOPs. Surprisingly, the Hth genetic and binding site loss-of-function assays revealed conflicting results. As shown in Fig. 3E, *RhoAAA-lacZ* activity was dramatically decreased in strong

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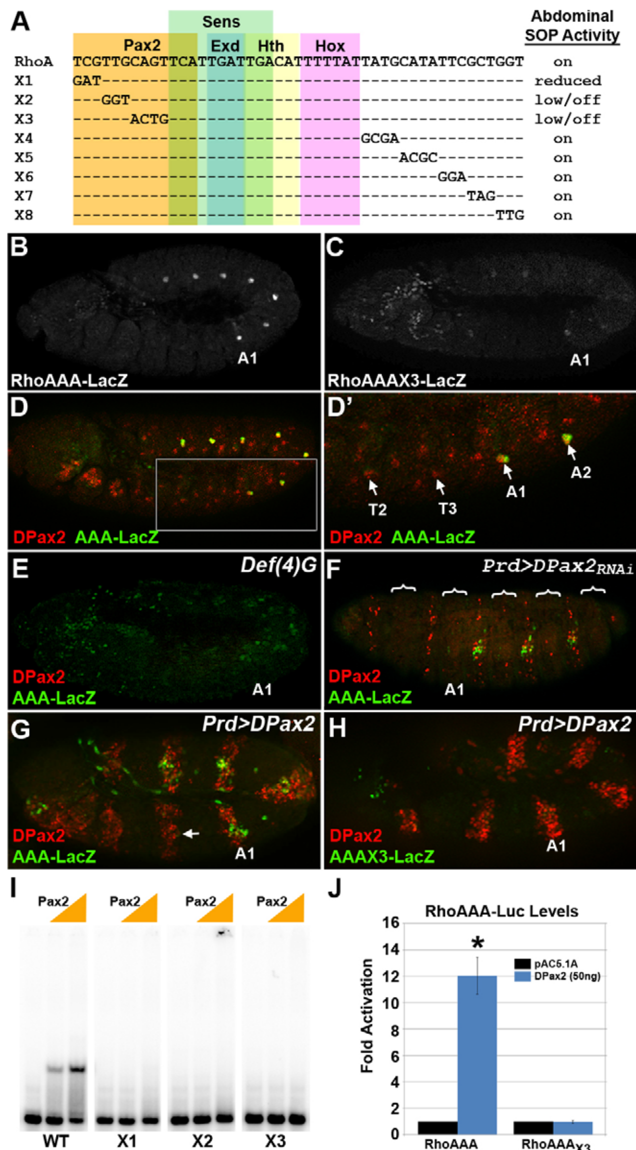

Fig. 2. DPax2 is an activator of the RhoA CRM. (**A**) The RhoA sequence and point mutations tested in transgenic reporter assays. The previously described Sens, Exd, Hth and Hox sites, as well as the newly characterized Pax2-binding site are highlighted. Column on the right summarizes the effect each mutation has on abdominal SOP reporter activity (see Fig. 2B,C; supplementary material Fig. S2). (**B**,**C**) Lateral views of stage 11 *RhoAAA-lacZ* (B) and *RhoAAA*$_{X3}$*-lacZ* embryos (C) immunostained for β-gal (white). The A1 segment is labeled. (**D**,**D′**) Lateral view of a stage 11 *RhoAAA-lacZ* embryo immunostained for β-gal (green) and DPax2 (red) reveals co-expression in abdominal SOPs. D′ is a higher magnification of two thoracic segments (T2 and T3) and two abdominal segments (A1 and A2), showing DPax2 is expressed in thoracic and abdominal SOPs (arrows), while *RhoAAA-lacZ* activity is restricted to the abdomen. (**E**) Lateral view of a stage 11 *RhoAAA-lacZ;Def(4)G* embryo immunostained for β-gal (green) and DPax2 (red) reveals a loss of *RhoAAA-lacZ* activity in abdominal SOPs. The abdominal SOPs still form in *Def(4)G* embryos, as evidenced by the expression pattern of Sens (supplementary material Fig. S4). (**F**) Lateral view of a stage 16 *PrdG4;UAS-DPax2RNAi;RhoAAA-lacZ* embryo immunostained for DPax2 (red) and β-gal (green). *PrdG4*-expressing segments have decreased DPax2 protein and *RhoAAA-lacZ* activity within the abdomen (first abdominal segment is marked, A1). (**G**,**H**) Lateral view of a stage 11 *RhoAAA-lacZ;PrdG4;UAS-DPax2* embryo (G) and a stage 11 *RhoAAA*$_{X3}$*-lacZ;PrdG4;UAS-DPax2* embryo (H) immunostained for DPax2 (red) and β-gal (green). DPax2 activates abdominal but not thoracic expression of *RhoAAA-lacZ* (arrow in G). DPax2 does not stimulate a reporter containing the X3 mutation that disrupts DNA binding. (**I**) EMSAs of wild type (WT) and mutant (X1, X2, X3) RhoA probes using two amounts of DPax2 protein (10 or 100 ng). (**J**) *Drosophila* S2 reporter assays reveal that DPax2 (50 ng, blue bars) significantly stimulates *RhoAAA-luciferase* (*$P<0.005$) but not a vector containing the X3 mutation (*RhoAAA*$_{X3}$*-luciferase*) that disrupts DPax2 binding. Cells transfected with empty pAc5.1 vector (black bars) had no effect on reporter activity.

hypomorphic mutant embryos (*hth*^{p2}), whereas Hth-binding site mutations that compromise Exd/Hth and Exd/Hth/Abd-A binding had little effect on abdominal SOP expression (Fig. 3A,F). To further assess the role of Hth DNA binding, we took advantage of a *hth* allele (*hth*$^{100-1}$) containing a premature stop codon that creates a homeodomain-less protein (HthΔhd) (Kurant et al., 2001). This protein mimics naturally occurring spliced isoforms of Hth that are unable to bind DNA but do interact with Exd (Noro et al., 2006). Consistent with Hth playing a DNA binding-independent role, *RhoAAA-lacZ* activity is relatively normal in abdominal SOPs of *hth*$^{100-1}$ mutant embryos (Fig. 3G). Unfortunately, similar genetic analysis cannot be meaningfully analyzed in *exd*-null mutants owing to segmentation defects and loss of sensory cells. However, RhoA point mutations that disrupt Exd binding have abdominal SOP activity, suggesting Exd DNA binding is not required for *RhoAAA-lacZ* activation (not shown). Altogether, these data indicate that while direct DNA binding by Pax2 and Abd-A is required for RhoAAA activity, Hth is likely to have a DNA binding-independent function in stimulating this reporter.

Integration of DPax2 and an Abd-A Hox complex stimulates cell-specific gene expression

To better investigate Hox specificity and the role of Exd/Hth in activating RhoA, we used a *RhoAAA-luciferase* assay in *Drosophila* S2 cells by transfecting limiting amounts of DPax2 with various combinations of Abd-A, Antp and either full-length (FL) or homeodomain-less (Δhd) Hth expression constructs (S2 cells express Exd). The expression of either Hox factor or Hth protein on its own or in combination had little effect on *RhoAAA-luciferase* activity (supplementary material Fig. S5A). By contrast, co-transfection of Abd-A with DPax2 resulted in a ninefold stimulation of *RhoAAA-luciferase* activity, whereas Antp and DPax2 had a more modest but significant fourfold increase (Fig. 4A). However, the inclusion of either the full-length Hth or HthΔhd protein with Abd-A and DPax2 dramatically enhanced *RhoAAA-luciferase* activity, whereas no additional stimulation was observed when Hth was expressed with Antp and DPax2 (Fig. 4A; supplementary material Fig. S5C,D). Moreover, the DPax2, Abd-A and Hth activation of *RhoAAA-luciferase* is dependent upon both the Hox- and DPax2-binding sites (supplementary material Fig. S5B). Thus, these cell culture findings correlate well with the in vivo genetic experiments demonstrating RhoAAA activity is dependent upon DPax2, Abd-A and either a full-length or DNA binding-deficient Hth protein.

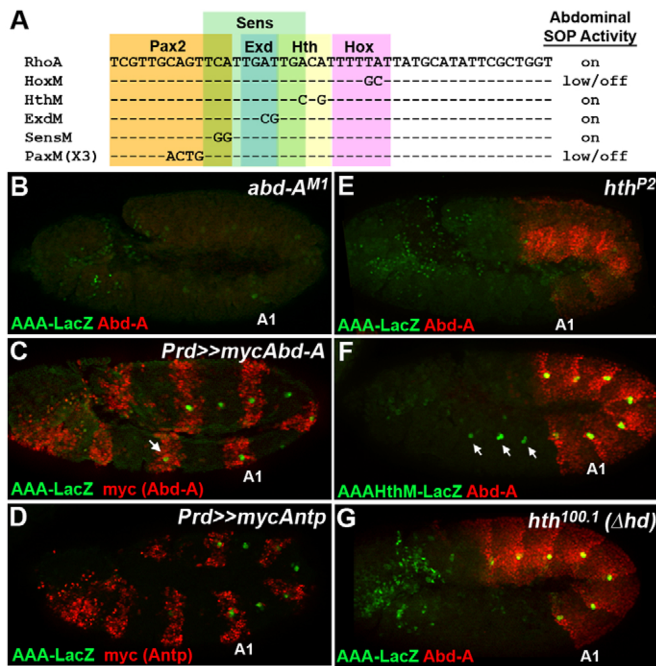

Fig. 3. *abd-A* and *hth* regulate *RhoAAA-lacZ* in abdominal SOPs. (**A**) The RhoA wild type and Hox, Exd, Hth and Sens mutations tested in DNA-binding and reporter assays. The Pax2, Sens, Exd, Hth and Hox sites are highlighted, and the column on the right summarizes the effect each mutation has on abdominal SOP reporter activity. (**B**) Lateral view of a stage 11 *RhoAAA-lacZ;abd-A^{M1}* embryo immunostained for β-gal (green) and Abd-A (red) reveals a loss of *RhoAAA-lacZ* activity (A1 segment indicated). (**C**,**D**) Lateral views of stage 11 *RhoAAA-lacZ;PrdG4;UAS-MycAbd-A* (C) or *RhoAAA-lacZ;PrdG4;UAS-MycAntp* (D) embryos immunostained for β-gal (green) and Abd-A (red). (**E**) Lateral view of a stage 11 *RhoAAA-lacZ;hth^{p2}* embryo immunostained for β-gal (green) and Abd-A (red) reveals a loss of *RhoAAA-lacZ* activity (A1 segment indicated). (**F**) Lateral view of a stage 11 *RhoAAA$_{HthM}$-lacZ* embryo immunostained for β-gal (green) and Abd-A (red) reveals relatively normal β-gal expression in the abdomen (A1 segment indicated) and de-repression in the thorax (white arrows). Thoracic de-repression correlates with a loss of DNA binding to the HthM site by the Sens repressor (Li-Kroeger et al., 2008). (**G**) Lateral view of a stage 11 *RhoAAA-lacZ;hth$^{100.1}$* embryo immunostained for β-gal (green) and Abd-A (red) reveals normal β-gal expression in the abdomen (A1 segment indicated).

As DPax2 and Abd-A synergistically activate gene expression in cell culture, we performed co-immunoprecipitation (co-IP) assays to assess whether they interact in S2 cells. An epitope-tagged DPax2 protein (DPax2:V5) was co-expressed with either a FLAG-tagged Abd-A or Antp. As shown in Fig. 4B, the IP of DPax2 using a V5 antibody revealed interactions with Abd-A but not Antp. Moreover, reciprocal experiments using anti-FLAG reagents revealed similar DPax2/Abd-A but not DPax2/Antp interactions (not shown). To determine whether Hth facilitates DPax2-AbdA interactions, we co-transfected an HA-tagged Hth protein with DPax2:V5 and FLAG:Abd-A and found no difference in the amount of DPax2/Abd-A precipitated (not shown). Thus, DPax2 interacts with Abd-A but not Antp, and Hth does not enhance this interaction.

We next investigated how DPax2 is integrated with Abd-A using EMSAs with purified DPax2 (V5-tagged) and Abd-A proteins. Our results reveal DPax2 and Abd-A form a complex on the RhoA probe and supershift assays confirmed both proteins are part of this complex (Fig. 4C,D). However, DPax2 and Abd-A do not cooperatively bind RhoA, as a similar amount of probe is bound by DPax2 in the presence and absence of Abd-A (supplementary material Fig. S5F). To ascertain the sequence dependence of DPax2/Abd-A complex formation on RhoA, we performed EMSAs on mutant probes and found the PaxM (X3) and HoxM mutations diminish complex formation in vitro (Fig. 4C), whereas SensM, ExdM and HthM do not affect DPax2/Abd-A binding (supplementary material Fig. S5E). Thus, the ability of DPax2 and Abd-A to form a complex on RhoA correlates well with *RhoAAA-lacZ* activity in abdominal SOPs.

To determine whether the DPax2/Abd-A/DNA complex is Hox specific, we compared the ability of equimolar amounts of Antp and Abd-A to form complexes on RhoA with DPax2 in the absence and presence of Exd/Hth(Δhd). For this assay, we used concentrations of Abd-A and Antp that do not significantly bind RhoA when added in isolation (Fig. 4E). When added with DPax2, we surprisingly found that Antp formed a complex on RhoA as efficiently as Abd-A (Fig. 4E). However, only Abd-A forms an additional complex upon adding Exd/HthΔhd (Fig. 4E). These data are consistent with the in vivo genetics and cell culture-based *RhoAAA-luciferase* data that revealed optimal activation requires DPax2, Abd-A and an Exd/Hth heterodimer. Last, we assessed the effect DPax2 and Abd-A binding has on the ability of the Sens repressor to interact with RhoA. We previously published that an Exd/Hth/Abd-A complex competes with Sens for overlapping RhoA sequences (Li-Kroeger et al., 2008). We similarly found that DPax2 and Abd-A compete for RhoA binding with Sens (Fig. 4F). Altogether, these data are consistent with Abd-A stimulating RhoA activity in two ways: (1) by forming a DPax2/Exd/Hth/Abd-A activation complex; and (2) by forming complexes with Exd/Hth and/or DPax2 to limit the binding of the Sens repressor.

The role of DPax2 DNA binding affinity in mediating abdomen-specific gene expression

Our genetic, cell culture and DNA-binding assays suggest that abdominal SOP specificity of *RhoAAA-lacZ* is mediated by interactions between Abd-A and DPax2. But how are these inputs integrated to mediate cell-specific activation? The lack of synergistic DNA binding between DPax2 and Abd-A suggests cooperative interactions do not account for abdominal specificity. However, a limitation of the in vitro DNA-binding studies is the use of proteins from bacteria, which may not fully represent the activities of DPax2 and Abd-A in *Drosophila* embryos. To circumvent this problem, we reasoned that if the primary role of Abd-A in vivo is to recruit/stabilize DPax2 binding to RhoA, then increasing the binding affinity of DPax2 should bypass the requirement for Hox input and result in all DPax2-positive cells activating reporter expression. To test this idea, we created a higher affinity DPax2-binding site by altering the RhoA sequence to match the consensus Pax2-binding sequence (Fig. 5A) (Jun and Desplan, 1996). Comparative DNA binding and competition assays revealed DPax2 binds this strong Pax site (PaxS) over 25-fold better than does wild-type RhoA, without affecting the binding of Sens or Abd-A (Fig. 5B-D and not shown). We subsequently analyzed transgenic flies carrying *RhoAAA$_{PaxS}$-LacZ* and found reporter activity was still abdominal SOP-specific (Fig. 5E). Moreover, even providing high levels of DPax2 using *PrdG4* activates *RhoAAA$_{PaxS}$-LacZ* in abdominal but not thoracic segments, suggesting that a complex with Abd-A is required for gene activation (Fig. 5F).

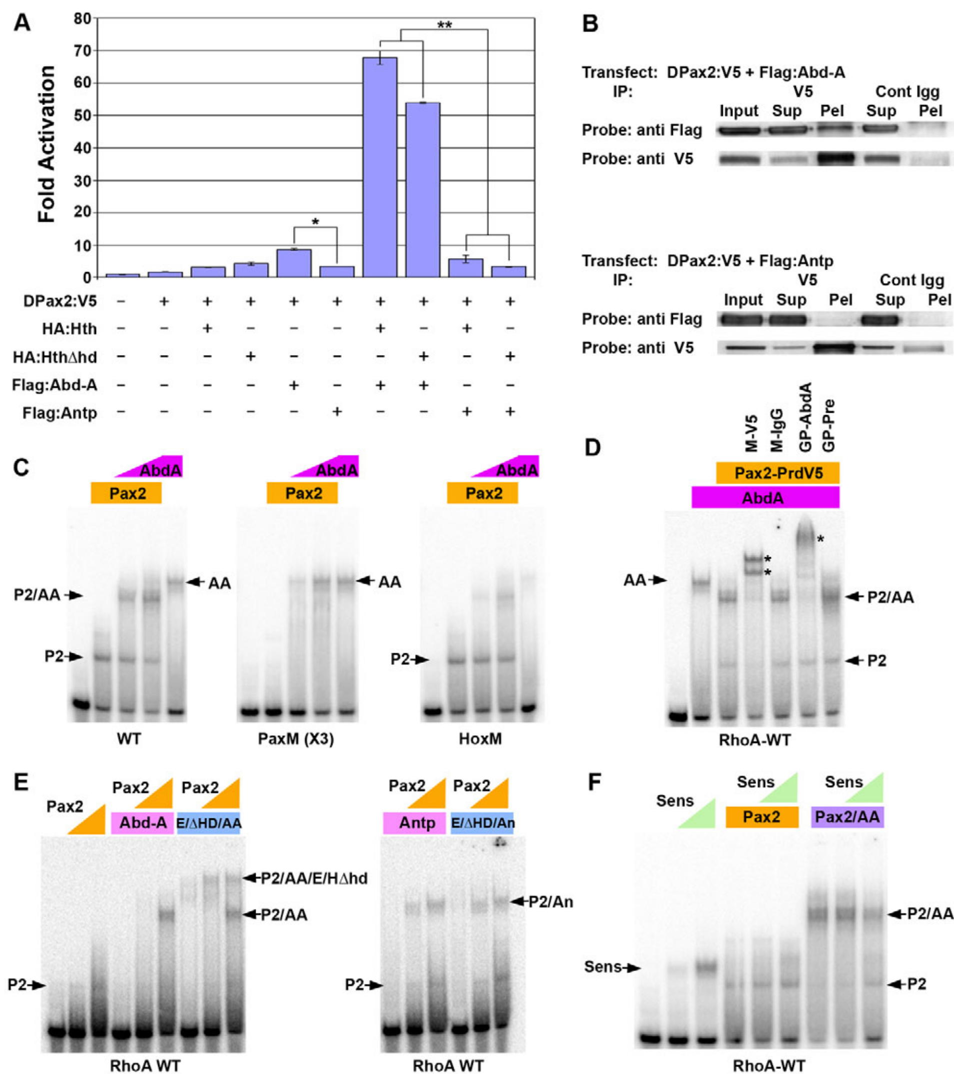

Fig. 4. The RhoA CRM integrates DPax2 and an Abd-A Hox complex to stimulate gene activation. (**A**) Luciferase assays from *Drosophila* S2 cells transfected with *RhoAAA-luciferase* and the indicated epitope-tagged constructs (25 ng) revealed optimal gene activation was achieved by the co-expression of DPax2 and Abd-A, and either a full-length Hth protein or a Hth protein lacking the homeodomain (HthΔhd). By contrast, equal expression of Antp failed to similarly enhance gene expression ($P<0.001$). (**B**) Co-immunoprecipitation (IP) assays of epitope-tagged DPax2 (V5-tag) and Hox factors (FLAG:Abd-A or FLAG-Antp) from transfected S2 cells reveals interactions between DPax2 and Abd-A (top) but not Antp (bottom). IPs were performed using a V5 antibody followed by western blot analysis of the input lysate (4% of total), the supernatant after IP (4% of total) and the pelleted complexes probed for either the FLAG or V5 epitopes. (**C**) EMSAs using a constant amount of DPax2 (150 ng) and two amounts of Abd-A (50 or 250 ng) on the wild-type (WT), PaxM (X3) and HoxM RhoA probes. Arrows indicate DNA-protein migration patterns for DPax2 (P2), Abd-A (AA) and DPax2/Abd-A (P2/AA). (**D**) Supershift assays using a RhoA probe with Abd-A (250 ng) and DPax2 (150 ng) in the presence of mouse-V5, mouse-IgG, guinea pig (GP)-AbdA or GP-preimmune serum. Both the V5 and Abd-A antibodies supershift the DPax2/Abd-A complex (asterisks). (**E**) Comparative EMSAs using a RhoA probe with two concentrations of DPax2 (10 and 100 ng) in the absence and presence of equimolar amounts of Abd-A (25 ng) or Antp (35 ng) and Exd/HthΔhd (50 ng). (**F**) EMSAs on a RhoA probe using Sens (25 or 250 ng), DPax2 (150 ng) and Abd-A (250 ng). Sens binding to RhoA is decreased in the presence of either DPax2 or DPax2 and Abd-A.

Thus, these data support the model that the independent binding of both DPax2 and Abd-A is required to form a functional activation complex on the RhoA CRM.

DPax2 contributes to strong RhoBAD activation and the induction of oenocytes

The RhoA sequence is part of the larger RhoBAD CRM that activates gene expression within abdominal SOPs. As DPax2 directly activates RhoAAA with Abd-A, we sought to determine whether it also regulates RhoBAD. We examined *RhoBAD-lacZ* activity in *Def(4)G* embryos that lack DPax2 and found reduced, but not absent, β-gal expression in abdominal SOPs (Fig. 6A,B). Quantification of β-gal levels within the abdominal segments of ten aged-matched embryos revealed a 40-50% reduction in reporter activity (Fig. 6C). By contrast, β-gal levels in thoracic segments were not significantly different between wild type and *Def(4)G* embryos (Fig. 6C). We further explored the role of DPax2 in regulating RhoBAD by analyzing the expression of a reporter containing the RhoA X3 Pax mutation ($RhoBAD_{PaxM}$-*lacZ*) and found a similar reduction in abdominal (~43% decrease) but not thoracic SOP expression (Fig. 6D). In contrast to the partial loss of RhoBAD activity in DPax2 mutants, the genetic removal of *abd-A*

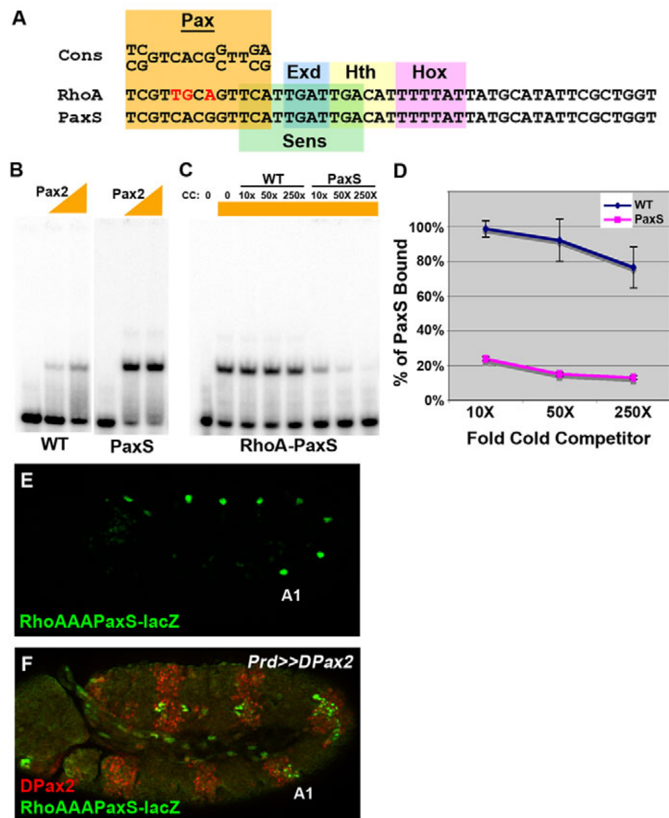

Fig. 5. A high-affinity DPax2 DNA-binding site in the RhoA CRM maintains abdominal SOP-specific transcriptional activity. (**A**) Alignment of a consensus Pax2 site (Cons) with the RhoA sequence. The RhoA nucleotides that do not match the consensus are in red and each has been changed to match the consensus in the PaxS probe (Jun and Desplan, 1996). (**B**) Comparative EMSAs using two concentrations of DPax2 (10 and 100 ng) with the wild type (WT) and PaxS probes. (**C**) DNA-binding competition assays using a constant amount of DPax2 (10 ng) with a radiolabeled RhoA-PaxS probe and 10×, 50× or 250× of cold RhoA-WT or PaxS competitors. (**D**) Results of three different DNA-binding competition assays using DPax2 protein and the RhoA-WT and RhoA-PaxS probes. The amount of RhoA-PaxS probe bound in the absence of any cold competitor was defined as 100%. (**E**) Lateral view of a stage 11 *RhoAAAPaxS-lacZ* embryo immunostained for β-gal (green) reveals strong reporter activity is restricted to abdominal SOPs. (**F**) Lateral view of a stage 11 *RhoAAAPaxS-lacZ;PrdG4;UAS-DPax2* embryo immunostained for β-gal (green) and DPax2 (red) reveals ectopic DPax2 only stimulates reporter activity in the abdomen.

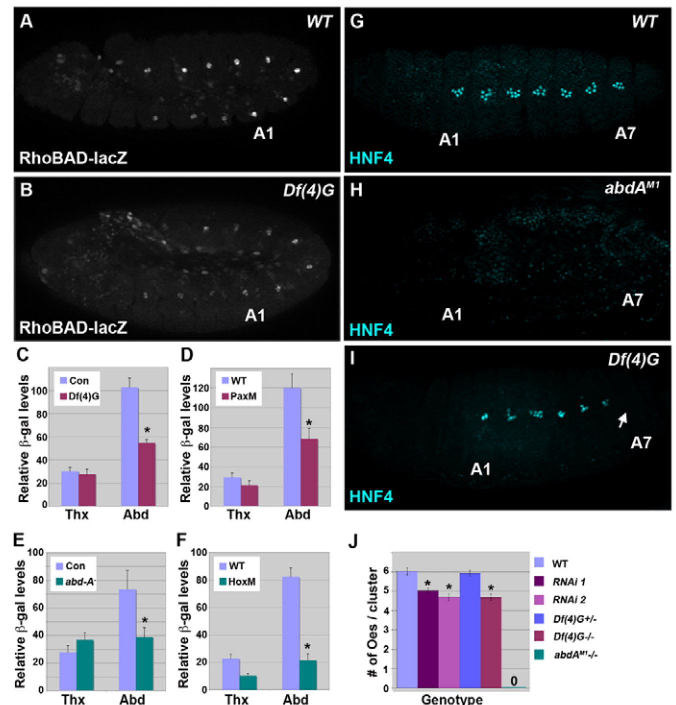

Fig. 6. DPax2 activity is required for proper RhoBAD activity and oenocyte numbers. (**A**,**B**) Lateral views of stage 11 wild-type *RhoBAD-lacZ* (A) and DPax2 mutant *RhoBAD-lacZ;Def(4)G* (B) embryos immunostained for β-gal (white) reveals strong β-gal is detected in abdominal segments of wild-type but not *Def(4)G* embryos, whereas similar weak thoracic expression is observed in both embryos. (**C-F**) Relative thoracic and abdominal expression levels of β-gal from 10 wild-type *RhoBAD-lacZ* (blue, C-F) and DPax2 mutant *RhoBAD-lacZ;Def(4)G* embryos (magenta, C), embryos carrying $RhoBAD_{PaxM}$*-lacZ* reporters (magenta, D), *abd-A* mutant *RhoBAD-lacZ;abd-A*^{M1} embryos (green, E) or embryos carrying $RhoBAD_{HoxM}$*-lacZ* reporters (green, F) (*P<0.01). (**G-I**) Lateral views of stage 16 wild-type (G), *abd-A*^{M1} (H) or *Def(4)G* (I) embryos immunostained with HNF4 (blue) to detect oenocytes within abdominal segments (A1 and A7are labeled). An abdominal segment in *Def(4)G* embryos lacks oenocytes (arrow). (**J**) Number of oenocytes that form in each abdominal segment of wild-type (WT), DPax2 knock-down (RNAi 1 and RNAi2), *Def(4)G* and *abd-A*^{M1} embryos (*P<0.01).

results in normalized *RhoBAD-lacZ* activity between thoracic and abdominal segments (Fig. 6E), and mutating the Hox-binding site within RhoBAD ($RhoBAD_{HoxM}$*-lacZ*) results in a more dramatic four-fold loss in reporter activity in abdominal SOPs (Fig. 6F). Thus, although DPax2 is required for some of the abdominal activity of RhoBAD, mutations that compromise Abd-A cause a complete loss of enhanced abdominal SOP activity. These findings are consistent with Abd-A using this Hox site to mediate the additional mechanism of limiting Sens repression on the RhoBAD CRM.

Ultimately, *rho* expression in abdominal SOPs leads to EGF secretion and the induction of oenocytes (Elstob et al., 2001; Rusten et al., 2001). Importantly, the number of oenocytes produced in each segment is dependent upon the level of EGF signaling, making oenocyte enumeration a functional readout of *rho* expression (Brodu et al., 2004). For example, the ectopic expression of Abd-A in thoracic segments activates *rho* expression to induce oenocytes, whereas the genetic removal of *abd-A* abolishes *rho* expression in abdominal SOPs and results in the complete loss of oenocytes (Fig. 6G,H; Brodu et al., 2002). To determine whether DPax2 can similarly affect oenocyte formation, we first attempted to use a gain-of-function approach using *PrdG4*. Unfortunately, however, ectopic DPax2 inhibited oenocyte formation even though their loss was not associated with a decrease of EGF signaling as revealed by phospho-ERK staining (data not shown). These findings suggest that expressing DPax2 within the EGF-receiving cells inhibits oenocyte differentiation, making gain-of-function approaches with DPax2 hard to interpret. Hence, we next examined DPax2 loss-of-function embryos using an oenocyte marker (HNF4) and found a small but significant reduction

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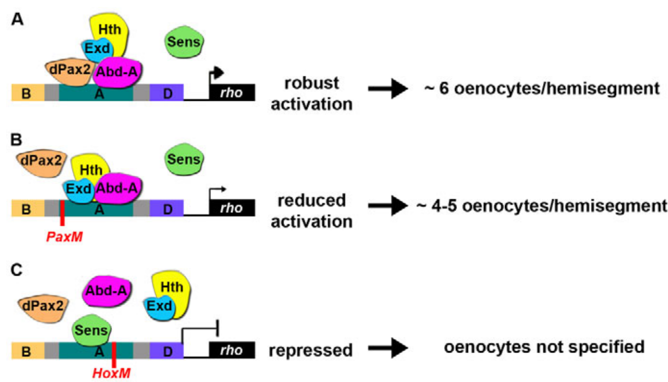

Fig. 7. Model of the Abd-A transcriptional mechanisms regulating RhoBAD activity in abdominal SOPs. (**A**) Schematic showing the DPax2/Abd-A/Exd/Hth complex on the RhoA CRM activates transcription and limits the binding of the Sens repressor to yield robust gene expression and the induction of six oenocytes per cluster. (**B**) Mutations in the Pax2-binding site disrupt the DPax2/Abd-A complex, resulting in diminished RhoBAD activity. However, an Abd-A/Hth/Exd complex can still bind RhoBAD to limit Sens-mediated repression and allow for some gene activation. Consistent with this model, DPax2 mutant embryos lose some, but not all, oenocytes in the *Drosophila* abdomen. (**C**) Mutations in the Hox-binding site disrupts both Abd-A complexes, allowing Sens to bind and the repression of RhoBAD activity; no oenocytes are specified.

compared with control embryos (Fig. 6I,J). A similar decrease in oenocytes was observed in segments expressing two different DPax2 UAS-RNAi constructs with *PrdG4* (Fig. 6J). Moreover, over 20% of *Def(4)G* mutant embryos have at least one abdominal segment that failed to form any oenocytes, a phenotype not observed in wild-type embryos. Thus, the loss of oenocytes in DPax2 mutants correlates well with the decreased *RhoBAD* activity observed in these embryos.

DISCUSSION

The activation of *rho* in abdominal SOPs provides a good example of how cell-specific gene expression can pattern the *Drosophila* embryo by inducing an abdominal-specific fate (oenocytes) (Elstob et al., 2001; Rusten et al., 2001). In particular, a subset of abdominal SOPs activates *rho* to promote EGF secretion, and the amount of *rho* expressed positively correlates with the number of oenocytes that develop (Brodu et al., 2004). We previously identified a *rho* CRM (RhoBAD) that is sufficient to recapitulate abdominal SOP expression and found that an Abd-A/Hth/Exd complex restricts the binding of the Sens repressor to RhoBAD (Li-Kroeger et al., 2008). Here, we further dissect the mechanisms used by Abd-A to ensure cell-specific RhoBAD activation. Using reporter assays, genetics and DNA-binding assays, we found that, in addition to Abd-A, DPax2 is a direct activator of RhoBAD. DPax2 is expressed by embryonic SOPs and the RhoA CRM mediates the formation of an activating complex composed of DPax2, Abd-A, Exd and Hth. Thus, Abd-A uses the same Hox-binding site to mediate robust cell-specific gene activation through two mechanisms: (1) by interacting with Exd/Hth to restrict the binding of Sens; and (2) by forming an activating complex with DPax2 (Fig. 7A). Hence, a RhoBAD mutation that selectively abolishes the DPax2-dependent mechanism (PaxM) has only a partial loss of abdominal SOP activity (Fig. 7B), whereas the HoxM mutation that disrupts both mechanisms results in a complete loss of enhanced abdominal SOP activity (Fig. 7C). Consistent with these findings on *rho* CRM function, *abd-A* mutants completely lack the EGF-dependent induction of oenocytes, whereas *DPax2* mutants have only a partial loss of oenocytes. Thus, our findings show that Abd-A uses both DPax2-dependent and DPax2-independent mechanisms to stimulate *rho* expression in SOPs.

Hox factors differentially regulate gene expression in specific cell and tissue types during development. For example, in addition to activating *rho* in abdominal SOPs, Abd-A activates *wingless* and represses *decapentaplegic* in the visceral mesoderm, and represses *distal-less* (*dll*) in the ectoderm (Grienenberger et al., 2003; Manak et al., 1994; Vachon et al., 1992). How does the same Hox factor regulate multiple target genes in a cell-specific manner? In general, Hox specificity could be achieved in multiple ways, including cell-specific post-translational modifications, recruitment of cell-specific co-activators/co-repressors, and/or the ability of CRMs to integrate Hox factors with additional transcriptional inputs. Our findings support the latter model, as the abdomen-specific activity of the RhoA CRM requires the binding of both DPax2 and Abd-A. As DPax2 expression is restricted to the PNS, this factor provides SOP specificity, whereas Abd-A provides abdominal specificity. Studies on Abd-A-mediated repression of a *Dll* CRM (DMXR) reveal a similar mechanism, albeit with different factors and binding sites. The DMXR integrates two additional transcription factors, Engrailed (En) and Sloppy-paired (Slp), which are expressed in subsets of the ectoderm (Gebelein et al., 2004). Importantly, like DPax2, En and Slp are also expressed in segments (the thorax) that express a different set of Hox factors and fail to either activate *rho* or repress *dll*. Hence, cell-specific gene regulation is achieved via the direct integration of a region-specific Hox factor with tissue-restricted transcription factors.

How does Abd-A synergize with DPax2 to stimulate RhoA CRM activity in abdominal SOPs, whereas a thoracic Hox factor (Antp) fails to perform this function in thoracic SOPs? Comparative genetics, reporter assays and biochemistry between these Hox factors reveal a model whereby Abd-A, but not Antp, activates *rho* in SOPs by forming a complex with DPax2, Exd and Hth on the RhoA CRM. There are two interesting aspects to this model: first, while direct binding of DPax2 and Abd-A to the RhoA CRM is required for gene activation, the direct binding of Hth to this CRM is not essential for this function (Fig. 7A). For example, RhoA CRM mutations that disrupt Hth DNA binding are active in vivo, and a *hth* mutation that generates a DNA-binding deficient protein can stimulate gene expression. By contrast, the complete absence of Hth results in a dramatic loss of reporter activity in abdominal SOPs, and the co-expression of DPax2 and Abd-A results in only weak reporter activation in cell culture without Hth. Hence, these data support Hth playing a non-DNA binding role in activating RhoA activity in abdominal SOPs. Second, comparative studies with Antp reveal this Hox factor fails to significantly stimulate the RhoA CRM with DPax2, Exd and Hth in vivo and in cell culture, fails to form a transcription factor complex with DPax2, Exd and Hth on the RhoA DNA, and fails to form a complex with DPax2 in co-IP assays. These findings suggest the mechanism underlying region- and cell-specific activation of *rho* in abdominal SOPs is due to the differential ability of Abd-A and Antp to form functional transcriptional complexes with DPax2 and the Exd/Hth Hox co-factors on the RhoA CRM.

Our findings that DPax2 forms complexes with Abd-A suggest they may cooperate to regulate additional target genes. In fact, published work demonstrates that a vertebrate transcription factor complex consisting of Pax2, the Hox11 paralogs, and the protein tyrosine phosphatase Eyes absent 1 (Eya1) regulate target genes

during mouse kidney development (Gong et al., 2007). Comparing the results of these two studies reveals interesting parallels and differences. First, in both cases Pax2 is collaborating with a posterior Hox factor to result in region- (abdomen) and tissue-specific (kidney in mouse, sensory cells in *Drosophila*) gene expression. Second, additional co-factor proteins are required for gene activation. In mammals, Eya1 is part of the Hox11-Pax2 complex that stimulates gene expression (Gong et al., 2007). *Drosophila* contains a single *eya* gene and expression analysis did not detect Eya protein in the SOPs that activate *RhoAAA-lacZ* (not shown). Moreover, eya^{D1}-null embryos have normal *RhoAAA-lacZ* expression (not shown), indicating Eya is not part of the activating complex in *Drosophila* SOPs. By contrast, Exd and Hth are an essential part of activating the RhoA CRM with DPax2 and Abd-A. Although vertebrate homologues of Exd (Pbx) and Hth (Meis) are expressed in the kidney (gudmap.org), their role in activating gene expression with Pax2-Eya1-Hox11 was not determined. Hence, future studies on Hox-Pax2 interactions are needed to determine whether only posterior/abdominal Hox factors collaborate with Pax2 and to test the role of different cofactors in regulating gene expression with these two factors.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References
Aspland, S. E. and White, R. A. (1997). Nucleocytoplasmic localisation of extradenticle protein is spatially regulated throughout development in Drosophila. *Development* **124**, 741-747.
Berger, M. F., Badis, G., Gehrke, A. R., Talukder, S., Philippakis, A. A., Pena-Castillo, L., Alleyne, T. M., Mnaimneh, S., Botvinnik, O. B., Chan, E. T. et al. (2008). Variation in homeodomain DNA binding revealed by high-resolution analysis of sequence preferences. *Cell* **133**, 1266-1276.
Brodu, V., Elstob, P. R. and Gould, A. P. (2002). abdominal A specifies one cell type in Drosophila by regulating one principal target gene. *Development* **129**, 2957-2963.
Brodu, V., Elstob, P. R. and Gould, A. P. (2004). EGF receptor signaling regulates pulses of cell delamination from the Drosophila ectoderm. *Dev. Cell* **7**, 885-895.
Carroll, S. B., Grenier, J. K. and Weatherbee, S. D. (2001). *From DNA to Diversity*. Malden, MA: Blackwell Science.
Charlton-Perkins, M., Whitaker, S. L., Fei, Y., Xie, B., Li-Kroeger, D., Gebelein, B. and Cook, T. (2011). Prospero and Pax2 combinatorially control neural cell fate decisions by modulating Ras- and Notch-dependent signaling. *Neural Dev.* **6**, 20.
Davidson, E. H. and Levine, M. S. (2008). Properties of developmental gene regulatory networks. *Proc. Natl. Acad. Sci. USA* **105**, 20063-20066.
Ekker, S. C., Jackson, D. G., von Kessler, D. P., Sun, B. I., Young, K. E. and Beachy, P. A. (1994). The degree of variation in DNA sequence recognition among four Drosophila homeotic proteins. *EMBO J.* **13**, 3551-3560.
Elstob, P. R., Brodu, V. and Gould, A. P. (2001). spalt-dependent switching between two cell fates that are induced by the Drosophila EGF receptor. *Development* **128**, 723-732.
Fu, W., Duan, H., Frei, E. and Noll, M. (1998). shaven and sparkling are mutations in separate enhancers of the Drosophila Pax2 homolog. *Development* **125**, 2943-2950.
Gebelein, B. and Urrutia, R. (2001). Sequence-specific transcriptional repression by KS1, a multiple-zinc-finger-Kruppel-associated box protein. *Mol. Cell. Biol.* **21**, 928-939.
Gebelein, B., Culi, J., Ryoo, H. D., Zhang, W. and Mann, R. S. (2002). Specificity of Distalless repression and limb primordia development by abdominal Hox proteins. *Dev. Cell* **3**, 487-498.
Gebelein, B., McKay, D. J. and Mann, R. S. (2004). Direct integration of Hox and segmentation gene inputs during Drosophila development. *Nature* **431**, 653-659.
Gong, K. Q., Yallowitz, A. R., Sun, H., Dressler, G. R. and Wellik, D. M. (2007). A Hox-Eya-Pax complex regulates early kidney developmental gene expression. *Mol. Cell. Biol.* **27**, 7661-7668.
Grienenberger, A., Merabet, S., Manak, J., Iltis, I., Fabre, A., Berenger, H., Scott, M. P., Pradel, J. and Graba, Y. (2003). Tgfbeta signaling acts on a Hox response element to confer specificity and diversity to Hox protein function. *Development* **130**, 5445-5455.
Gutzwiller, L. M., Witt, L. M., Gresser, A. L., Burns, K. A., Cook, T. A. and Gebelein, B. (2010). Proneural and abdominal Hox inputs synergize to promote sensory organ formation in the *Drosophila* abdomen. *Dev. Biol.* **348**, 231-243.
Jun, S. and Desplan, C. (1996). Cooperative interactions between paired domain and homeodomain. *Development* **122**, 2639-2650.
Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78**, 191-201.
Kurant, E., Eytan, D. and Salzberg, A. (2001). Mutational analysis of the Drosophila homothorax gene. *Genetics* **157**, 689-698.
Levine, M. (2010). Transcriptional enhancers in animal development and evolution. *Curr. Biol.* **20**, R754-R763.
Levine, M. and Davidson, E. H. (2005). Gene regulatory networks for development. *Proc. Natl. Acad. Sci. USA* **102**, 4936-4942.
Lewis, E. B. (1978). Gene complex controlling segmentation in Drosophila. *Nature* **276**, 565-570.
Li-Kroeger, D., Witt, L. M., Grimes, H. L., Cook, T. A. and Gebelein, B. (2008). Hox and senseless antagonism functions as a molecular switch to regulate EGF secretion in the Drosophila PNS. *Dev. Cell* **15**, 298-308.
Manak, J. R., Mathies, L. D. and Scott, M. P. (1994). Regulation of a decapentaplegic midgut enhancer by homeotic proteins. *Development* **120**, 3605-3619.
Mann, R. S., Lelli, K. M. and Joshi, R. (2009). Hox specificity unique roles for cofactors and collaborators. *Curr. Top. Dev. Biol.* **88**, 63-101.
McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.
Noro, B., Culi, J., McKay, D. J., Zhang, W. and Mann, R. S. (2006). Distinct functions of homeodomain-containing and homeodomain-less isoforms encoded by homothorax. *Genes Dev.* **20**, 1636-1650.
Noyes, M. B., Christensen, R. G., Wakabayashi, A., Stormo, G. D., Brodsky, M. H. and Wolfe, S. A. (2008). Analysis of homeodomain specificities allows the family-wide prediction of preferred recognition sites. *Cell* **133**, 1277-1289.
Pearson, J. C., Lemons, D. and McGinnis, W. (2005). Modulating Hox gene functions during animal body patterning. *Nat. Rev. Genet.* **6**, 893-904.
Rauskolb, C., Peifer, M. and Wieschaus, E. (1993). extradenticle, a regulator of homeotic gene activity, is a homolog of the homeobox-containing human proto-oncogene pbx1. *Cell* **74**, 1101-1112.
Rusten, T. E., Cantera, R., Urban, J., Technau, G., Kafatos, F. C. and Barrio, R. (2001). Spalt modifies EGFR-mediated induction of chordotonal precursors in the embryonic PNS of Drosophila promoting the development of oenocytes. *Development* **128**, 711-722.
Shilo, B. Z. (2005). Regulating the dynamics of EGF receptor signaling in space and time. *Development* **132**, 4017-4027.
Uhl, J. D., Cook, T. A. and Gebelein, B. (2010). Comparing anterior and posterior Hox complex formation reveals guidelines for predicting cis-regulatory elements. *Dev. Biol.* **343**, 154-166.
Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M. E., Botas, J. and Cohen, S. M. (1992). Homeotic genes of the Bithorax complex repress limb development in the abdomen of the Drosophila embryo through the target gene Distal-less. *Cell* **71**, 437-450.
Witt, L. M., Gutzwiller, L. M., Gresser, A. L., Li-Kroeger, D., Cook, T. A. and Gebelein, B. (2010). Atonal, Senseless, and Abdominal-A regulate rhomboid enhancer activity in abdominal sensory organ precursors. *Dev. Biol.* **344**, 1060-1070.
Xie, B., Charlton-Perkins, M., McDonald, E., Gebelein, B. and Cook, T. (2007). Senseless functions as a molecular switch for color photoreceptor differentiation in Drosophila. *Development* **134**, 4243-4253.