Development 134, 3585-3592 (2007) doi:10.1242/dev.009522

Collaboration between Smads and a Hox protein in target gene repression

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Hox proteins control the differentiation of serially iterated structures in arthropods and chordates by differentially regulating many target genes. It is yet unclear to what extent Hox target gene selection is dependent upon other regulatory factors and how these interactions might affect target gene activation or repression. We find that two Smad proteins, effectors of the *Drosophila* Dpp/TGF-β pathway, that are genetically required for the activation of the *spalt* (*sal*) gene in the wing, collaborate with the Hox protein Ultrabithorax (Ubx) to directly repress *sal* in the haltere. The repression of *sal* is integrated by a cis-regulatory element (CRE) through a remarkably conserved set of Smad binding sites flanked by Ubx binding sites. If the Ubx binding sites are relocated at a distance from the Smad binding sites, the proteins no longer collaborate to repress gene expression. These results support an emerging view of Hox proteins acting in collaboration with a much more diverse set of transcription factors than has generally been appreciated.

KEY WORDS: Hox proteins, Repression, Smad proteins, Collaboration, Combinatorial regulation

INTRODUCTION

The formation and differentiation of many animal body parts are controlled by a special class of transcription factors termed selector proteins, the most prominent of which are the Hox proteins (Mann and Morata, 2000). It is thought that the Hox proteins regulate many target genes within individual developing body parts and cells (Pearson et al., 2005). However, it is not clear how the Hox proteins selectively regulate a broad spectrum of target genes, nor is it understood how individual Hox proteins either activate or repress target gene expression.

Two DNA-binding co-factors, Extradenticle (Exd) (Chan et al., 1994) and Homothorax (Hth) (Rieckhof et al., 1997) of *Drosophila*, have been demonstrated to interact with and cooperate with Hox proteins in the regulation of certain target genes in vivo (Gebelein et al., 2004; Mann and Affolter, 1998; Ryoo et al., 1999). However, these two co-factors are not expressed in many tissues, such as the appendages (Azpiazu and Morata, 1998; Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995). Furthermore, Hox-Exd-Hth complexes activate some of their target genes but repress others. Thus, the binding of Hox-Exd-Hth complexes to target sites is not sufficient to account for their biological activity.

Although much focus has been placed on Exd and Hth as co-factors, it has recently been shown that certain Hox proteins can also collaborate with other transcription factors, specifically Engrailed (En) and Sloppy paired (Slp), in the selection of a target gene in vivo (Gebelein et al., 2004). In the case of Slp, collaboration occurs in the absence of a physical interaction. Beyond these few proteins and target genes, the prevalence of collaboration is unknown, and the diversity of collaborating factors and their impact on Hox protein activity has not been explored.

In *D. melanogaster*, the Ultrabithorax (Ubx) protein is the sole Hox protein that shapes the differentiation of the hindwing (haltere). Removal of Ubx activity from the developing haltere results in the homeotic transformation from hindwing to forewing (wing) morphology (Lewis, 1978). Ubx patterns the haltere by modulating the expression of a variety of genes in the wing morphogenetic program (Crickmore and Mann, 2006; Weatherbee et al., 1998).

The molecular requirements for Ubx target gene regulation are not well understood. The simple TAAT core nucleotide sequence of the Ubx and most other Hox binding sites is a very common motif within gene regulatory regions that are not Hox-responsive (Ekker et al., 1991). It is possible that the number and/or affinity of Hox binding sites in regulatory DNA must reach some threshold to elicit a response (Galant et al., 2002), or that the topology of Hox binding sites in association with other transcription factor binding sites might be critical for Hox target gene selection.

Here, we performed genetic and biochemical analyses to identify the transcription factors and regulatory sequences required for Ubx regulation of the *spalt* [*sal*; also known as *spalt major* (*salm*) – FlyBase] gene, which is directly repressed by Ubx in the haltere (Galant et al., 2002). Surprisingly, we found that whereas the Dpp/TGF-β pathway is required for *sal* activation in the developing wing, *sal* is directly repressed by a combination of Dpp signaling input and Ubx in the developing haltere. Furthermore, we show that the close proximity of Ubx and Smad binding sites in the *sal* cis-regulatory element (CRE) is critical for target gene repression. These results, together with recent findings (Gebelein et al., 2004), suggest that the Hox proteins collaborate with, and might depend upon, a wide variety of transcription factors for target gene regulation.

MATERIALS AND METHODS

Drosophila genotypes for clonal analysis

For the induction of clones, crosses were reared at 25°C. For *Mad* clones, flies of genotype *w*; $Mad^{[7\text{-}2]}$/CyO were crossed to *f z* FLP; FRT40A Ubi-GFP(nls)/CyO. For *Med* clones, flies of genotype FRT82B Med^{13}/TM6b were crossed to hsFLP; FRT82B Ubi-GFP(nls)/MKRS. For *shn* clones, flies of genotype *w*; FRT42D shn^{1B}/SM6a were crossed to *f z* FLP; FRT42D Ubi-GFP(nls)/CyO. Clones were induced in progeny 72 to 98 hours after egg laying (AEL) by heat shock at 37°C for 45 minutes. Larvae were aged an additional 48 hours. Third instar haltere imaginal discs were dissected, fixed and immunostained using previously described methods (Galant et al., 2002). The primary antibodies and their dilutions were: rabbit anti-Sal

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Accepted 7 August 2007

(1:1000; provided by R. Barrio) (de Celis et al., 1996), rat anti-Brk (1:100; provided by G. Campbell) (Campbell and Tomlinson, 1999) and mouse anti-Ubx FP3.83 (1:20; provided by R. White) (Kelsh et al., 1994).

Protein expression and EMSA

GST-MadN and GST-MedMH1 were purified from *Escherichia coli* as described (Kim et al., 1997; Xu et al., 1998). Full-length Ubx1a protein was produced by in vitro transcription and translation as described (Promega) (Galant and Carroll, 2002). Double-stranded oligonucleotide probes with GATC overhangs at the 5′ and 3′ ends were end-filled with [α-^{32}P]dNTPs using the Klenow fragment (Roche). Electrophoretic mobility shift assays (EMSAs) were performed using previously reported methods with the following modifications (Galant et al., 2002): the conditions for binding were 20 mM HEPES pH 7.8, 50 mM KCl, 0.25 mg/mL BSA, 1 mM DTT, 4% (w/v) Ficoll. Binding reactions were incubated on ice for 30 minutes and polyacrylamide gel electrophoresis was performed at 4°C.

Reporter constructs for the *sal*1.1 CRE

The sequence of the *sal*1.1 CRE is available in GenBank (accession AF46408712). Mutant variants of the *sal*1.1 CRE were created by site-directed mutagenesis via either two-step PCR or the QuikChange Multi Site-directed Mutagenesis Kit (Stratagene). Primer sequences are available upon request. Mutated *sal*1.1 CREs were cloned into the *hsp-lacZ CaSpeR* reporter plasmid (Nelson and Laughon, 1993). At least four independent lines for each construct were analyzed for expression level. Representative lines are included in figures.

Phylogenetic analysis of the *sal*1.1 CRE

D. melanogaster sequence of the *sal*1.1 CRE was aligned with *D. virilis* genomic sequence using BLAST and by eye. Regions of high nucleotide conservation flanking the *sal*1.1 CRE were used in the design of PCR primers. These primers were used to PCR amplify homologous *sal*1.1 CREs from *D. subobscura* and *D. malerkotliana*. Collected *sal*1.1 CREs were aligned using MacClade and by eye (Maddison and Maddison, 1989). The *sal*1.1 CRE from *D. pseudoobscura* was cloned into *hsp-lacZ CaSpeR*, used in P-element mediated transgenesis and tested for expression in three independent lines.

RESULTS

Mad/Med/Shn are required genetically for *sal* repression in the haltere imaginal disc

Sal is expressed in the 'pouch' of the wing imaginal disc, where it regulates the formation and position of longitudinal veins L2 and L5 (Barrio and de Celis, 2004; Sturtevant et al., 1997). *sal* is not expressed in the corresponding region of the haltere imaginal disc, owing to its direct repression by Ubx (Fig. 1A,B) (Galant et al., 2002). Ubx may repress *sal* or other target genes through a number of mechanisms. One possibility is that Ubx blocks the binding of an activator to *sal* regulatory DNA. A second possibility is that Ubx acts independently of activators or other proteins to repress target gene expression.

Activation of *sal* expression in the wing imaginal disc has been shown to require the Dpp/TGF-β signaling pathway (de Celis et al., 1996; Lecuit et al., 1996; Nellen et al., 1996). The Mothers against Dpp (Mad) protein is the *Drosophila* ortholog of Smad1/5 and is required for the transduction of Dpp signaling in the wing disc (Kim et al., 1997; Raftery et al., 1995; Sekelsky et al., 1995). Homozygous *Mad* mutant clones lack *sal* expression, indicating that Mad is genetically required for *sal* activation in the wing disc (Fig. 1E,F) (Lecuit et al., 1996; Marty et al., 2000).

We were therefore surprised to observe that *sal* was expressed in *Mad* mutant clones in the haltere disc (in 24% of clones). Mad is therefore required to repress *sal* expression in this tissue (Fig. 1I,J), and is not required for *sal* activation in the haltere disc. Either the perdurance of activated, phosphorylated Mad (pMad) in cells or a restricted temporal requirement for Mad activity might account for the clones in which *sal* is not derepressed. *sal* is derepressed in larger clones further from the source of Dpp signaling along the anterior-posterior compartment boundary and these cells have lower levels of pMad. *sal* expression in *Mad* mutant clones in the haltere disc could be due to either a direct requirement for Mad to repress *sal* or to an indirect effect of the cell-autonomous loss of Mad activity on the expression of some other repressor of *sal*.

In order to test whether *sal* expression in *Mad* mutant clones could be an indirect effect, we examined the effect of loss of pMad activity on the expression of two repressors of *sal*, *brinker* (*brk*) (Barrio and de Celis, 2004) and *Ubx* (Galant et al., 2002). In wild-type wing and haltere imaginal discs, *brk* is expressed in cells along the lateral edges of each disc and is repressed in the central region by the Dpp morphogen gradient emanating from the anterior-posterior compartment boundary (Fig. 1C,D) (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). In *Mad* clones in the wing disc, *brk* was expressed and in turn repressed *sal* (Fig. 1E,G). In the haltere disc, *brk* was also expressed in *Mad* clones; however, it did not repress *sal* expression (Fig. 1I,L). Furthermore, *Ubx* expression was not altered in *Mad* mutant clones in the haltere, demonstrating that the derepression of *sal* is not due to a loss of *Ubx* expression (data not shown). Therefore, the derepression of *sal* due to the loss of Mad activity is not a secondary effect on known repressors of *sal* in the haltere.

It is well established that R-Smads interact with a co-Smad in target gene regulation (Feng and Derynck, 2005). In *Drosophila*, Mad, an R-Smad, interacts with the co-Smad Medea (Med), the ortholog of Smad4 (Das et al., 1998). Schnurri (Shn) is a co-repressor known to interact in a trimeric complex with Mad and Med (Pyrowolakis et al., 2004). In order to test whether Med and Shn are also required to repress *sal* in the haltere, we examined *sal* expression in *Med* and *shn* hypomorphic clones in the haltere disc. *sal* was found to be derepressed in *Med* hypomorphic clones (26% of clones) and in *shn* hypomorphic clones (29% of clones) in the haltere disc (Fig. 1M-P). These results suggest that the trimeric repressor complex of Mad-Med-Shn is required to repress *sal* in the haltere and raises the possibility that the complex acts directly upon a regulatory element of the *sal* gene.

A Mad/Med binding site is required for *sal* repression in the haltere imaginal disc

The activation of *sal* in the wing and its repression in the haltere are regulated by a 1.1 kb CRE, *sal*1.1 (Galant et al., 2002). Previously, we have shown that *sal*1.1 is directly repressed by Ubx in the haltere (Galant et al., 2002). In order to test whether Mad/Med binds to and directly represses the activity of the *sal*1.1 CRE in the haltere, we searched for candidate Mad/Med binding sites in the *sal*1.1 CRE. We identified one candidate Mad/Med binding site, M1 (5′-AGACGGGCAC-3′), which lies between Ubx binding sites 5 and 6 in *sal*1.1, using binding site prediction and electrophoretic mobility shift assays (EMSAs) (Fig. 2A). The sequence of M1 deviates somewhat from published Mad/Med silencer consensus binding sites (5′-AGAC-5 bp-GNCGYC-3′) (Gao et al., 2005; Pyrowolakis et al., 2004), and Mad and Med bound with >10-fold and >25-fold lower affinities, respectively, to the M1 site than to the *bam* (Gao et al., 2005) and *brk* (Pyrowolakis et al., 2004) silencer elements (data not shown).

In order to test whether Mad/Med bound specifically to the M1 site, we introduced a series of point mutations within the M1 site and examined their effect on protein binding in vitro. Of four point

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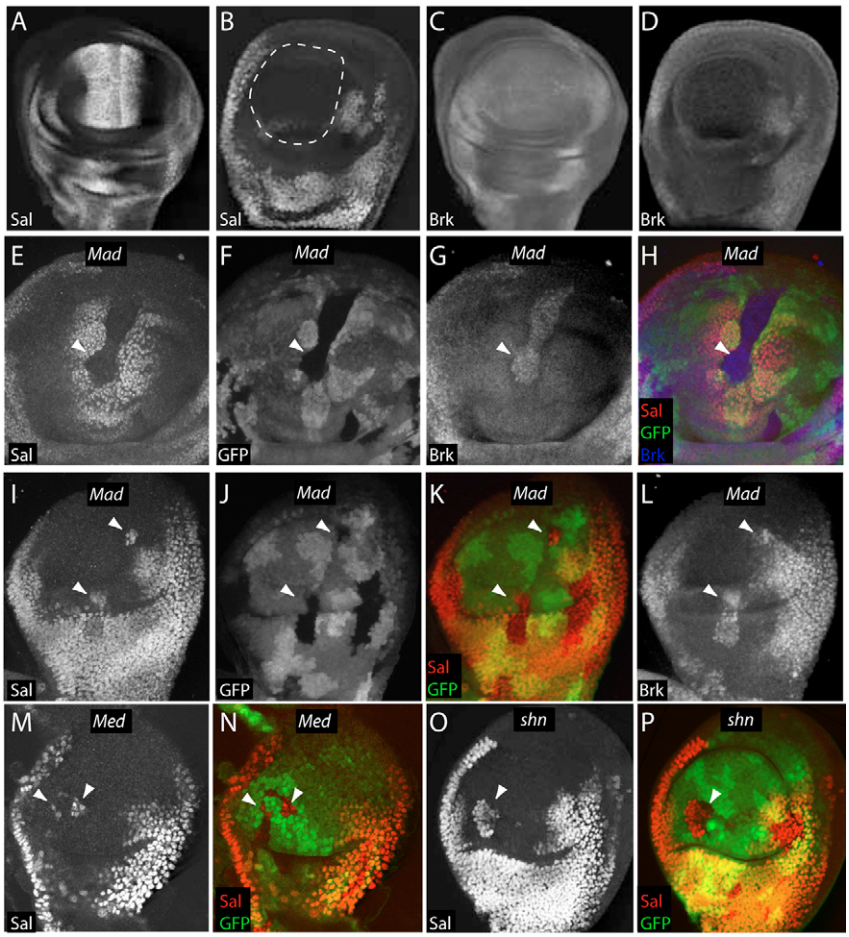

Fig. 1. *Mad*, *Med* and *shn* are required to repress *sal* expression in the *Drosophila* haltere. The proteins visualized are indicated at the bottom left of each panel; in E-P, the mutant gene in clones is indicated at the top of each panel. The absence of GFP expression indicates the position of homozygous mutant clones. (**A**,**B**) Wild-type Sal expression in the wing (A) and haltere (B) imaginal discs. Sal is repressed in the haltere pouch (dashed outline). (**C**,**D**) Brk is expressed in both the wing (C) and haltere (D) imaginal discs. (**E-H**) Sal is not expressed in *Mad* homozygous mutant clones in the wing (E,F, arrowheads), owing, at least in part, to the repression of *sal* by the upregulation of Brk in clones (G, arrowhead). (H) Merged image of E-G. (**I-P**) Sal is derepressed in *Mad* (I-K, arrowheads), *Med* (M,N, arrowheads), and *shn* (O,P, arrowheads) mutant clones in the haltere. (L) Brk is expressed in *Mad* mutant clones but is insufficient to repress *sal* expression in the haltere. (K) Merged image of I,J. (N) Merged image of M and GFP channel (data not shown). (P) Merged image of O and GFP channel (data not shown).

mutations to the M1 site, the single mutation at position 808 reduced the binding of a Med fusion protein (GST-MedMH1) to M1 as compared with the wild-type sequence (Fig. 2B, lanes 1-4 and 5-8). The remaining three point mutations did not affect the affinity of GST-MedMH1 for the probe (Fig. 2B, lanes 9-20). These results suggest that Med might contact the sequence 5′-AGAC-3′ in *sal*1.1 (Fig. 2A). By contrast, the four individual point mutations each decreased, but did not abolish, binding of a Mad fusion protein (GST-MadN) in vitro, with the point mutation at bp 814 having the strongest effect (Fig. 2C, lanes 25-49). The weaker effect of the individual point mutations in M1 on Mad binding affinity in vitro is likely to be due to the affinity of MadN for both 5′-AGAC-3′ Smad sites and GC-rich sequence. Combining these four mutations (sal798-824 kM1) had the greatest effect on GST-MadN binding to the probe (Fig. 2C, lanes 25-29 and 50-54). This analysis of individual point mutations indicates a putative orientation for a Mad/Med compound-binding site in the *sal*1.1 CRE (Fig. 2A).

Most importantly, in transgenic flies, each point mutation of M1 introduced into an otherwise wild-type *sal*1.1 reporter construct caused derepression of the reporter gene *lac*Z in the haltere imaginal disc (Fig. 2D-H). The strength of derepression correlates with the decreased affinity of Mad for its binding site with the pm814 mutation, the strongest point mutation in vitro, showing the strongest level of derepression in vivo (Fig. 2H). We observed full derepression when all four point mutations were combined into a *sal*1.1 reporter construct (Fig. 2I). We did not observe an effect of mutations in M1 on *sal*1.1-driven reporter gene expression in the wing as compared with the wild-type *sal*1.1 element or with endogenous *sal* expression, indicating that this site is not required for gene activation in the wing or haltere disc (data not shown). Together, the biochemical, reporter gene and genetic evidence indicate that Mad/Med/Shn are directly required for *sal* repression in the haltere imaginal disc.

Juxtaposition of Ubx and Mad/Med binding sites is required for *sal* repression

The Mad/Med M1 binding site and the Ubx U5 and U6 binding sites lie adjacent to one another. This proximity raises the possibility that mutations in one site could affect the binding of the other protein and/or that the proteins might contact each other. We tested whether mutations in the M1 site affected the affinity of Ubx for its neighboring sites U5 and U6 by EMSA in vitro (Fig. 3A,B). Initially, we examined the activity of the Ubx homeodomain (HD) and found that it bound similarly to wild-type (Fig. 3A, lanes 2-7) and mutated (Fig. 3A, lanes 16-21) M1 probes in EMSAs. However, we considered that the truncated Ubx-HD protein might be less sensitive than full-length Ubx1a to mutations in sites flanking its TAAT binding site core sequence. Therefore, we also examined the binding of full-length Ubx1a to probes in which the M1 site was mutated. Ubx1a bound equally well to U5 and U6 even when all four mutations were introduced into the M1 site (Fig. 3B, lanes 12-15), but did not bind to probes when the U5 and U6 sites were mutated (Fig. 3B, lanes 7-10). Similarly, binding of GST-MedMH1 (Fig. 3C, lanes 1-8) and GST-MadN (Fig. 3D, lanes 9-18) to the M1 site was unaffected when Ubx binding sites U5 and U6 were mutated. We

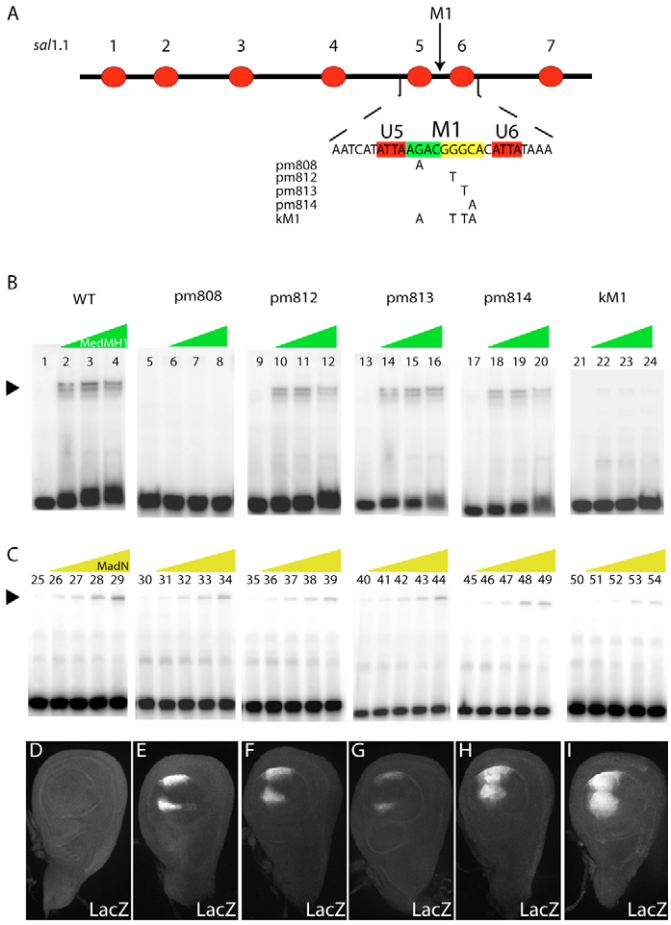

Fig. 2. Mad and Med directly repress *sal* expression in the haltere through binding sites in the *sal*1.1 CRE. (**A**) Schematic of the *Drosophila sal*1.1 CRE with Ubx binding sites 1-7 (red circles). Putative Med (green box) and Mad (yellow box) binding sites are located between Ubx binding sites 5 and 6 (red boxes). Binding site mutations introduced into the M1 site are indicated below the wild-type *sal*1.1 sequence. The binding of proteins to the probes was quantitated with ImageQuant software to assess the effect of mutations on their affinity for specific sites. (**B-I**) The effect of mutations in the putative M1 site on Mad and Med binding in vitro and their effect on reporter gene expression in vivo are aligned in columns. (B) Electrophoretic mobility shift assays (EMSAs) with GST-MedMH1. In each set of lanes, the protein concentration increases from left to right. The point mutation at bp 808 (lanes 5-8) eliminates Med binding as compared with the wild-type M1 site (lanes 1-4). Mutations at positions 812 (lanes 9-12), 813 (lanes 13-16) and 814 (lanes 17-20) have little to no effect on Med binding. (C) EMSAs with GST-MadN on wild-type and mutant M1 probes. In each set of lanes, the protein concentration increases from left to right. Each point mutation (808, 812, 813 and 814) causes a decrease in the strength of Mad binding (2.2-, 2.7-, 2.6- and 3.6-fold, respectively) as compared with the wild-type sequence (compare lanes 25-29 with 30-49). Combining the four point mutations in kM1 causes an 8.6-fold reduction in Mad binding affinity (lanes 50-54). (D-I) Haltere imaginal discs of transgenic *sal*1.1 reporter lines that are either wild-type or that carry mutations in the M1 site, immunolabeled for *lacZ* expression.

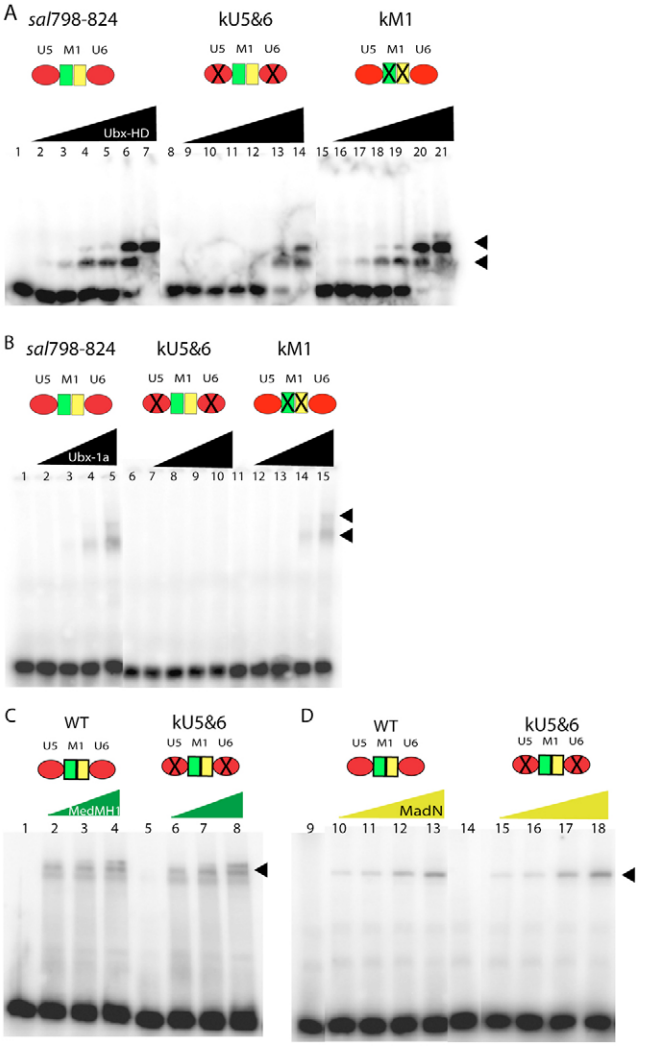

Fig. 3. Discrete Ubx and Smad binding sites are closely juxtaposed, but independent of one another, in the *sal*1.1 CRE. Probes with Ubx binding sites 5 and 6 (red circles) and Med and Mad binding sites (green and yellow boxes, respectively) and mutated binding sites (marked with an 'X') are depicted above each EMSA. Protein-DNA complexes are indicated by arrowheads. (**A**,**B**) EMSAs with either purified Ubx homeodomain (Ubx-HD) (A) or full-length Ubx1a (B) on wild-type and mutated *sal* probes. (A) Mutations in Ubx binding sites 5 and 6 cause a 10-fold decrease in Ubx-HD binding affinity for probe (compare lanes 1-7 with 9-14), but mutations in the kM1 site (lanes 16-21) have no effect on binding. (B) Mutations in Ubx binding sites 5 and 6 eliminate the binding of full-length Ubx protein to the probe (compare lanes 1-5 with 6-10), but mutations in the kM1 site (lanes 11-15) have no effect on binding. (**C**) Mutations in Ubx binding sites 5 and 6 do not affect Med binding affinity for its site, as compared with the wild-type probe (compare lanes 1-4 with 5-8). (**D**) Mutations in Ubx binding sites 5 and 6 do not affect Mad binding affinity for its site, as compared with the wild-type probe (compare lanes 9-13 with 14-18).

also tested whether the requirement in vivo for both Ubx binding sites 5 and 6 and the Smad M1 site were equal or additive by comparing *sal*1.1 reporter constructs with mutations in either the pair of Ubx binding sites or the Smad M1 site or both. We found that the strength and pattern of derepression were equivalent if either the pair of Ubx binding sites 5 and 6 or the Smad M1 site or both were mutated (Fig. 4A,B; data not shown). These results indicate that both Mad/Med and Ubx are binding to distinct sites in very close proximity and confirm that a reduction in the binding of any of these proteins to the CRE leads to derepression of *sal* in the haltere disc.

The proximity of collaborator binding sites is critical for *sal* repression

The proximity of the Ubx and Mad/Med binding sites in *sal*1.1 raises the question of the nature of the collaboration between these proteins in *sal* repression. One possibility is that Ubx and Mad-Med-Shn bind cooperatively to DNA to repress *sal*. We tested this possibility in a wide variety of biochemical assays following established protocols. These included EMSAs with (1) either *Drosophila* S2 or mammalian 293T cell lysates transfected with constructs driving expression of the activated Thickveins receptor (Tkv^{QD}), Mad, Med, Shn and Ubx; (2) full-length proteins produced by coupled in vitro transcription and translation; and (3) bacterially expressed purified fusion proteins. We found no evidence of a physical interaction between Mad-Med-Shn and Ubx on a probe containing Ubx binding sites 5 and 6 and the M1 site under conditions in which Mad-Med-Shn formed complexes on well-characterized high affinity target sites such as the *brk* silencer element (Pyrowolakis et al., 2004) (data not shown), nor did we detect co-occupancy of the probe by either of the Smads and Ubx together. It is certainly possible that our failure to detect a Hox-Smad interaction or a tripartite complex was because the conditions we tested were insufficient for the assembly of such complexes. Because mutations that affect Smad or Ubx binding do not affect the binding of the other protein (Fig. 3), we have no evidence that their respective binding sites overlap and that the binding of one protein might occlude binding of the other protein, nor do steric considerations indicate that the M1 site cannot accommodate both proteins. We suspect that the low affinity of Smads for the M1 site has hampered our ability to detect complexes of both proteins on DNA. Nonetheless, the possibility remains that the Smads and Ubx bind sequentially, but not simultaneously, to the *sal* CRE.

As we found no evidence for a direct physical interaction between Ubx and Smads, we tested whether the topology of binding sites U5, U6 and M1 was necessary for *sal* repression. Previously, we have shown that mutations in U5 and U6 derepress the activity of a subfragment of *sal*1.1 (*sal*328) in the haltere imaginal disc (Fig. 4C) (Galant et al., 2002). In order to test whether the position of the Ubx binding sites relative to the M1 site was critical for *sal* repression, we added one copy of the binding site U5 (5′-CATATTAAGA-3′) to both the 5′ and 3′ ends of a *sal*328 element (178 bp 5′ and 132 bp 3′ from their native positions) in which both native sites (U5 and U6) had been mutated. The addition of two copies of U5 to the mutated *sal*328 CRE (*sal*328kU5&6) did not restore repression to this element (*sal*328kU5&6+2U5) (Fig. 4D). Although the *sal*328kU5&6+2U5 CRE, like wild-type *sal*328CRE, has three natural Ubx binding sites, we conclude that Ubx does not repress gene expression because its sites are placed too far from the collaborating Mad/Med binding site M1.

We further tested the dependence of Ubx regulation upon Ubx monomer binding site topology by attempting to impart Ubx regulation upon a naive CRE. We tested whether the *vestigial* boundary enhancer (*vg*B), which drives reporter gene expression along the dorsal-ventral compartment boundary in both the wing and haltere imaginal discs (Fig. 4E), could be specifically repressed by Ubx in the haltere. The Ubx binding site topologies tested included the addition of a cassette of four copies of Ubx binding site 5 (5′-CATATTAAGA-3′) from the *sal*1.1 CRE to both the 5′ and 3′ ends of the *vg*B CRE. Each copy of Ubx binding site 5 was one helical turn from its neighboring Ubx binding site. The arrays of Ubx binding sites had no effect on reporter gene expression in the haltere (Fig. 4F).

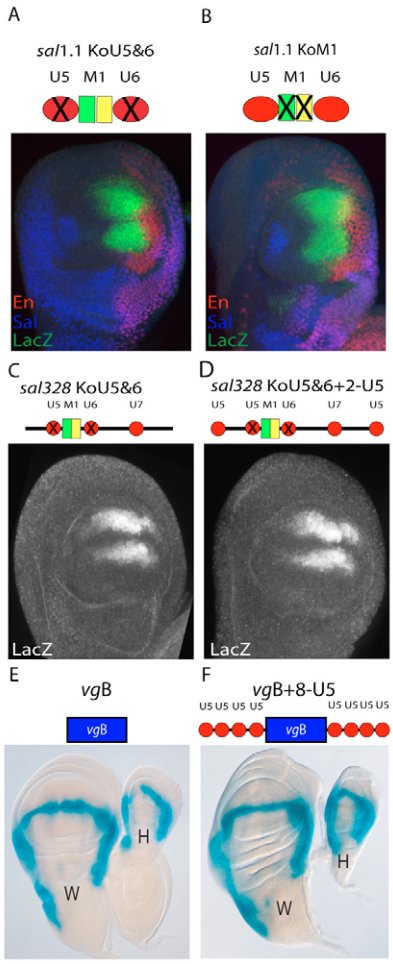

Fig. 4. Ubx and Mad/Med binding sites collaborate to repress *sal* expression in the haltere. Schematics above each panel highlight the relevant changes to CREs. (**A**,**B**) Haltere imaginal discs from transgenic *Drosophila* carrying *sal*1.1 reporter constructs with mutations either in Ubx binding sites 5 and 6 or in the M1 site. The level and pattern of derepression is identical whether Ubx sites 5 and 6 or the M1 site are mutated. (**C**) Mutations in Ubx binding sites 5 and 6 in a subelement of the *sal*1.1 CRE, *sal*328, derepress the reporter gene *lacZ* in haltere discs. (**D**) The addition of one copy of Ubx binding site 5 to both the 5′ and 3′ ends of *sal*328koU5&6 (*sal*328koU5&6+2-U5) is insufficient to repress reporter gene expression in the haltere. (**E**) The *vg*B CRE is expressed along the dorsal-ventral compartment boundary in both the wing (W) and haltere (H) imaginal discs. (**F**) The addition of two cassettes, each containing four copies of Ubx binding site 5, to the 5′ and 3′ ends of the *vg*B is not sufficient to impart Ubx-dependent repression in the haltere imaginal disc.

These results and the functional requirement for the proximity of the Mad/Med M1 site to Ubx binding sites U5 and U6 in the *sal* CRE indicate that there might be selective constraints on the sequence and arrangement of the binding sites. Indeed, alignments of the orthologous regions of the *sal*1.1 CRE from diverse *Drosophila* species revealed that not only are these crucial binding sites conserved, but a region of 37 bp encompassing these three sites is perfectly conserved (Fig. 5). This is an exceptional degree of sequence conservation among the sampled taxa and is very strong additional evidence that this Mad-Med-Shn- and Ubx-responsive CRE requires the integrity and close spacing of these binding sites to be maintained for *sal* repression in the haltere.

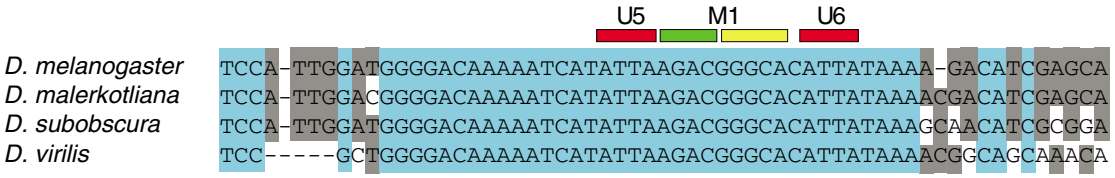

Fig. 5. The conserved topology of Ubx binding sites 5 and 6 and the M1 site in the *sal*1.1 CRE. Alignment of a deeply conserved block of 37 nucleotides (shaded blue, indicating 100% nucleotide conservation) containing Ubx binding sites 5 and 6 and the collaborating M1 site in the *sal*1.1 CRE from four species of *Drosophila*. Nucleotides conserved in three out of four species are shaded in gray.

DISCUSSION

We have demonstrated that Mad/Med and Ubx bind to adjacent sites in the *sal*1.1 CRE and that each protein is required for the direct repression of *sal* expression in the haltere. Furthermore, the sequence and spacing of Ubx and Smad binding sites are highly conserved and their proximity is required for target gene repression in the haltere. Because we found no evidence that these proteins interact directly, we suggest this is an example of 'collaboration' or target gene co-regulation without direct cooperative interaction. These results have general implications for understanding how Hox proteins regulate diverse sets of target genes in animal development.

Collaboration between Smads and Ubx

The direct role for Smads in the repression of *sal* in the haltere is surprising in the light of previous genetic (de Celis et al., 1996; Lecuit et al., 1996; Marty et al., 2000; Nellen et al., 1996) and molecular studies (Barrio and de Celis, 2004) that had indicated that the Dpp pathway and Mad/Med were involved in *sal* activation in the wing. We find no direct evidence that this is the case and the fact that *sal* is activated in *Mad* and *Med* clones in the haltere indicates that *sal* is activated independently of Mad/Med in the flight appendages. The requirement for Mad/Med/Shn in shaping the pattern of *sal* expression in the wing appears to be indirect – the protein complex represses the expression of *brk*, a repressor of *sal*, in cells in the central region of the developing wing and thereby permits *sal* expression (Marty et al., 2000; Muller et al., 2003; Pyrowolakis et al., 2004).

The Mad-Med-Shn complex is also active within cells in the central region of the haltere as a consequence of Dpp signaling (Fig. 6) (Muller et al., 2003; Pyrowolakis et al., 2004). However, whereas *sal* is expressed and the *sal*1.1CRE is active in the wing, *sal* and the *sal*1.1 CRE are repressed in the haltere. These observations raise the question of how the Mad-Med-Shn complex selectively represses *sal* in the haltere but not in the wing disc? Our results suggest that there are two key determinants in the selective repression of *sal* in the haltere. The first is collaboration with Ubx, which is expressed in the haltere and not in the wing disc. The second key determinant might be the affinity of Mad/Med binding to the *sal* CRE.

The different responses of the *brk* and *sal* genes to Mad/Med/Shn suggests how the different affinities of proteins for binding sites might determine how available transcriptional regulatory inputs are integrated by CREs (Fig. 6). Mad/Med binding to the *brk* CRE is of high affinity (Pyrowolakis et al., 2004) and apparently sufficient to impart repression, whereas that to the *sal* CRE is of much lower affinity and insufficient to impart repression in the wing. In the haltere, although Mad-Med-Shn or Ubx binding are alone insufficient, they act together either via simultaneous or sequential occupancy of their binding sites to repress *sal*.

Collaboration as a distinct mode of combinatorial regulation

The requirement for two or more regulators to act together to control gene expression, i.e. combinatorial regulation, is fundamental to the generation of the great diversity of gene expression patterns by a finite set of transcription factors. Several previous studies have revealed the dual requirement for Hox and Smad functions for the activation of a target gene (Grieder et al., 1997; Grienenberger et al., 2003; Marty et al., 2001). These studies suggested a general combinatorial mechanism for gene activation in which apparently separate transcriptional inputs act synergistically in gene activation and, in at least one case, the Hox response element and Dpp response element are separable (Marty et al., 2001). Here, however, we have observed a requirement for and strict evolutionary conservation of the close topology of Hox and Smad binding sites in the *sal* CRE. We suggest that collaboration is a distinct mode of combinatorial regulation in which two or more regulatory proteins must bind to nearby sites, but not necessarily to each other.

The integration of Hox and Smad inputs could work through a number of possible mechanisms (Guss et al., 2001; Marty et al., 2001) in the absence of direct physical interaction. One appealing possibility that might explain the requirement for the close proximity of binding sites is that Ubx and Mad-Med-Shn might interact with, and could therefore cooperatively recruit, the same co-repressor(s) for the repression of *sal*. Alternatively, if Mad-Med-Shn and Ubx bind sequentially to *sal*1.1, they might recruit different co-repressors and thereby orchestrate the assembly of a co-repressor complex. A third possibility is that because the Ubx and Mad/Med sites are embedded within a larger block of conserved regulatory DNA sequence in the *sal*1.1 CRE, the binding of other interacting transcription factors might also be involved in the repression of *sal* by Ubx and Mad-Med-Shn.

The general role of collaboration in Hox target gene selection and activity regulation

These and recent results raise the question of whether collaboration is a general feature of target gene selection by Hox proteins (Gebelein et al., 2004). We suggest that collaboration might be a widespread requirement for Hox function in vivo.

Our proposal is prompted by three observations. First, Hox proteins alone have low DNA-binding specificity (Ekker et al., 1991). Second, some, and perhaps all, Hox proteins might act as both repressors and activators. Third, Hox proteins regulate a great diversity of target genes that are also regulated by other transcription factors. In order to be such versatile regulators, it would be too great a constraint to require that Hox proteins always interact cooperatively with the diverse repertoire of transcription factors with which they act. Indeed, it may be argued that too much weight has been ascribed to the cooperative binding of Hox proteins and co-factors to DNA.

DEVELOPMENT

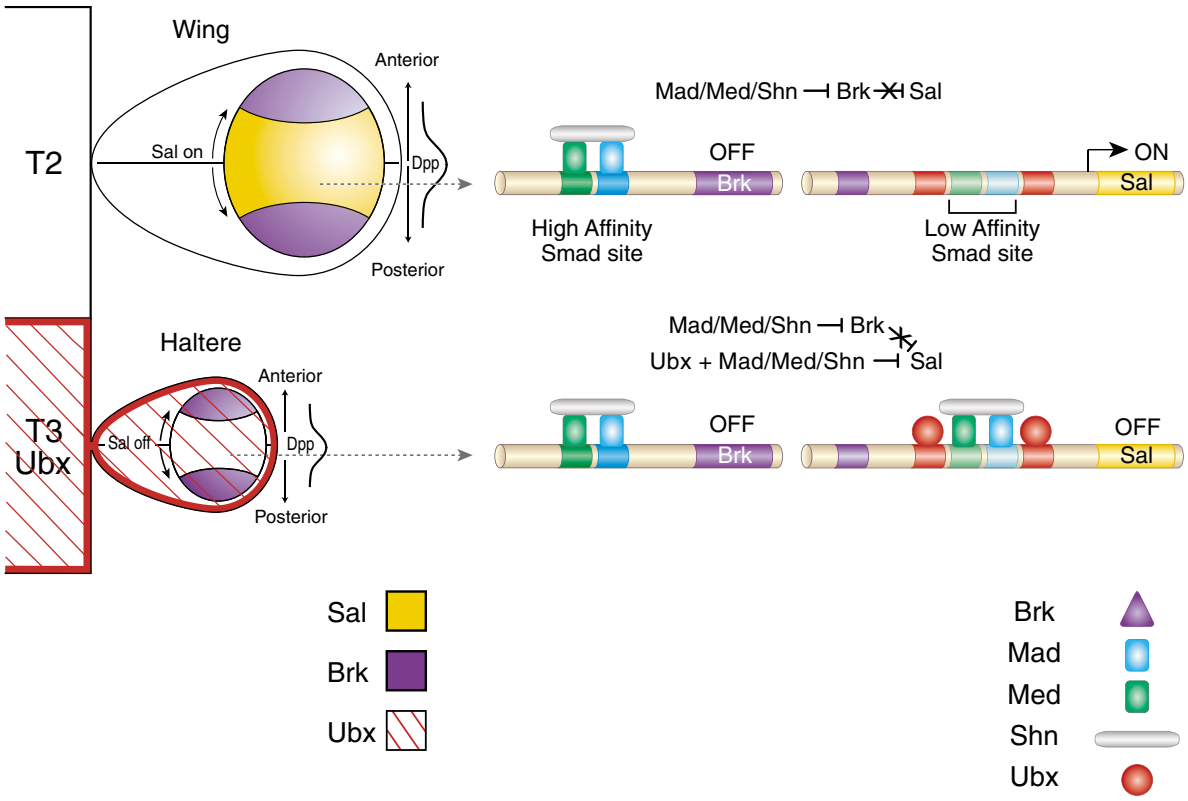

Fig. 6. Collaboration between Ubx and Smads in the selective repression of the *sal* gene in the *Drosophila* haltere disc. (Left) A depiction of the wing and haltere imaginal discs of the second and third thoracic segments (T2 and T3, respectively). Sal is expressed (yellow) where high levels of Dpp signaling emanate from the anterior-posterior compartment boundary. Brk (purple) represses *sal* and restricts its expression to the center of the wing imaginal disc. Ubx (red stripes) is expressed in the haltere disc, where it regulates target genes such as *sal*. (Right) The genetic circuitry and molecular mechanisms allowing *sal* expression in the wing and repressing *sal* in the haltere. In the wing, Sal is expressed in cells where the Dpp-responsive Mad-Med-Shn repressor complex binds to a high-affinity Smad site in a *brk* CRE to directly repress Brk expression. The *sal*1.1 CRE contains a low-affinity Smad site but this is insufficient to repress *sal* in the wing. However, in the haltere, *sal* is repressed via the collaborative action of Ubx and Mad-Med-Shn binding to the *sal*1.1 CRE. Both the affinity and topology of repressor binding sites are critical to the selective repression of *sal* in the T3 flight appendage.

Previously, much attention has focused on Exd and Hth, which interact with Hox proteins and bind cooperatively to DNA, thereby increasing Hox DNA-binding selectivity (Chan et al., 1994; Chan et al., 1997; Mann and Carroll, 2002; Pederson et al., 2000). However, it was only recently shown that the binding of these complexes alone was not sufficient to regulate target gene expression. Rather, Hox-Exd-Hth collaborate with and require the segmentation proteins Slp and En to repress the target gene *Dll* (Gebelein et al., 2004). Here, we have shown that the Exd- and Hth-independent target gene repression of *sal* requires collaboration between Ubx and Mad-Med-Shn. Although still a tiny sample of target genes, we now have cases of transcription factors of various structural types acting as collaborators with Hox proteins. The picture of Hox proteins relying on dedicated interacting co-factors such as Exd and Hth is expanding to a larger pool of collaborating transcription factors that modulate target gene selection.

Indeed, collaboration might be the key to another unresolved mystery of the Hox proteins – the regulation of Hox protein activity. Some Hox proteins appear to act in both gene activation and repression; this is certainly the case for Ubx. This versatility would appear to be crucial to their role as sculptors of major features of body patterns, but how does the same transcription factor act positively in some contexts but negatively in others? There is evidence to suggest that the identity of the collaborating proteins and/or CRE sequences determines the 'sign' of Hox action.

For instance, there is no evidence that the mere binding of Hox-Exd-Hth to a site determines the sign of Hox activity. These co-factors are involved in both Hox target gene activation (e.g. *dpp* in the midgut) and target repression (e.g. *Dll* in the embryonic abdomen). But, in the latter case, En and Slp, two proteins that each harbor motifs for interaction with the co-repressor Groucho (Alexandre and Vincent, 2003; Andrioli et al., 2004; Kobayashi et al., 2003; Lee and Frasch, 2005), are required collaborators for *Dll* repression. The roles of En and Slp in this instance might not be so much a matter of facilitating Hox target selection, but rather in regulating the sign of the output of the collaboration.

Similar to the Hox proteins, the Smads can either activate or repress target genes (Feng and Derynck, 2005). Furthermore, it has been demonstrated that the topology of Smad binding sites on DNA appears to be critical for determining whether a target gene is activated or repressed. In *Drosophila*, the topology of Mad and Med binding sites is critical for the recruitment of the co-repressor Shn (Gao et al., 2005; Pyrowolakis et al., 2004). The recruitment of Shn was shown here to be necessary for *sal* repression. These two examples suggest that the positive or negative regulatory

activity of a Hox protein depends on the context of surrounding binding sites and how they influence the activity of collaborating factors.

The dependence of Hox proteins upon co-factors and collaborators indicates that, at the molecular level, Hox proteins are not 'master' regulatory proteins that dictate how target genes behave. Rather, they exert their great influence by virtue of their simple binding specificity, broad domains of expression and versatile, collaborative properties.

We thank A. Laughon, E. Ferguson, R. Barrio, G. Campbell, R. White and the Bloomington Stock Center for antibodies and fly stocks and A. Laughon, B. Prud'homme, J. Yoder and T. Williams for comments on the manuscript. This work was supported by the Howard Hughes Medical Institute (S.B.C.).

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