Nuclear integration of positive Dpp signals, antagonistic Wg inputs and mesodermal competence factors during *Drosophila* visceral mesoderm induction

Hsiu-Hsiang Lee* and Manfred Frasch†

Brookdale Department of Molecular, Cell and Developmental Biology, Box 1026, Mount Sinai School of Medicine, New York, NY 10029, USA

*Present address: Genetics, Development and Behavioral Sciences Building, 1550 4th Street, UCSF, San Francisco, CA 94158, USA
†Author for correspondence (e-mail: manfred.frasch@mssm.edu)

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Summary

Tissue induction during embryonic development relies to a significant degree on the integration of combinatorial regulatory inputs at the enhancer level of target genes. During mesodermal tissue induction in *Drosophila*, various combinations of inductive signals and mesoderm-intrinsic transcription factors cooperate to induce the progenitors of different types of muscle and heart precursors at precisely defined positions within the mesoderm layer. Dpp signals are required in cooperation with the mesoderm-specific NK homeodomain transcription factor Tinman (Tin) to induce all dorsal mesodermal tissue derivatives, which include dorsal somatic muscles, the dorsal vessel and visceral muscles of the midgut. Wingless (Wg) signals modulate the responses to Dpp/Tin along anteroposterior positions by cooperating with Dpp/Tin during dorsal vessel and somatic muscle induction while antagonizing Dpp/Tin during visceral mesoderm induction. As a result, dorsal muscle and cardiac progenitors form in a pattern that is reciprocal to that of visceral muscle precursors along the anteroposterior axis. Our present study addresses how positive Dpp signals and antagonistic Wg inputs are integrated at the enhancer level of *bagpipe* (*bap*), a NK homeobox gene that serves as an early regulator of visceral mesoderm development. We show that an evolutionarily conserved *bap* enhancer element requires combinatorial binding sites for Tin and Dpp-activated Smad proteins for its activity. Adjacent binding sites for the FoxG transcription factors encoded by the Sloppy paired genes (*slp1* and *slp2*), which are direct targets of the Wg signaling cascade, serve to block the synergistic activity of Tin and activated Smads during *bap* induction. In addition, we show that binding sites for yet unknown repressors are essential to prevent the induction of the *bap* enhancer by Dpp in the dorsal ectoderm. Our data illustrate how the same signal combinations can have opposite effects on different targets in the same cells during tissue induction.

Key words: *Drosophila*, Dpp, Wg

Introduction

Tissues and organs develop from primordial cells that arise in precisely defined spatial and temporal patterns within a particular germ layer of the early embryo. These patterns are typically generated by combinatorial cues, whose restricted domains of activity intersect at specific positions within a larger field of cells. In order to understand early organogenesis, it is necessary to identify these cues and to determine the mechanisms by which they cooperate at the developmental and molecular level to elicit their responses.

Tissue development in the *Drosophila* mesoderm has been a favorable system in which to study these events. Upon the spreading of the mesodermal cell layer underneath the embryonic ectoderm, the progenitor cells of different organs, such as the dorsal vessel, somatic and visceral muscles, are generated at stereotyped locations within the mesoderm. Specifically, in the dorsal region of the mesoderm (which has been studied in most detail), the progenitors of cardioblasts, pericardial cells, specific dorsal somatic muscles and circular midgut muscles are generated (Campos-Ortega and Hartenstein, 1997; Frasch and Nguyen, 1999). Signals from the dorsal ectoderm mediated by Dpp are required, but not sufficient, for the induction of all of these progenitor cells in the dorsal mesoderm (Staehling-Hampton et al., 1994; Frasch, 1995). Importantly, the Dpp signals need to act in concert with mesoderm-intrinsic regulators, which make the mesodermal cells competent to respond. One of the key regulators intrinsic to the mesoderm is the NK homeobox gene *tinman*, which, like *dpp*, is required for the induction of all dorsal mesodermal cell types (Azpiazu and Frasch, 1993; Bodmer, 1993; Yin and Frasch, 1998). *tinman* itself is initially activated in the early mesoderm by *twist* and, just prior to cell specification events, its expression is prolonged by Dpp signals specifically in the dorsal mesoderm (Bodmer et al., 1990; Frasch, 1995; Yin et al., 1997).

In addition to these dorsal cues, differentially active cues modulate the specific responses in the mesoderm along the anteroposterior (AP) axis. Notably Wg, which is expressed in transversely striped domains within the A compartments of the ectoderm, is required in combination with Dpp for the

specification of the progenitors of cardioblasts, pericardial cells and dorsal somatic muscles (Baylies et al., 1995; Wu et al., 1995). Conversely, the precursors of the midgut visceral mesoderm are induced by Dpp but suppressed by Wg (Frasch, 1995; Azpiazu et al., 1996) (see http://www.eurekah.com/abstract.php?chapid=2028&bookid=162&catid=20). Hence, visceral mesoderm precursors arise in domains that are alternating with those of cardiac and somatic muscle progenitors along the AP axis in the dorsal mesoderm. Additional cues, which include signals through various receptor tyrosine kinases (RTKs) and the FGF receptor Heartless, then generate further subdivisions within the visceral mesoderm as well as diverse identities among the progenitors of cardiac and somatic muscle tissues (Carmena et al., 1998; Michelson et al., 1998; Englund et al., 2003; Lee et al., 2003). Mutual repression among induced regulatory genes also plays a role (Han et al., 2002; Jagla et al., 2002). The combined actions of these regulators results in the spatially restricted transcriptional activation of target genes, which drive genetic programs controlling the specification and/or differentiation of individual cells.

Recently, significant progress has been made in resolving the issue of how combinatorial inputs are integrated at the level of enhancers of target genes in this system. A relatively simple situation exists for the Dpp-responsive enhancer of *tin*, which does not receive any differential inputs along the AP axis. This enhancer has been shown to contain several copies of binding sites for Smads, which function as nuclear Dpp signaling effectors, as well as binding sites for Tin protein. Each of the two types of binding sites are essential for enhancer activity (Xu et al., 1998). Thus, it appears that combinatorial binding of Dpp-activated Smads and mesoderm-intrinsic Tin, together with protein interactions between Smads and Tin (Zaffran et al., 2002), provides the synergism required for the active state of the enhancer. A more complex situation, when compared with *tin*, is found for *even-skipped* (*eve*), a homeobox gene that is induced in specific segmentally repeated progenitors of pericardial cells and dorsal somatic muscles within the dorsal mesoderm (Frasch et al., 1987; Su et al., 1999). This pattern of *eve* expression requires not only Dpp but also Wg signals and RTK signals that are active in smaller areas within the fields where Dpp and Wg intersect (Frasch, 1995; Wu et al., 1995; Azpiazu et al., 1996; Carmena et al., 1998). As for the induction of *tin*, these external signals require Tin as a mesoderm-intrinsic activity for the induction of *eve* (Azpiazu and Frasch, 1993; Bodmer, 1993). Consistent with these identified inputs, the corresponding enhancer region of *eve* has been found to contain functionally important binding sites for Tin, Smads, the Wg effector dTCF/Lef-1 and Ets-domain protein-binding sites that are presumed targets of RTK signals (Halfon et al., 2000; Knirr and Frasch, 2001; Han et al., 2002). A comparison between the situation in the *eve* versus the *tin* enhancer raises the question: why are the Smad and Tin sites in the *tin* enhancer sufficient for its induction when the *eve* enhancer requires additional inputs from Wg via the dTCF/Lef-1 sites? Additional functional studies have provided answers to this question. A model was proposed, in which bound dTCF/Lef-1 acts as a repressor that abrogates the activity of the bound Tin and Smad proteins in the absence of Wg signals, whereas in the presence of Wg signals the repressive activity of dTCF/Lef-1 is abolished (Knirr and Frasch, 2001). Consequently, Wg signals allow Tin/Smads (together with RTK signal effectors) to induce *eve* in segmentally repeated clusters of cells within the dorsal mesoderm.

Herein, we define the distinct molecular inputs into a third enhancer, namely that of the NK-homeobox gene *bagpipe* (*bap*), which is induced by Dpp in the early dorsal mesoderm during the same period when *tin* and *eve* are being induced (Staehling-Hampton et al., 1994; Frasch, 1995). *bap* is a crucial regulator of the development of the trunk visceral mesoderm and, hence, of midgut muscle development (Azpiazu and Frasch, 1993; Zaffran et al., 2001) (see http://www.eurekah.com/abstract.php?chapid=2028&bookid=162&catid=20). *bap* is induced in metameric clusters of cells within the dorsal mesoderm that alternate with those expressing *eve* and other early cardiac and dorsal muscle markers along the AP axis (see Fig. 1). This pattern is explained by the finding that the activity of Dpp to induce *bap* is abrogated by Wg signals (Azpiazu et al., 1996), which contrasts with the situation for *eve*, where Wg synergizes with Dpp. Recent studies have shown that Wg signals act indirectly during this process and function by inducing the forkhead domain genes *sloppy paired 1* and *sloppy paired 2* (*slp1 and slp2*) in striped domains within the mesoderm (Lee and Frasch, 2000). Slp proteins, in turn, act as segmental repressors of *bap* induction (Riechmann et al., 1997; Lee and Frasch, 2000) (see Fig. 1). In common with *eve* (and *tin*), the induction of *bap* expression by Dpp signals also require synergism with mesodermal *tin* (Azpiazu and Frasch, 1993) (see Fig. 1). Our functional dissection of the corresponding *bap* enhancer reveals interesting similarities and differences to the mesodermal *tin* and *eve* enhancers. Specifically, we show that a 267 bp element, bap3.2, which recapitulates the endogenous *bap* pattern in the dorsal trunk mesoderm, includes combinatorial binding sites for Tin and Smad proteins that are essential for its induction. By contrast,

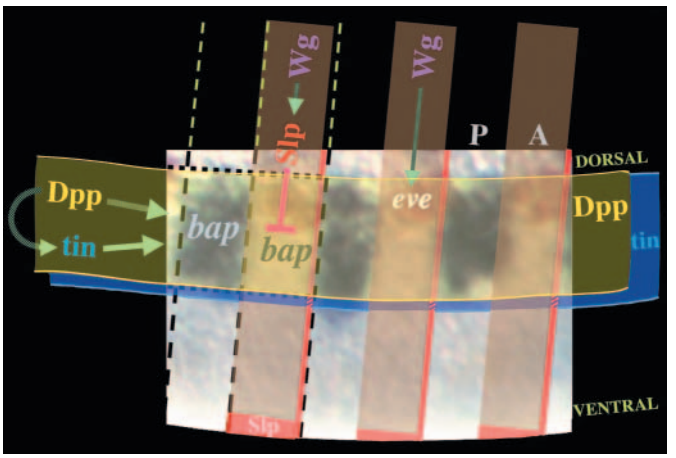

Fig. 1. Summary of signaling and transcriptional pathways during the induction of trunk visceral mesoderm. Shown are three parasegments of the mesoderm (divided into P and A domains) (Azpiazu et al., 1996) of a stage 10 embryo stained for *bap* mRNA (purple) and Eve protein (brown). The expression domains of Tin (schematically shown in blue) and the relevant Slp domains (red) are within the mesoderm, whereas Dpp (yellow) and Wg (brown) are secreted from the overlying ectoderm. Inductive signals are represented by hatched arrows and transcriptional interactions are represented by solid arrows.

binding sites for Slp are required for the segmental repression of *bap* enhancer activity. We also show that the Slp-binding sites exert an additional, positive function during *bap* induction, in part through binding of Biniou (Bin), a forkhead domain protein that is activated downstream of *bap* but provides positive feedback on *bap* (Zaffran et al., 2001). Finally, we show that this *bap* enhancer includes elements that prevent the induction of *bap* by Dpp in the dorsal ectoderm and, thereby, contribute to the observed germ layer specific response. Similar elements were previously found in the Dpp-responsive enhancer of *tin* (Xu et al., 1998), and we provide data to indicate that the same repressing mechanism prevents induction of both *tin* and *bap* in the dorsal ectoderm. Altogether, the data presented illustrate how Wg signals can either antagonize or cooperate with Dpp signals at the molecular level. More generally, they extend our knowledge of how the molecular integration of combinatorial signals at the level of target enhancers generates mesoderm-specific outputs with precisely defined spatial patterns, which prefigure specific tissue primordia.

Materials and methods

Construction of P-transformation plasmids

The *bap* gene of *Drosophila virilis* was isolated from a genomic library obtained from W. Hanna-Rose through J. D. Licht (Hanna-Rose et al., 1997) using *bap* cDNA of *Drosophila melanogaster* as a probe. For the P-transformation constructs, upstream and downstream DNA fragments of *bap* (*D. melanogaster* and *D. virilis*) were cloned into the pCaSpeRhs43-βgal vector with reversed orientations relative to the basal promoters, unless mentioned otherwise. The following DNA constructs of *D. melanogaster* were made and tested in vivo: For bapDS3.5, a 3.5 kb *Sal*I/*Hinc*II DNA downstream fragment; for bapH2-1.2, a 1.2 kb *Hinc*II/*Hinc*II DNA downstream fragment; and for bap3, a 460 bp *Hinc*II/*Xho*I DNA downstream fragment were cloned into the vector. Constructs bap1 (432 bp), bap2 (460 bp), bap3.1 (255 bp), bap3.2 (267 bp) and bap3.2.1 (180 bp) were generated by PCR reaction with *Bam*HI/*Eco*RI site-containing primers and bapH2-1.2 as a template, and cloned into the vector. For *D. virilis*, the following constructs were generated and tested in vivo: for bapUS3.5-R, a 3.5 kb *Sal*I/*Sal*I DNA upstream DNA fragment; for bapDS2.7-R, a 2.7 kb *Eco*RI/*Bam*HI downstream fragment; for bapDS2.7-F, the same 2.7 kb fragment was cloned into the vector with native (forward) orientation relative to the basal promoters; for bapDS4.6-R, a 4.6 kb *Eco*RI/*Eco*RI downstream fragment was cloned with inverted and for bapDS4.6-F the same fragment with native (forward) orientation into the vector. Constructs bapV1 (545 bp) and bapV2 (165 bp) were made by PCR with primers containing *Bam*HI/*Eco*RI sites and bapDS2.7 as a template.

For site-directed mutagenesis and deletion within the bap3.2.1 DNA fragment, PCR reactions were performed with bap3.2.1 as a template and with different primer sets, which were designed to introduce new restriction sites to mutate or delete specific sites. Detailed information on the sequences of primers used for PCR can be obtained upon request. All constructs were sequenced to confirm that only the intended mutations were introduced, and were then cloned into the transformation vector. The mutated DNA sequences of each construct are shown in Figs 5, 7.

To identify the DNA elements mediating the ectodermal repression, the following constructs were made by PCR, cloned into vectors and tested in embryos: for bap3.2ΔR3, a 237 bp DNA fragment from nucleotides 1-237 of bap3.2 (for sequences see Fig. 5); for bap3.2ΔR1-2, a 210 bp DNA fragment from nucleotides 58-267 of bap3.2; and for bap3.2ΔR1, a 248 bp DNA fragment from nucleotides 20-267 of bap3.2. For bap3.2R1-3mut, PCR reactions were performed with primers to mutate the core sequences of R1, R2 and R3 sites. *Pst*I, *Nsi*I and *Bgl*II were introduced to mutate R1, R2 and R3, respectively (for R1 site, CGTCCCGctgcaGATGG; for R2 site, GAGGAGGAtgcatAACGG; for R3 site, TGTGCCCAGatctAATTG; for wild-type sequence, see Fig. 8). For bap3.2.1-tinD1.5′ and bap3.2.1-tinD1.3′, the DNA fragments around tinD1a and tinD1b (sequences shown in Fig. 8B) were added to the 5′- and the 3′-ends of bap3.2.1, respectively.

Embryo stainings

In situ hybridizations were performed as described by Lo and Frasch (Lo and Frasch, 1997), antibody stainings as described by Azpiazu et al. (Azpiazu et al., 1996) and double fluorescent staining as described by Knirr et al. (Knirr et al., 1999). Rabbit anti-βGal antibodies (Cappel) and guinea pig anti-Slp antibody (Kosman et al., 1998) were used in this study.

DNase I footprinting assays

Footprinting assays were performed essentially as described by Yin et al. (Yin et al., 1997) with single-end-labeled bap3.2.1 probes. Different amounts of GST-Tin, GST-Mad, GST-Medea (Xu et al., 1998), GST-Bin (Zaffran et al., 2001), GST-Bap and GST-Slp were added to the reaction. For producing GST-Bap, a *Ssp*I/*Eco*RI DNA fragment from the *bap* cDNA (filled in by Klenow reaction), containing the full coding sequences, was cloned into the *Sma*I site of pGEX3X. For GST-Slp, a DNA fragment (*Eco*RV/*Not*I) from a *slp1* cDNA (a gift from L. Pick) containing the full protein-coding sequence was cloned into the *Sma*I site of pGEX3X.

Results

Functional and positional conservation of *bap* enhancer elements in *Drosophila*

Typically both the coding and regulatory sequences of developmentally important genes are conserved through evolution among related species. For example, the genes and enhancer elements of *tin* and *Mef2* are highly conserved between *Drosophila melanogaster* (*D. melanogaster*) and *Drosophila virilis* (*D. virilis*), two related species that separated evolutionarily about 60 million years ago (Yin et al., 1997; Cripps et al., 1998). As shown in Fig. 2A,B, the embryonic expression pattern of *bap* is also fully conserved between these two *Drosophila* species, suggesting that the corresponding regulatory elements of *bap* are also conserved. DNA sequence comparisons of these regulatory elements between the two species would be expected to facilitate the identification of important regulatory sites, as these should display the highest degrees of sequence conservation. Based upon this premise, flanking genomic sequences were isolated from both *D. melanogaster* and *D. virilis*, and tested for their ability to drive *lacZ* reporter gene expression in embryos. Constructs with upstream sequences of the *bap* gene, baplac4.5 in *D. melanogaster* and bapUS3.5-R in *D. virilis*, direct metameric *lacZ* expression within the visceral mesoderm from stage 11, after the segmented *bap*-expressing cell clusters have merged into continuous visceral mesoderm bands (Fig. 3, ‘late TVM’) (Azpiazu and Frasch, 1993) (data not shown). In addition, the flanking genomic DNA fragments downstream of *bap*, bapDS3.5 in *D. melanogaster* and bapDS4.6-R in *D. virilis*, are able to activate *lacZ* expression strongly in the primordia of foregut and hindgut visceral mesoderm (FVM and HVM) from stage 11 until late embryogenesis, and very weakly in the trunk

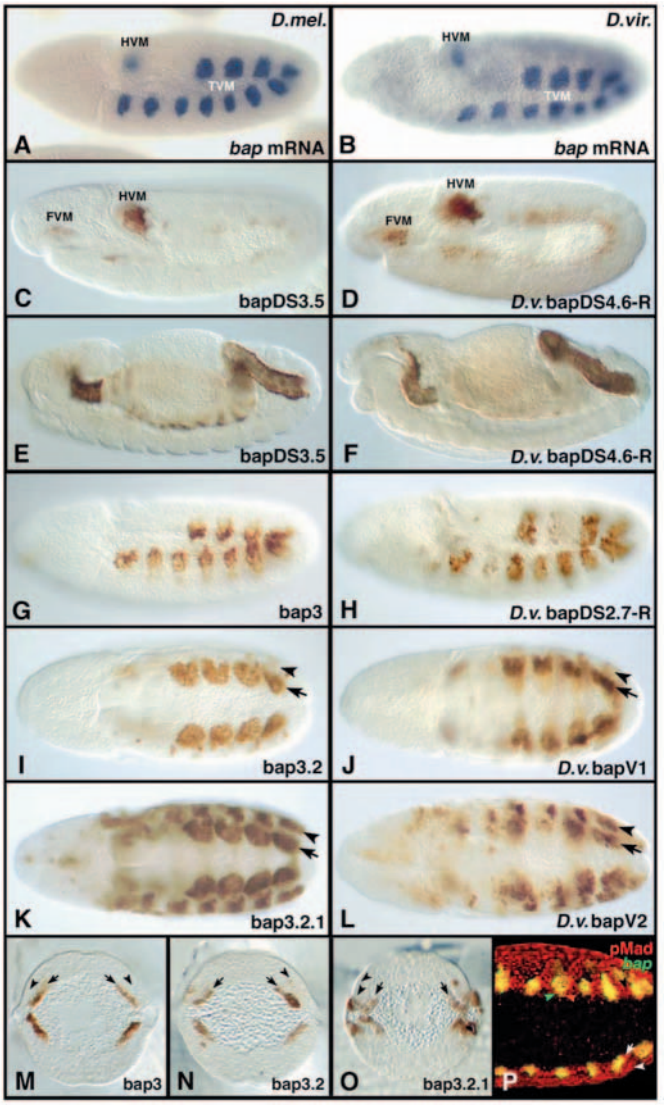

Fig. 2. Conserved *bap* expression and activities of *bap* enhancers from *D. melanogaster* and *D. virilis*. (A-H) Lateral views, (I-L) dorsal views, (M-O) cross-sections and (P) ventral view. (I-P) Arrows indicate the dorsal mesoderm and arrowheads indicate the dorsal ectoderm. (A) Stage 10 *D. melanogaster* embryo hybridized with a *D. melanogaster bap* probe, which detects *bap* mRNA expression in the trunk visceral mesoderm (TVM) and hindgut visceral mesoderm (HVM) primordia (foregut/FVM expression out of focus). (B) Hybridization of a stage 10 *D. virilis* embryo with a *D. virilis bap* probe shows an identical expression pattern as in *D. melanogaster.* (C) *D. melanogaster bapDS3.5-lacZ* (stage 10) recapitulating *bap* expression in HVM and FVM. Low levels of TVM expression are also seen. (D) *D. virilis bapDS4.6-R-lacZ* activity is nearly identical to that of *D. melanogaster bapDS3.5-lacZ*. (E,F) Stage 14 embryos carrying the same constructs as embryos in C,D, respectively, show foregut and hindgut visceral mesoderm expression. (G) *D. melanogaster bap3-lacZ* recapitulates the TVM pattern of *bap* mRNA expression during stage 10. (H) *D. virilis bapDS2.7-R-lacZ* activity is similar to *D. melanogaster bap3-lacZ* activity. (I) *D. melanogaster bap3.2-lacZ* activity in stage 11 embryo is largely confined to the TVM primordia, although there are traces of ectopic activity in the dorsal ectoderm (arrowhead). (J) *D. virilis bapV1-lacZ* activity is similar to *D. melanogaster bap3.2-lacZ* activity. (K) *D. melanogaster bap3.2.1-lacZ* embryo (stage11) showing ectopic segmented enhancer activity in the dorsal ectoderm in addition to normal mesodermal expression. (L) *D. virilis bapV2-lacZ* showing ectopic ectodermal and largely normal mesodermal enhancer activity, similar to *D. melanogaster bap3.2.1.* (M) Cross-sectioned *bap3-lacZ* embryo (stage 10) showing exclusive dorsal-mesodermal enhancer activity. (N) *bap3.2-lacZ* with largely mesodermal expression but weak ectopic ectodermal expression. (O) *bap3.2.1-lacZ* showing equally strong activity in dorsal mesoderm and dorsal ectoderm. (P) Stage 9 embryo stained for *bap* mRNA (green) and phospho-Mad (red), showing coincidence of the ventral borders of nuclear pMad and *bap* mRNA expression.

visceral mesoderm (Fig. 2C-F, Fig. 3). The focus of our studies presented herein is on the regulation of *bap* expression in the early trunk visceral mesoderm (TVM) precursors, which is driven by regulatory sequences within a 1.2 kb genomic DNA fragment, bapH2-1.2, downstream of the *bap*-coding region in *D. melanogaster* (Fig. 3A). This enhancer, as well as a truncated version of it, bap3 (460 bp), is active in 11 metameric domains on either side of the dorsal mesoderm at stage 10-11 (Fig. 2G, Fig. 3A; data not shown). A similarly active element, bapDS2.7-R, was also found downstream of *bap* in *D. virilis* (Fig. 2H, Fig. 3B). Hence, the activity of these elements recapitulates the early endogenous *bap* expression pattern of segmented domains in the dorsal mesoderm, i.e. in the presumptive trunk visceral mesoderm (TVM). Overall, the similar spatial and temporal activities of the different *bap* regulatory elements as well as their genomic arrangements with respect to the coding sequences in *D. melanogaster* and *D. virilis* illustrate the high degree of evolutionarily conservation of *bap* regulatory elements and suggest that the regulatory mechanisms between the two species are also conserved.

To further dissect this early *bap* regulatory element, bap3, from *D. melanogaster*, several overlapping shorter constructs were made to examine their enhancer activities in embryos. A minimal 267 bp DNA fragment, termed bap3.2, was able to drive reporter expression similar to the endogenous *bap* expression pattern in the TVM precursors (Fig. 2I, Fig. 3A). DNA sequence alignments of the 267 bp bap3.2 element of *D. melanogaster* with the 2.7 kb bapDS2.7-R element of *D. virilis*, as well as with corresponding genomic DNA sequences from *D. yakuba*, *D. pseudoobscura* and *D. ananassae* (obtained from http://hgsc.bcm.tmc.edu/projects/drosophila/), revealed high levels of similarities within a stretch of ~150 to 200 bp of genomic sequences in all five species (Fig. 5). In particular, the DNA sequences from *D. melanogaster* and *D. pseudoobscura* share ~90% identity within a DNA stretch from nucleotide 85 to 230 of the *D. melanogaster* bap3.2 element (Fig. 5). Consistent with this strong sequence conservation, a 540 bp genomic DNA fragment within the bapDS2.7-R element, bapV1 of *D. virilis*, which contains the highly conserved 150 bp sequences in its center, was capable of driving *lacZ* expression in the dorsal mesoderm similar to *bap3.2-lacZ* from *D. melanogaster* (Fig. 2J, Fig. 3B).

Repressing sequences prevent ectopic induction of *bap* enhancer in the dorsal ectoderm

A shorter regulatory DNA fragment, bap3.2.1, was derived from the bap3.2 regulatory element by removing the first 57 bp

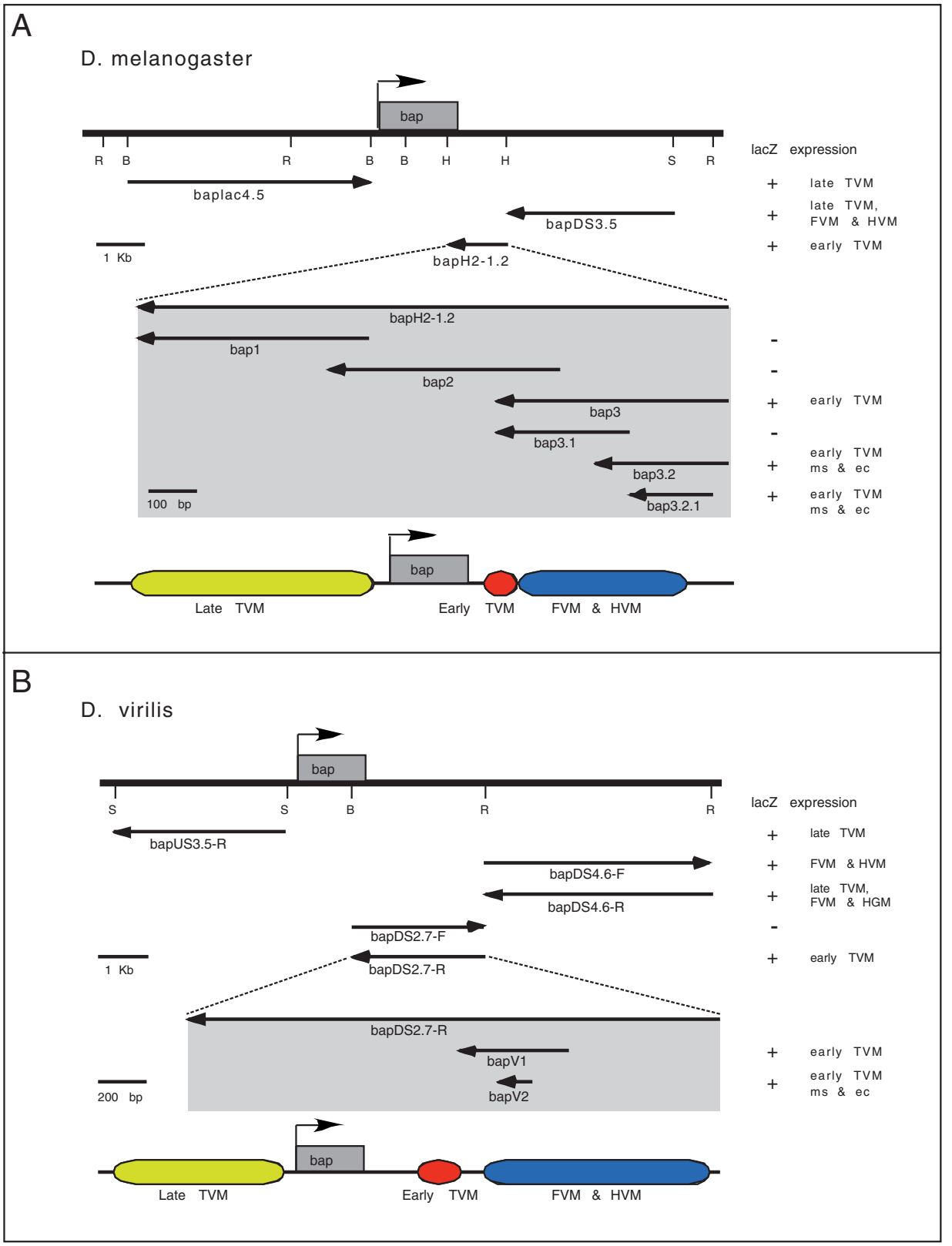

Fig. 3. *bap* enhancer regions and reporter constructs from *D. melanogaster* (A) and *D. virilis* (B). A restriction map of the genomic locus including the *bagpipe* (*bap*) transcription unit (5′ to the left) is shown at the top of each panel. Shown below are the genomic fragments tested in reporter constructs, with the arrowhead indicating their orientations in the construct (arrowheads point towards basal promoter). *bap*H2-1.2 (*D. melanogaster*), *bap*DS2.7-R (*D. virilis*) and their respective subfragments are shown at higher magnification. The in vivo reporter gene expression patterns are indicated on the right-hand side. Identified enhancer regions are summarized at the bottom of each panel. B, *Bam*HI; R, *Eco*RI; H, *Hinc*II; S, *Sal*I; ec, ectoderm; ms, mesoderm; FVM, foregut visceral mesoderm; HVM, hindgut visceral mesoderm; TVM, trunk visceral mesoderm.

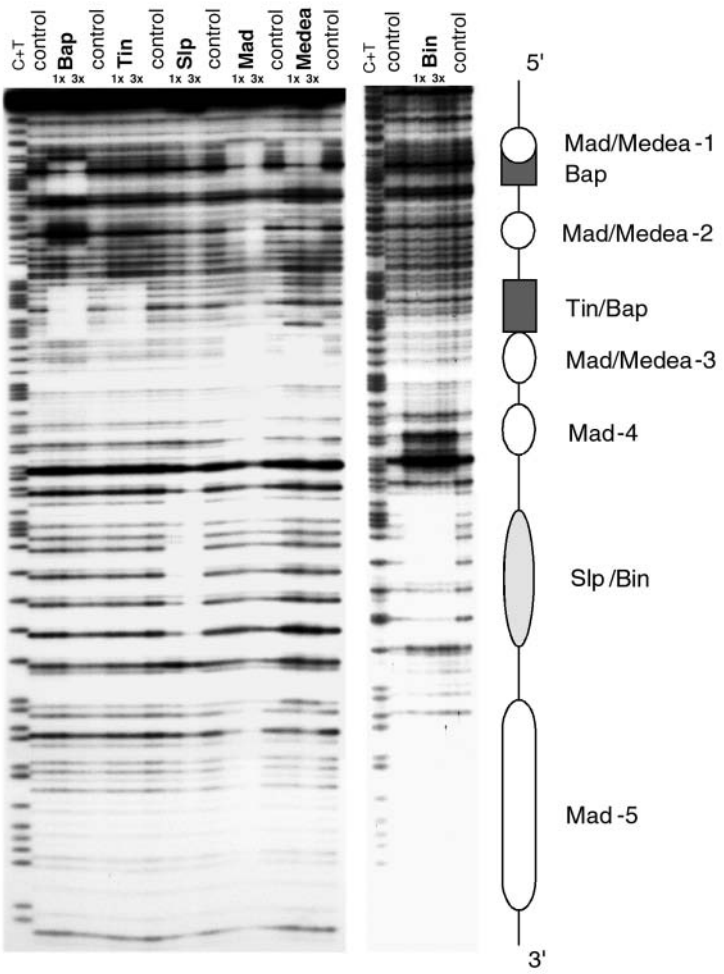

Fig. 4. DNAseI protection experiments with candidate transacting factors on *bap*3.2.1 enhancer DNA. γ^{32}P-labelled probe was tested with two different amounts (1× and 3×, see Materials and methods) of bacterially expressed GST-fusion proteins of Bap, Tin, Slp, Mad, Medea and Bin, as well as BSA as a control. C+T sequencing ladder is shown on the left of each blot and a schematic drawing of protected regions on the right.

from the 5′-end and the last 30 bp from the 3′-end of bap3.2 (see Fig. 3A, Fig. 5). bap3.2.1 drives expression in the same segmented pattern as bap3.2; interestingly, however, this expression occurs not only in the dorsal mesoderm but also in the dorsal ectoderm (Fig. 2K, compare with 2I). The *lacZ* expression patterns of *bap3.2.1-lacZ* in dorsal ectoderm and mesoderm can largely be superimposed onto one another. The only major difference between the two germ layers is observed in parasegments 13 and 14, where *bap3.2.1-lacZ* produces two additional expression clusters in the ectoderm that is neither seen with any of the reporter constructs nor with endogenous *bap* (Fig. 2K, see also 2A). Likewise, a 165 bp genomic DNA fragment, bapV2 from *D. virilis*, which corresponds to the bap3.2.1 element of *D. melanogaster* (see Fig. 3B and Fig. 5), also displays enhancer activity in both dorsal mesoderm and dorsal ectoderm with this pattern (Fig. 2L). The presence of ectopic *lacZ* expression in the ectoderm with bap3.2 derivatives was further confirmed in embryo cross-sections. Whereas *bap3-lacZ* embryos show no detectable *lacZ* expression in the ectoderm (Fig. 2M), there are traces of ectodermal *lacZ* expression in *bap3.2-lacZ* embryos (Fig. 2N) and strong dorsal ectodermal *lacZ* expression in *bap3.2.1-lacZ* embryos (Fig. 2O).

Altogether, these observations imply that the first 57 bp and the last 30 bp of the bap3.2 regulatory element have a key role in the repression of *bap* enhancer induction in the dorsal ectoderm and show that the mechanism of repression of ectodermal *bap* induction is evolutionarily conserved. The similar patterns of enhancer activity in both germ layers upon deletion of these repressor sequences support the notion, based on our genetic data, that the major spatial inputs regulating *bap* expression in the mesoderm are also active in the ectoderm (Azpiazu et al., 1996; Lee and Frasch, 2000). Indeed, one of these candidate inputs from the ectoderm, namely Dpp, leads to the activation of Mad in a dorsal domain in the mesoderm (and ectoderm) the ventral border of which coincides with the ventral borders of *bap* induction (Fig. 2P).

Early TVM enhancer of *bap* contains combinatorial binding sites for key signaling effectors and mesodermal regulators

To investigate whether the *bap* regulators identified genetically, including *tin*, *dpp*, *slp* (downstream of *wg*) and *bin*, can act directly on the early TVM regulatory element of *bap*, DNaseI protection experiments with recombinant Tin, Bap, Smad (Mad and Medea), Slp and Bin proteins was performed on the 180 bp bap3.2.1 DNA sequence from *D. melanogaster*. The DNA footprinting results demonstrate that both Tin and Bap proteins can bind to the predicted Tin-binding site, which includes a perfect match to the canonical Tin-binding motif TCAAGTG (Fig. 4; Fig. 5) (Chen and Schwartz, 1995; Gajewski et al., 1997). In addition to the Tin-binding site, a site with a TAAG core motif can strongly bind Bap but not Tin (CTTA in opposite strand; Fig. 4 and Fig. 5; note that the same core motif is found in binding sites of a Bap ortholog, Nkx3.2) (Kim et al., 2003). With regard to Dpp signaling mediators, there are five Mad-protected regions, three of which are also protected by recombinant Medea (Mad/Medea-1 to -3; Fig. 4 and Fig. 5). Site 1 includes an AGAC motif that was initially identified as a Smad binding motif in vertebrates (Zawel et al., 1998; Shi et al., 1998) whereas sites 3-5 contain GC-rich sequences with CGGC motifs that were first shown to bind Smad proteins in *Drosophila* (Kim et al., 1997; Shi, 2001). Site 2 may be a combination of the two types (TGAC motif and CG-rich sequences). We do not observe a clear correlation of either type of site with the binding of Mad versus Medea. Finally, recombinant Slp proteins protect a wide stretch that includes an inverted repeat of core binding motifs for forkhead transcription factors (TAAACA) (Pierrou et al., 1994; Kaufmann et al., 1995), but extends further downstream (Fig. 5 and Fig. 4). During the course of our work, it was reported that Slp can bind to tandem repeats of CAAA sequences, which are present in three copies in the 3′ region of the protected region (Andrioli et al., 2002). Gel mobility shift and competition assays with Slp using wild-type oligonucleotides and a version in which the TAAACA motifs were mutated

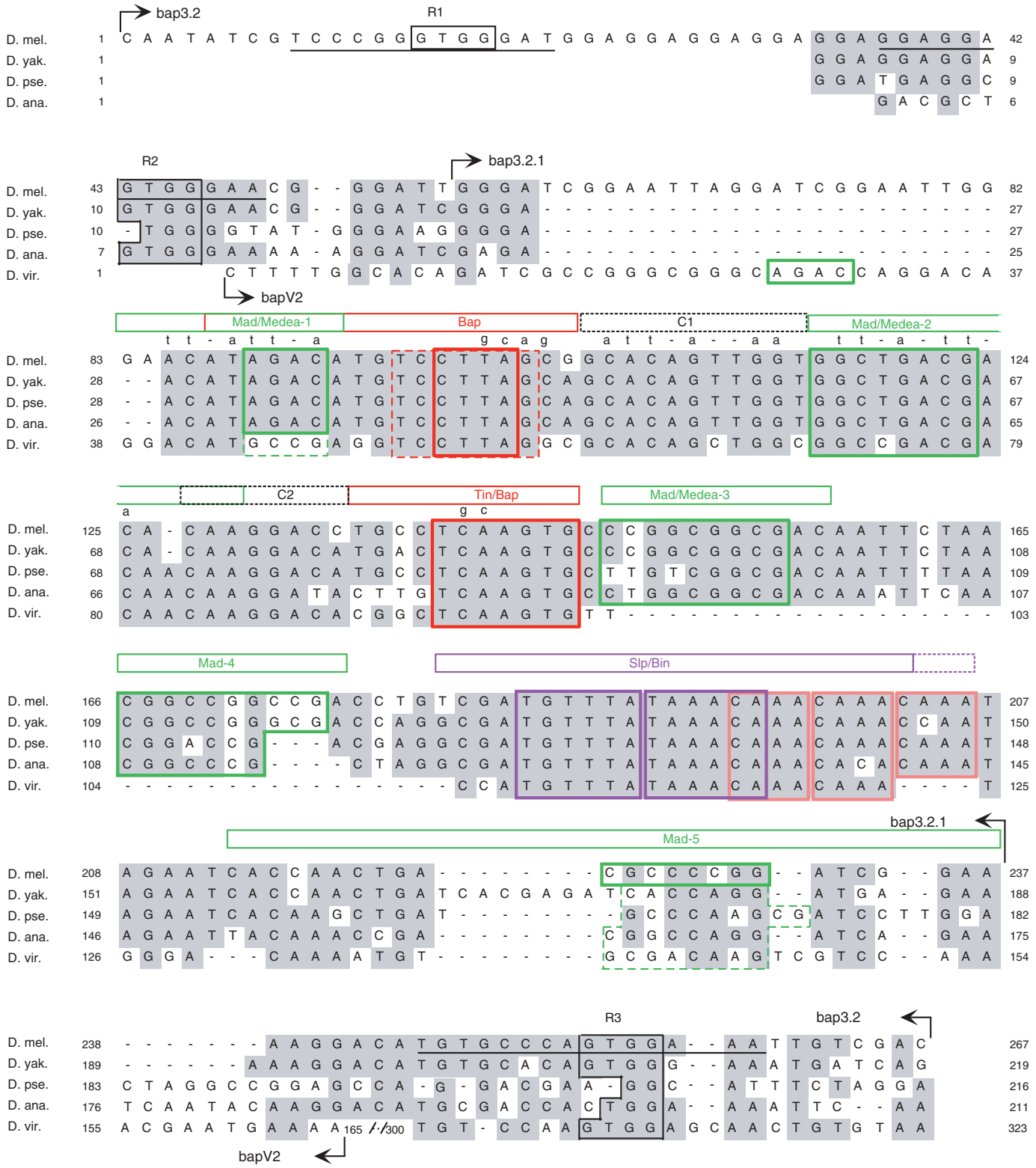

Fig. 5. Evolutionary conservation of *bap* enhancer sequences and binding motifs. Shown are alignments of enhancer sequences of *bap*3.2 (*D. melanogaster*), *bap*V2 (*D. virilis*) and corresponding genomic sequences from *D. yakuba* (*D. yak.*), *D. pseudoobscura* (*D. pse.*) and *D. ananassae* (*D. ana.*). Colored boxes above the sequences with unbroken lines indicate the extent of DNAseI footprints on *D. melanogaster* sequences, boxes with black broken lines delineate highly conserved DNA stretches (C1 and C2), and colored boxes within the sequences denote core binding motifs for the respective binding factors. Nucleotides altered by in vitro mutagenesis for in vivo testing of binding site activities are shown on top of the *D. melanogaster* sequence (for Slp/Bin site mutations, see Fig. 7). For R1-R3 motifs, see text and Fig. 8. The R1 sequence is not readily detectable in the other species but the 5′ region of bap3 (not shown) contains additional R-related motifs that are conserved and may have functionally redundant activities.

indicated that Slp can bind to both the TAAACA and the CAAA motifs with roughly equal affinity (data not shown). In addition, the FoxF family protein Bin binds to the TAAACA inverted repeat region, but less well to the CAAA repeat region when compared with Slp (Fig. 4). Taken altogether, these binding data are consistent with the hypothesis that the known mesodermal regulators of *bap*, namely Tin and Bin (and possibly autoregulatory Bap), as well as the signaling inputs from Dpp and Wg (through Smads and Slp, respectively) are integrated via direct binding to the early TVM enhancer of *bap*.

Binding sites for Tin, Smads, and additional conserved sequences are required for activation of the *bap* TVM enhancer in the mesoderm

The very high degree of sequence conservation of the binding sites for Tin, Bap, Slp/Bin and Smads (except for Mad site 5) in different *Drosophila* species (Fig. 5) is indicative of the biological importance of these sites. In addition, two other sequence stretches, C1 and C2, without any known candidates for binding factors, are also highly conserved (Fig. 5). To test for the relevance of these sites in vivo, transgenes carrying either mutations or deletions of these sequences were examined for their enhancer activities in embryos. These assays were performed within the context of the 180 bp regulatory element bap3.2.1, which shows ectopic activity in stripes within the dorsal ectoderm (Fig. 2K, Fig. 6B). The use of bap3.2.1 rather than bap3.2 allowed us to determine whether any particular site is required for receiving inputs from a mesoderm-specific factor or a factor active in both germ layers.

Mutations at the Bap-specific binding site result in a delayed onset of *lacZ* expression in the mesoderm and reduced levels of expression at early stage 11 (bap3.2.1-bap-m; Fig. 5, Fig. 6A-C), but by late stage 11 an almost normal expression level and pattern is observed in the TVM (data not shown). The reduced activity of bap3.2.1-bap-m in the mesoderm at early stages suggests a contribution of Bap to the regulation of *bap* enhancer activity in the TVM precursors. The full contribution of Bap autoregulation may be masked by the presence of an intact Tin/Bap-binding site in this construct. Mis-expression of *bap* in the whole mesoderm does not result in any expansion of *bap3.2.1-lacZ* expression, suggesting that Bap alone is not sufficient to autoactivate its own enhancer in the mesoderm (data not shown).

The construct bap3.2.1-tin-m with a mutated Tin/Bap-binding site completely fails to activate *lacZ* expression in the dorsal mesoderm (Fig. 6A,D). By contrast, and as predicted because there is no Tin, the ectodermal *lacZ* expression remains unaffected (Fig. 6D; the ectodermal expression is also unperturbed in bap3.2.1-bap-m, Fig. 6C). *twist*-driven ectopic expression of *tin* in the whole mesoderm does not cause any ectopic expression of *bap* or *bap3.2.1-lacZ* ventrally (data not shown), suggesting that Tin binding to this *bap* enhancer element is essential, but also not sufficient to activate *bap* in the mesoderm. Most likely, Tin needs to cooperate with other localized activators that bind to the same regulatory element to activate *bap* expression in the mesoderm. However, bap3.2.1 enhancer activity in the dorsal ectoderm, which lacks Tin, does not require an intact Tin-binding site. These results point to an intricate molecular mechanism that makes bap3.2.1 enhancer activity differentially sensitive to regulators in the mesoderm versus ectoderm.

To study the in vivo function of Smad-binding sites in the early *bap* regulatory element, a series of mutation or deletion constructs were generated. A reporter with bap3.2.1-Smad1-m, with mutations in the AGAC core sequence of the 5′ most Mad/Medea-binding site (Mad/Medea-1), does not display any *lacZ* expression in either mesoderm or ectoderm (Fig. 6A,E).

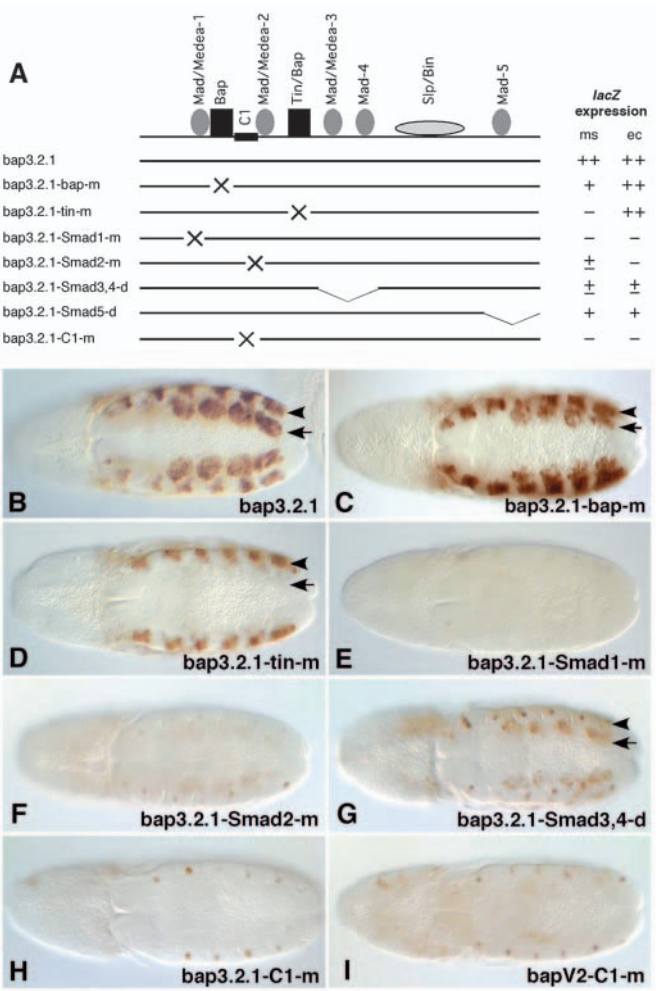

Fig. 6. In vivo requirements for binding sites of Smad, Tin and Bap proteins, and for other conserved sequences. (A) Schematic representations of *bap*3.2.1 and its mutated derivatives with a summary of their in vivo activities (ms, dorsal mesoderm; ec, dorsal ectoderm). (B-I) Dorsal views of stage 11 embryos. Arrow indicates mesodermal layer and arrowhead indicates ectodermal layer. (B) Activity of the parental *bap3.2.1-lacZ* construct. (C) Mutations in the Bap-binding site cause a slight and transient reduction of mesodermal enhancer activity. (D) Mutations in the Tin-binding site cause a loss of enhancer activity in the mesoderm. (E) Mutations in the Mad/Medea-binding site 1 cause a loss of enhancer activity in both ectoderm and mesoderm. (F) Mutations in the Mad/Medea-binding site 2 nearly abolish enhancer activity in both ectoderm and mesoderm. (G) Deletion of DNA sequences containing the Mad/Medea-binding sites 3 and 4 causes a strong reduction of ectodermal and mesodermal enhancer activity. (H,I) Mutations within the conserved sequence C1 of the enhancer from *D. melanogaster* (H) and *D. virilis* (I) cause a loss of enhancer activity in both ectoderm and mesoderm. (The observed expression within single ectodermally derived cells in each hemisegment is an artificial effect from the transformation vector.)

Hence, this Smad-binding site (Mad/Medea-1) is essential for bap3.2.1 enhancer activity in both mesoderm and ectoderm. In addition, mutations in the second Mad/Medea-binding site (Mad/Medea-2; derivative bap3.2.1-Smad2-m) result in a loss of *lacZ* expression in the ectoderm and a near-loss of expression in the mesoderm (Fig. 6A,F). The above results and the highly conserved sequences of Mad/Medea-1 and -2 among different *Drosophila* species (Fig. 5) suggest that both Smad-binding sites have essential and non-redundant functions in regulating *bap* expression during embryogenesis. Deletion of both the third and fourth Smad-binding sites (Mad/Medea-3 and Mad-4; derivative bap3.2.1-Smad3,4-d) causes weak *lacZ* expression in both mesoderm and ectoderm (Fig. 6A,G). Thus, these two Smad-binding sites are not quite as crucial as sites 1 and 2, but have additive or synergistic effects in inducing high levels of enhancer activity. Consistent with this notion, these two Smad-binding sites are absent from the homologous *bap* regulatory element of *D. virilis* (Fig. 5). The most 3′ Smad-binding site (Mad-5) does not closely match the GC-rich Mad/Medea-binding motif and is not well conserved among the five *Drosophila* species (Fig. 5). Deletion of this Smad-binding site (derivative bap3.2.1-Smad5-d) does not cause any change of *lacZ* expression in either mesoderm or ectoderm (data not shown), implying that it does not have an essential function in vivo even though Mad can bind to it in vitro.

Mutations in the C1 region from *D. melanogaster* (bap3.2.1-C1-m) and, likewise, within the bapV2 element from *D. virilis* were also examined for their effects in embryos. In both cases, *lacZ* expression is absent in both mesoderm and ectoderm (Fig. 6H,I), suggesting that the highly conserved C1 sequence plays an essential role in mediating the function of *bap* activators that function in conjunction with Dpp in both germ layers. By contrast, mutation of the C2 region does not affect enhancer activity (data not shown).

From the above data we conclude that the activation of *bap* in the mesoderm normally requires combinatorial binding of mesodermal Tin and Dpp-activated Smad proteins. Binding of Bap (and Bin, see below) increases enhancer activity via a feedback regulatory loop. In addition, yet unidentified activating binding factors are required, potentially as general DNA-binding Smad co-activators.

The Slp-binding site mediates segmental repression of *bap* enhancer induction and overlaps with an essential mesodermal activation site

Based on the observation that Slp represses *bap* expression within the *slp*-expressing domains of the mesoderm (Riechmann et al., 1997; Lee and Frasch, 2000), we predicted that mutations made at the Slp-binding site would result in uniform *lacZ* expression along the AP axis, similar to the endogenous *bap* expression in *slp* mutant embryos. However, several different mutations, particularly within the forkhead domain consensus sites, caused a complete loss (bap3.2.1-slp-m1, Fig. 7A-C) or severe reduction (bap 3.2.1-slp-m2, Fig. 7A,D; bap 3.2.1-slp-m3, Fig. 7A,E) of *lacZ* expression in the mesoderm. The levels of ectodermal enhancer activity are not affected by these mutations. These observations show that there are one or several activators that require this site and whose function is mesoderm specific. These activators are likely to include Bin, which is needed for prolonged expression of *bap* at stage 11 and binds to this site (Fig. 4) (Zaffran et al., 2001). However, there must be at least one additional, yet unidentified, binding factor that is required for initiation of enhancer activity through this site.

In spite of this complication, the observed ectopic ectodermal expression of these enhancer derivatives and the

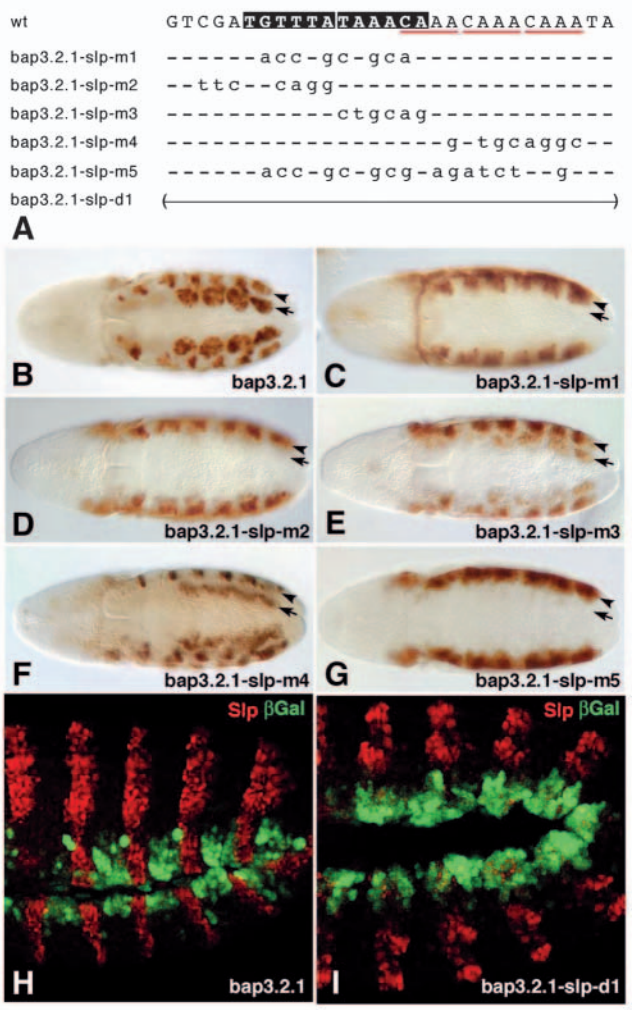

Fig. 7. Functional dissection of the Slp binding sites in the *bap* enhancer. (A) Wild-type and mutated sequences within the region protected by Slp. The inverted repeat of canonical forkhead domain-binding motifs is in black boxes and the CAAA type of Slp-binding motifs are underlined in red. Unaltered sequences are represented by dashes below, and deleted sequences are indicated as a bracketed unbroken line. (B) Activity of the parental *bap3.2.1-lacZ* construct used as a control. (C) *bap3.2.1-slp-m1-lacZ* is not active in the mesoderm, while in the dorsal ectoderm it is active in metameric domains and there is weak ectopic activity between these domains. (D) *bap3.2.1-slp-m2-lacZ* shows very weak activity in the mesoderm and similar ectodermal activity as with *bap3.2.1-slp-m1-lacZ*. (E) *bap3.2.1-slp-m3-lacZ* shows weakened activity in the mesoderm and similar ectodermal activity as with *bap3.2.1-lacZ*. (F) *bap3.2.1-slp-m4-lacZ* activity is similar to that of the parental *bap.3.2.1-lacZ* (mesodermal clusters have physically merged at this slightly later stage). (G) *bap3.2.1-slp-m5-lacZ* shows lack of mesodermal activity and largely uniform dorsal ectodermal activity along the anteroposterior axis. (H,I) Fluorescent double staining for Slp (red) and βGal (green) in stage 10 embryos. *bap3.2.1-lacZ* expression (H) is complementary to that of Slp, whereas *bap3.2.1-slp-d1-lacZ* expression (I) overlaps with Slp.

known presence of Slp in the same pattern in both mesoderm and ectoderm enabled us to study the potential repressive activity of the Slp-binding sequences further. With bap3.2.1-slp-m1, in which both of the canonical forkhead domain-binding sites are mutated, reporter gene expression in the ectoderm is expanded only slightly along the AP axis compared with the strictly segmented *bap3.2.1-lacZ* expression (Fig. 7B,C). As Slp is able to bind CAAA sequence repeats (Andrioli et al., 2002), which are present in three copies within the 3′ half of the protected sequence stretch, it was possible that Slp can still bind to bap3.2.1-slp-m1 and repress *lacZ* expression in the ectoderm. Indeed, electrophoresis mobility shift assays (EMSA) with recombinant Slp proteins and bap3.2.1-slp-m1 DNA oligo probes showed that Slp was still able to bind, presumably through the CAAA sequence motifs (data not shown). No segmental de-repression is observed when only one of the two canonical forkhead domain sites is mutated (bap3.2.1-slp-m2, Fig. 7D; bap3.2.1-slp-m3, Fig. 7E), presumably because of the unaffected binding of Slp to the intact site and/or the CAAA sequence motifs. Similarly, mutations in the CAAA repeats still allow segmental repression in both ectoderm and mesoderm (Fig. 7F and data not shown). Presumably, Slp is able to repress enhancer activity through binding to the canonical forkhead domain sites in this situation, which can also bind the unknown mesodermal activator. By contrast, the introduction of mutations in both types of Slp-binding sequences (bap3.2.1-slp-m5, Fig. 7A) or the deletion of the entire sequence protected by Slp (bap3.2.1-slp-d1, Fig. 7A), results in almost complete segmental de-repression of reporter gene expression in the ectoderm (Fig. 7G-I). The inability of Slp to repress these enhancer derivatives is further confirmed by the observed co-expression of enhancer-driven *lacZ* with Slp in the dorsal ectoderm (Fig. 7I, compare with the normal mutually exclusive expression, 7H). Taken together, the above results suggest that in the normal context, the Slp-protected DNA fragment mediates segmental repression by Slp proteins in the mesoderm both through the canonical forkhead domain sites and the Slp-specific CAAA motifs. Conversely, the activation of the enhancer in the mesoderm requires binding of Bin and a yet unidentified activator to the canonical forkhead domain sites or sequences overlapping with them.

Characterization of sequence elements preventing the induction of *bap* and *tin* in the dorsal ectoderm

Information from several different enhancer derivatives has shown that the germ layer-specific induction of *bap*, as well as of *tin*, relies in part on repressive sequences that prevent ectopic induction of both genes in the dorsal ectoderm. For example, the *bap* enhancer derivative bap3.2.1, which differs from bap3.2 by the absence of 57 bp from the 5′-end and 30 bp from the 3′-end, is induced ectopically in the dorsal ectoderm with the same pattern as its normal expression in the dorsal mesoderm (Fig. 2K, Fig. 6B, Fig. 8D). Similarly, a shortened version of the *bap* enhancer from *D. virilis*, bapV2, drives segmental *lacZ* expression ectopically in the dorsal ectoderm, in contrast to a longer version, bapV1, which is largely mesoderm specific (Fig. 2J,L). An analogous situation was previously described for *tin*. In this case, it was observed that the deletion of two identical sequence motifs, tinD1a and tinD1b, within the Dpp-responsive enhancer of *tin* causes ectopic enhancer induction by Dpp in the dorsal ectoderm (Xu et al., 1998). Together, these observations suggest that the mechanisms for the repression of ectopic induction of

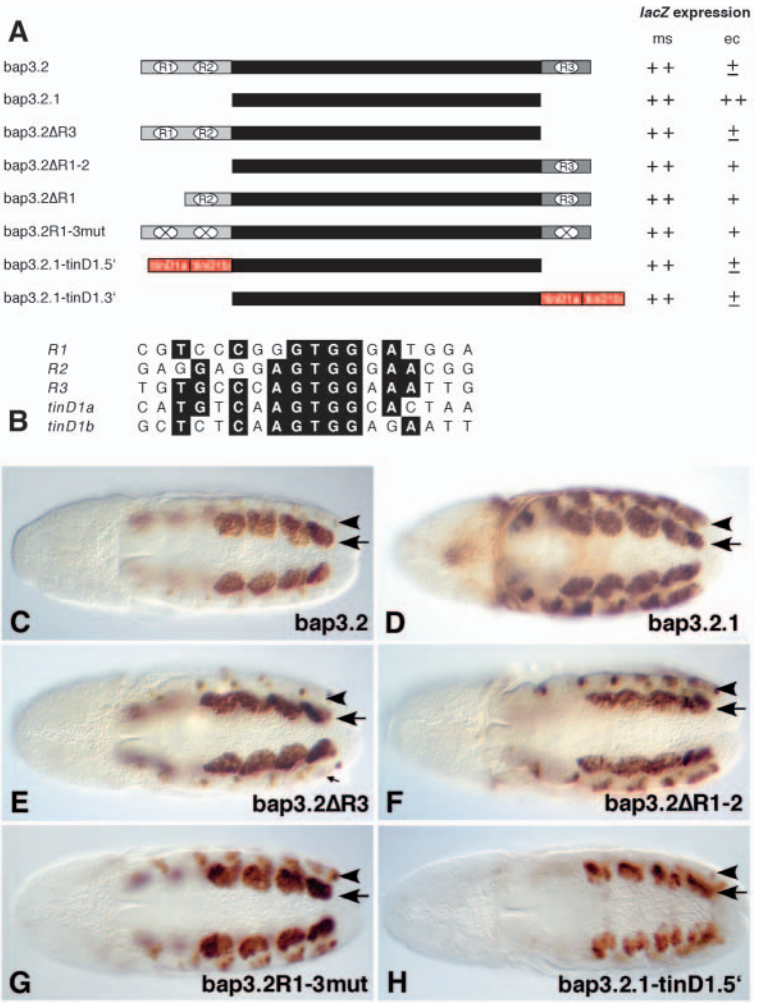

Fig. 8. Sequences required for preventing the induction of *bap* enhancer activity in the dorsal ectoderm. (A) Summary of tested enhancer derivatives and their activities in the dorsal mesoderm (ms) and ectoderm (ec). R1, R2 and R3 denote native motifs with putative repressing activities, whereas tinD1a and tinD1b denote related sequence motifs from the tinD enhancer of *tinman*. (B) Sequence alignments of motifs thought to confer ectodermal repression from the *bap3.2* and tinD enhancers. (C-H) Dorsal views of early stage 11 βGal-stained embryos carrying various reporter constructs (arrow, mesoderm; arrowhead, ectoderm). (C) *bap3.2-lacZ* shows almost complete repression in the dorsal ectoderm. (D) *bap3.2.1-lacZ* shows complete de-repression in the dorsal ectoderm. (E) *bap3.2ΔR3-lacZ* shows low levels of de-repression in the dorsal ectoderm (small arrow; see comment in Fig. 6 legend regarding large ectodermal cells). (F) *bap3.2ΔR1-2-lacZ* and (G) *bap3.2R1-3mut-lacZ* show strong de-repression of enhancer activities in the dorsal ectoderm. (H) The addition of *tin*D1 sequences to *bap*3.2.1 prevents ectopic induction in the ectoderm.

mesodermal genes by Dpp in the ectoderm have been conserved in different *Drosophila* species, and that different Dpp targets in the mesoderm use closely related mechanisms to ensure their germ layer-specific induction.

DNA sequence comparison among the 5′-57 bp and the 3′-30 bp sequences of bap3.2, as well as tinD1a and tinD1b from the *tin* enhancer, identified three regions, termed R1, R2 and R3, in bap3.2 that showed sequence similarities with one another and with tinD1a and tinD1b (Fig. 8B). Four to five additional copies of this type of motifs are found at conserved positions within ~200 bp of sequences upstream of bap3.2 in different *Drosophila* species (data not shown). These observations raise the possibility that these motifs might represent binding sites for a yet unknown repressor preventing mesodermal gene induction in the ectoderm. To further characterize the DNA elements mediating the ectodermal repression, various derivatives of bap3.2 were generated that were either truncated or contained mutated or swapped sequences in the identified repressing regions (Fig. 8A; Materials and methods). All three putative binding sites for the ectodermal repressor were found to contribute to the repression, albeit with slightly different degrees of inhibitory activities. Comparisons of the ectodermal enhancer activities of bap3.2ΔR3, bap3.2ΔR1-2 and bap3.2ΔR1 suggest that the three sites have partially redundant activities, with R1 having the strongest effect in ectodermal repression (Fig. 8E,F; data not shown). When mutations are introduced all three sites, R1, R2 and R3, within bap3.2 (bap3.2R1-3mut), the resulting de-repression in the ectoderm is almost as complete as with deletions of these sequences (Fig. 8G, compare with 8D). Hence, the identified sequence motifs appear to be largely responsible for the repression of induction in the ectoderm. The sequence similarities of tinD1a and tinD1b, and their analogous biological activities within the *tin* enhancer would suggest that these sequences are able to replace the R1, R2 and R3 sequences functionally within the *bap* enhancer. To test this possibility, both tinD1a and tinD1b were added to either the 5′- or the 3′-end of bap3.2.1 (bap3.2.1-tinD1.5′ and bap3.2.1-tinD1.3′, respectively). As predicted, tinD1a and tinD1b strongly repress bap3.2.1 enhancer activity in the ectoderm, without affecting it in the mesoderm (Fig. 8H and data not shown). These results suggest that similar mechanisms, probably via binding of identical repressor factor(s), are involved in preventing the ectopic induction of *bap* and *tin* in the ectoderm. The ectopic ectodermal expression of the enhancers of *bap* and *tin* is directly controlled by Dpp signals, as shown with mutations of Smad-binding sites, which prevent the induction in the dorsal ectoderm of the enhancers that lack the repressing sequences (Fig. 6E-G) (Xu et al., 1998). Consequently, the putative repressors must normally interfere with Dpp signaling outputs at the level of the target enhancers.

Discussion

It has become evident that enhancers of target genes provide crucial platforms for the integration of diverse signaling inputs and germ layer or tissue-specific nuclear factors during inductive events in development. As a result, specific transcriptional responses of combinatorial signals are triggered in restricted domains within a target tissue, but usually not in the cells that send the signals. In several systems, particularly during *Drosophila* and *Xenopus* embryogenesis, TGFβ or BMP signals have been shown to act in concert with Wnt signals to achieve particular inductive responses of this type. Although in most cases described to date, TGFβ/BMP and Wnt signals act in a synergistic manner, there are also a few known situations in which Wnt signals antagonize TGFβ or BMP signals (Hazelett et al., 1998; Yu et al., 1998; Kopp et al., 1999; Nishita et al., 2000; Morata, 2001; Waltzer et al., 2001; Zaffran and Frasch, 2002). However, the molecular basis of these signal interactions, particularly of antagonistic ones, is largely unknown.

The induction of mesodermal tissues in *Drosophila* is a process during which Wg signals can modulate the responses to Dpp signals by either synergizing with them or antagonizing them. Whereas previous studies have described the functional architecture of mesodermal enhancers that are targeted either by Dpp alone or by synergistic Dpp and Wg signals, our present study describes an example of an enhancer whose response to Dpp is suppressed by Wg signals. A comparison of the functional organization of these enhancers provides new insight into molecular strategies of nuclear signal integration to produce differential developmental responses.

Nuclear Dpp signaling outputs and their suppression by Wg-induced Slp

Our data show that *bap* is a direct target of Dpp signals. Thus, we can rule out an indirect pathway of *bap* being activated solely by *tin*, whose mRNA expression is known to depend on Dpp inputs during the time of *bap* activation (Azpiazu and Frasch, 1993). Rather, *tin* acts simultaneously and synergistically with Dpp. In fact, recent data with *tin* alleles lacking the Dpp-responsive enhancer show that *bap* can be induced in the absence of Dpp-induced *tin* products, as long as the *twist*-activated *tin* products are present (S. Zaffran and M.F., unpublished). We show that the molecular basis for this observed synergism of *tin* and *dpp* relies on the combinatorial binding of Tin and Dpp-activated Smad proteins to the *bap* enhancer. Several possible molecular mechanisms could underlie the strict requirement for combinatorial binding of Tin and Smads. For example, the relatively low binding affinity and specificity of Smads might be enhanced by bound Tin, which can engage in protein interactions with Mad and Medea (Zaffran et al., 2002). The combined presence of Tin and Smads in close vicinity or in complexes may also be a prerequisite for the assembly of higher order complexes with transcriptional co-activators such as CBP/p300 (Liu et al., 1997; Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998; Waltzer and Bienz, 1999). In addition, Tin may counteract the function of yet unknown repressors of nuclear Dpp signaling activity so that they can only repress in the ectoderm.

Unlike Dpp, Wg signals act indirectly upon the early *bap* enhancer. Previous genetic and molecular data showed that Wg induces the expression of the forkhead domain-encoding gene *slp* via crucial dTCF/Lef-1 binding sites in both mesoderm and ectoderm (Lee and Frasch, 2000). *slp*, in turn, functions as a repressor of *bap* (Riechmann et al., 1997; Lee and Frasch, 2000). Our present data show that *slp* products exert this function by direct binding to the Dpp-responsive *bap* enhancer, which obviously results in a suppression of the synergistic activity of bound Tin and Smad complexes. Slp proteins

contain eh1 motifs that can potentially bind the Groucho co-repressor and Slp has known repressor activities in other contexts (Lee and Frasch, 2000; Andrioli et al., 2002; Gebelein et al., 2004). In addition, the vertebrate counterpart of Slp, FoxG (BF-1), is known to interact with Groucho and histone deacetylases (Yao et al., 2001). Thus, we propose that Slp overrides nuclear Dpp signaling activities by dominantly establishing an inactive state of the chromatin at the *bap* locus.

It is likely that additional components are involved in the antagonistic interaction of Slp with Tin/Smad complexes. As we have shown, the Slp-binding site includes sequences that are also required positively for the mesodermal response to Dpp, although not for ectopic responses in the ectoderm. In a genome-wide expression analysis, we did not find any forkhead domain genes other than *bin* that are mesoderm specific (Lee and Frasch, 2004). However, the function of an essential co-activator in the mesoderm interacting with this site could be fulfilled by a ubiquitously expressed forkhead domain protein, and in part by Bin, which is required for the prolongation of the Dpp response (Zaffran et al., 2001). In the yeast one-hybrid screens with this site that yielded Slp clones we also isolated a clone of *fd68A*, a uniformly expressed ortholog of vertebrate *FoxK1* (*Myocyte Nuclear Factor*), but genetic confirmation of its involvement in *bap* induction is currently lacking (Lee and Frasch, 2004). Regardless of the identity of this factor, Slp could either compete with this protein and with Bin for DNA binding, or it could disrupt their productive functional interactions with the Tin/Smad complexes. Interestingly, the latter type of mechanism has been proposed to operate during the interference of the *slp* ortholog *BF-1* with TGFβ signaling in the vertebrate cerebral cortex (Dou et al., 2000).

Nuclear mechanisms guaranteeing germ layer-specific signaling outputs

Inductive responses that are germ layer- or cell type-specific and exclude the signal-producing cells are a recurring theme in developmental systems. Although this type of target specificity can involve different levels of the signaling cascade, including the tissue-specific expression of receptors or signaling effectors, we have shown that germ layer-specific induction of *bap* is controlled by nuclear events. This is crucial because activated Smads are present in dorsal nuclei of both germ layers (Knirr and Frasch, 2001) (Fig. 2P). We have identified two mechanisms, which are probably functionally intertwined, that ensure mesoderm-specificity of the response to Dpp. The first is the requirement for Tin to synergize with activated Smads, as discussed above. Tin is present exclusively in the mesoderm and is therefore not available to fulfill such a function in the ectoderm. Hence, in developmental terms, Tin provides the mesoderm with the unique competence to respond to Dpp and induce *bap*. Perhaps surprisingly then, there is an additional component involved, which actively prevents induction of the *bap* enhancer by Dpp in the ectoderm. As we have shown, the Dpp-responsive core enhancer of *bap* is flanked by sequences that appear to function as binding sites for yet unidentified repressor(s), which keep the enhancer silent in the ectoderm. A very similar situation was previously described for the Dpp-responsive enhancer of *tin* (Xu et al., 1998) and, as shown herein by sequence comparisons as well as functional swapping of the putative ectodermal repressing sequences from the *tin* and *bap* enhancers, they appear to bind the same repressor(s). Brinker, a known nuclear repressor of Dpp signaling, can be excluded as a candidate because of its different sequence preference and absent expression in the dorsal ectoderm (Jazwinska et al., 1999; Sivasankaran et al., 2000; Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001; Zhang et al., 2001). The situation is reminiscent of an endodermal *labial* enhancer, in which a homeotic response element and a repressor element interact to control the spatially restricted activity of a minimal Dpp response element (Marty et al., 2001).

Why would induction of *tin* and *bap* in the mesoderm require Tin as a co-factor of Smads, whereas in the ectoderm, which lacks Tin, the induction of *tin* and *bap* needs to be actively repressed? In the case of the *tin* enhancer, the ectodermal repressor elements are overlapping with the Tin-binding sites. Based upon this situation, we proposed a model in which the repressor would be present in both germ layers, but in cells of the mesoderm it is competed away from binding to the enhancer by Tin (Xu et al., 1998). This model is compatible with data showing that ectopic expression of Tin in the ectoderm is able to activate the Dpp-responsive enhancer of *tin*, even in the presence of the putative repressor binding elements. However unlike full-length Tin, an N-terminally truncated version with an intact homeodomain is not able to allow induction of the *tin* enhancer in the ectoderm (Zaffran et al., 2002). Furthermore, the putative repressor binding sites in the *bap* enhancer are separate from the Tin site. Hence, Tin does not compete for binding but may rather block or override the repressor factor(s) functionally. Thus, the positive activity of Tin would dominate over the negative action of this repressor in the mesoderm. By contrast, the repressing activity of Slp dominates over the positive action of Tin. Through this intricate balance of positive and negative switches, Tin could ensure that *bap* is induced by Dpp only in the mesoderm, while bound Slp prevents Tin from promoting Dpp inputs towards *bap* in striped domains within this germ layer. However, we can still not fully explain why the absence of both the functional Tin and ectodermal repressor sites allows enhancer induction in the ectoderm, while preventing it in the mesoderm. The additional positive and negative binding factors involved will need to be identified to gain a full understanding of the germ layer-specific induction of these Dpp-responsive enhancers.

Mesodermal enhancers of *tin*, *eve* and *bap* – variations on a theme

The *bap* enhancer described herein represents the third example of well-characterized Dpp-responsive enhancers from mesodermal control genes. The other two are from *tin*, which is induced in the entire dorsal mesoderm, and *eve*, which is active in a small number of somatic muscle founder cells and pericardial progenitors in the dorsal mesoderm. The activities of the *bap* and *eve* enhancers along the anteroposterior axis are reciprocal, which is due to the fact that the *eve* enhancer requires inputs from Wg, whereas *bap* enhancer activity is suppressed by Wg. A comparison of the molecular architecture of these three enhancers reveals that they all share a number of important features (Fig. 9) (Xu et al., 1998; Halfon et al., 2000; Knirr and Frasch, 2001; Han et al., 2002). Most notably, all three enhancers feature several Tin- and Smad-binding sites in close vicinity that are essential for the activation of the

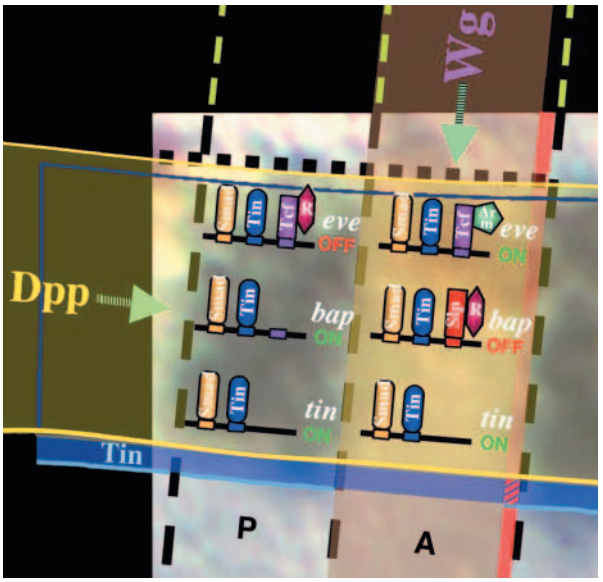

Fig. 9. Molecular switching of Dpp-responsive enhancer activities in the dorsal mesoderm. The schematic diagram summarizes the molecular basis for the reciprocal activities of the *bap* and *eve* enhancers in the A and P domains of the dorsal mesoderm. In the A domains, the differential activities are due to the Wg-dependent relief of dTCF-associated co-repressors at the *eve* enhancer and to the Wg-dependent loading of Slp/co-repressor complexes at the *bap* enhancer. Conversely, in the P domains where Wg signaling is absent *slp* is not induced, which allows the *bap* enhancer to be active, while dTCF/co-repressor complexes keep the *eve* enhancer 'off'. Owing to the lack of Wg-responsive sequences, the *tin* enhancer is 'on' in both domains of the dorsal mesoderm. For simplicity, only one site of each type is depicted and various sites binding yet unknown factors involved in activation or ectodermal repression are not depicted.

enhancer in the mesoderm. Each enhancer includes both types of known Smad-binding motifs, which have 'AGAC' and 'CG'-rich cores, respectively. Hence, the basic activation mechanisms of each of the three enhancers downstream of Dpp are likely to be closely related. As discussed above, in the enhancers of both *tin* and *bap*, binding sites for a nuclear repressor of Dpp signals are key for the germ layer specificity of the inductive response. Although we do not know whether the same repressive mechanism operates at the *eve* enhancer, we note that motifs related to the presumed repressor binding motifs are present and their function can now be tested in vivo (M.F., unpublished). As in the case of *bap*, the *tin* enhancer includes also additional sites that are required for Dpp-inducible enhancer activity, which may bind essential Smad co-factors. However, based upon the divergent sequences of these sites (C1 site in the *bap* and 'CAATGT' motifs in the *tin* enhancer) (Xu et al., 1998), they appear to bind different types of factors in each case.

On top of this basic arrangement that allows the enhancer to be active in the dorsal mesoderm, the enhancers from *bap* and *eve*, but not *tin*, include binding sites that make them respond to Wg inputs in an opposite fashion (Fig. 9). In the case of *bap*, Wg-induced Slp binds and dominantly suppresses the activity of bound Smad effectors. For the *eve* enhancer we have proposed that there is an analogous repressive activity; however, in this case, it is exerted by bound Wg signal effectors, i.e. dTCF/Lef-1, in the absence of Wg signals (Knirr and Frasch, 2001). In the domains with active Wg signaling, the repressive activity of dTCF/Lef-1 is neutralized by the Wg signaling cascade, which allows the Dpp effectors to be active at the *eve* enhancer (as it lacks Slp binding sites). Through these switches, the *bap* and *eve* enhancers become induced in reciprocal AP patterns. In addition, the *eve* enhancer includes binding sites for activators and repressors downstream of receptor tyrosine kinases and Notch, respectively, which serve to restrict its activity to specific subsets of cells within the domains of overlapping Dpp and Wg activities (Halfon et al., 2000). Clearly, many of the molecular details still need to be clarified. Nevertheless, we are now beginning to understand the basic principles of how differential inputs from inductive signals and tissue-specific activities can be integrated at the enhancer level to achieve distinct patterns of target gene expression during early tissue induction in the *Drosophila* mesoderm.

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