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Response to the BMP gradient requires highly combinatorial inputs from multiple patterning systems in the *Drosophila* embryo

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SUMMARY

Pattern formation in the developing embryo relies on key regulatory molecules, many of which are distributed in concentration gradients. For example, a gradient of BMP specifies cell fates along the dorsoventral axis in species ranging from flies to mammals. In *Drosophila*, a gradient of the BMP molecule Dpp gives rise to nested domains of target gene expression in the dorsal region of the embryo; however, the mechanisms underlying the differential response are not well understood, partly owing to an insufficient number of well-studied targets. Here we analyze how the Dpp gradient regulates expression of *pannier* (*pnr*), a candidate low-level Dpp target gene. We predicted that the *pnr* enhancer would contain high-affinity binding sites for the Dpp effector Smad transcription factors, which would be occupied in the presence of low-level Dpp. Unexpectedly, the affinity of Smad sites in the *pnr* enhancer was similar to those in the *Race* enhancer, a high-level Dpp target gene, suggesting that the affinity threshold mechanism plays a minimal role in the regulation of *pnr*. Our results indicate that a mechanism involving a conserved bipartite motif that is predicted to bind a homeodomain factor in addition to Smads and the Brinker repressor, establishes the *pnr* expression domain. Furthermore, the *pnr* enhancer has a highly complex structure that integrates cues not only from the dorsoventral axis, but also from the anteroposterior and terminal patterning systems in the blastoderm embryo.

KEY WORDS: Morphogen, BMP gradient, Brk repressor

INTRODUCTION

A recurring theme in development is the use of morphogen gradients, where different concentrations of a regulatory molecule specify different cell fates (reviewed by Ashe and Briscoe, 2006; Rogers and Schier, 2011). For example, patterning of the dorsoventral (DV) axis of the *Drosophila* blastoderm embryo utilizes two morphogen gradients. A ventral-to-dorsal gradient of nuclear-localized Dorsal (Dl) divides the blastoderm embryo into three basic domains: mesoderm, neuroectoderm and dorsal ectoderm; and a dorsal-to-ventral gradient of Dpp further subdivides the dorsal ectoderm into the dorsal-most amnioserosa, and dorsomedial and dorsolateral epidermis.

Dl is a transcription factor related to mammalian NFκB, whereas Dpp is an extracellular ligand of the TGFβ family and is most related to vertebrate BMP2. Dpp interaction with transmembrane receptors results in the phosphorylation of cytoplasmic Smad (Mad-*P*), which in combination with Smad4 (Medea) enters the nucleus and functions as a transcription factor. Hence, both the Dl and Dpp gradients are interpreted by downstream target genes. Several candidate target genes for Dl and Dpp have been identified and classified into categories depending on the level of morphogen that they respond to: high, intermediate and low, also referred to as Type I, II and III, respectively (Ashe et al., 2000; Stathopoulos and Levine, 2004).

How do target genes interpret and respond to morphogen gradients? One widely accepted mechanism is the affinity threshold model, whereby target enhancers respond to low levels of morphogen if they contain high-affinity binding sites. For example, the *sog* enhancer contains high-affinity Dl binding sites and is thereby activated in regions of low-level Dl protein (the lateral neuroectoderm). By contrast, the *twi* enhancer contains low-affinity Dl binding sites and requires high levels of Dl for activation. Conversion of the low-affinity sites in the *twi* enhancer to high-affinity sites prompted reporter expression in regions containing lower levels of Dl (Jiang and Levine, 1993).

The mechanisms utilized by Dpp target genes in the early embryo are not well understood. Peak levels of Dpp and Mad-*P* are present along the dorsal midline in a 5- to 6-cell wide domain, the presumptive amnioserosa, while lower levels are found in the 3-4 cells to either side; beyond this, Dpp and Mad-*P* drop precipitously to very low levels (Rushlow et al., 2001; Dorfman and Shilo, 2001; Sutherland et al., 2003; Wang and Ferguson, 2005). Using the same approach of changing binding site affinities it was shown that the target gene *Race* (*Ance* – FlyBase), which is expressed in the presumptive amnioserosa, could respond to lower levels of Dpp if high-affinity sites were introduced into its enhancer (Wharton et al., 2004), indicating that differential binding site affinity could underlie threshold responses to the Dpp gradient.

Additional studies on *Race* regulation by Xu et al. (Xu et al., 2005) revealed that *Race* activation relies on a feed-forward loop, whereby peak levels of Dpp/Smads first activate *zen* expression in the dorsal-most region, and then both Smads and Zen bind and activate *Race*. The Dpp/Zen feed-forward motif provided a mechanism by which all amnioserosa-specific genes might be activated (Xu et al., 2005).

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Analysis of a second Dpp target gene, *C15* (Lin et al., 2006), which is expressed in a wider domain (8-10 cells) than *Race* that extends into the region of intermediate Dpp levels, revealed a different mechanism for threshold responses. The *C15* enhancer contains multiple clusters of Smad binding sites that contribute cumulatively to the *C15* expression domain, and, importantly, although it contains higher affinity Smad sites than those in *Race*, abolishing the highest affinity Mad sites had little effect on the width of the expression domain. By contrast, decreasing the number of Smad sites resulted in a narrow and sporadic expression domain (Lin et al., 2006). Using multiple Smad sites to ensure robust and precise spatial expression might be a distinguishing feature of enhancers that respond to intermediate levels of Dpp.

It remains unclear how target genes respond to low levels of Dpp in the dorsolateral region. Do they also contain multiple clusters of Smad binding sites and/or do they use the affinity threshold mechanism? Here we analyze the regulation of the *pannier* (*pnr*) gene, which is expressed in a broad dorsal domain of ~32-35 cells (Jaźwińska et al., 1999a; Ashe et al., 2000). To our surprise, the *pnr* enhancer structure resembled that of *Race*, containing a similar number of Smad sites with the same relative affinity as those in the *Race* enhancer, suggesting that the affinity threshold mechanism plays a minimal role, if any, in the response of *pnr* to low levels of Dpp. Instead, we found that sequences that lie adjacent to a crucial Smad site are required for proper *pnr* activation, and that the extent of *pnr* expression is limited by Brinker (Brk)-mediated repression. We also found that the *pnr* expression pattern is influenced by anteroposterior (AP) genes. Thus, *pnr* gene regulation relies on a complex combinatorial mechanism that integrates spatial cues from diverse patterning systems.

MATERIALS AND METHODS

Fly strains

y^1w^{67c23} embryos were used as wild type. dpp^{H46} is a null allele balanced over *CyO23, P[dpp+]* (Wharton et al., 1993). Homozygous dpp^{H46} embryos were identified by lack of *Race* staining. The null alleles zen^{w36}, brk^{M68}, kni^{301}, tll^1, gt^{x11}, hb^{12} and Kr^1 were balanced over *lacZ*-marked balancer chromosomes to distinguish hemizygous or homozygous mutant embryos from their heterozygous and homozygous balancer siblings. bcd^{E1} is a null allele.

In vitro mutagenesis and transgenic analysis

DNA fragments (summarized in Fig. 2A) were prepared by PCR using genomic DNA as template (Clontech) and the Expand High Fidelity PCR System (Roche Molecular Biochemicals). Nucleotide changes in the Mad binding sites and the HD site were introduced by PCR mutagenesis (see Figs 3 and 5 for DNA sequences). Amplified DNA fragments were first cloned into the pCRII vector using the TOPO cloning system (Invitrogen) and then subcloned into the *Eco*RI site of the pCasPeRhs43-β-gal transformation vector (Thummel and Pirrotta, 1992). Plasmid constructs were mixed with helper 'turbo' DNA and injected into *yw* embryos to make transgenic flies. At least three transformant lines for each construct were analyzed.

Bacterial expression of Zen, Mad, Medea and Brk

Expression plasmids encoding GST-Mad and GST-Medea fusion proteins containing the N-terminal MH1 domains were obtained from A. Laughon (Kim et al., 1997) and M. Frasch (Xu et al., 1998), respectively. GST-Brk^N contains the N-terminal 266 amino acids (Rushlow et al., 2001). GST-Zen contained the full-length protein (Xu et al., 2005). The expression and purification of the recombinant proteins were carried out as described (Kirov et al., 1993). The concentration of the isolated proteins was determined by SDS-PAGE with defined amounts of bovine serum albumin and staining with Coomassie R-250.

In vitro DNA binding assays

DNase I footprint analyses and electrophoretic mobility shift (gel shift) assays were carried out as described (Kirov et al., 1993; Rushlow et al., 2001). All DNA fragments were generated from the *pnr* P3 and P4 enhancers by restriction digestion or PCR (summarized in Fig. 2B) or prepared as oligonucleotides (Integrated DNA Technologies). In each gel shift reaction, 10 ng or 30 ng of GST-Mad, GST-Medea or 5 ng Brk recombinant protein was used; about ten times more was used for footprinting reactions.

In situ hybridization and antibody staining

Wild-type, mutant and transgenic embryos were fixed, hybridized with *pnr* or *lacZ* antisense RNA probes labeled with digoxigenin (Roche Molecular Biochemicals) and mounted in Aqua Polymount (Polysciences) as described (Rushlow et al., 2001).

RESULTS

pnr is regulated by Dpp and Brk

pnr encodes a GATA factor that is required for several developmental processes including proper subdivision of the embryonic dorsal ectoderm (Ramain et al., 1993; Winick et al., 1993; Heitzler et al., 1996; Herranz and Morata, 2001). In addition, *pnr* mutant embryos do not undergo proper dorsal closure, leaving holes in the dorsal cuticle (Heitzler et al., 1996). The *pnr* expression pattern is consistent with these embryonic phenotypes. *pnr* transcripts are first detected in the dorsal region of the cellularizing embryo from future parasegment 3 (labial) to 14 (A9) (Fig. 1A) (Winick et al., 1993; Heitzler et al., 1996; Herranz and Morata, 2001). In older embryos, expression becomes segmentally repeated and is lost from the amnioserosa, but the AP limits of the pattern remain the same (Herranz and Morata, 2001). Thus, *pnr* appears to be highly regulated on both the DV and AP axes.

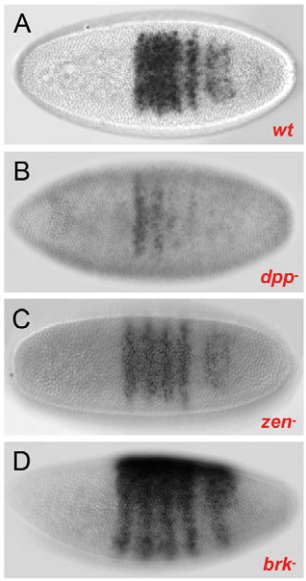

Fig. 1. *pnr* is regulated by Dpp, Zen and Brk. Wild-type (wt) (A) and mutant (B-D) *Drosophila* embryos hybridized with *pnr* RNA probes. Dorsal views, except D in lateral view, with anterior to the left. (**A**) The *pnr* expression domain at the late blastoderm stage comprises ~30% of the DV circumference and extends from 20-60% egg length. Note that six stripes of different width can be distinguished in the pattern. (**B**,**C**) Stripes become more visible in dpp^{H46} (B) and zen^{w36} (C) embryos, although there is a much greater effect on the overall pattern in the absence of Dpp (B). (**D**) *pnr* extends into the ventrolateral region in brk^{M68} mutants.

To determine how *pnr* responds to DV cues, we examined *pnr* in different mutant backgrounds. *pnr* expression was reduced to a few faint stripes in *dpp* mutants (Fig. 1B), but was only slightly affected in *zen* mutants (Fig. 1C). *pnr* expression expanded into the ventrolateral region in *brk* mutant embryos (Fig. 1D). Interestingly, the 'stripey' nature of *pnr* expression that can be seen in wild type (Fig. 1A) becomes more apparent in the absence of Brk. These results suggest that *pnr* is activated by Dpp, but that additional activators are also required, and that Brk establishes the *pnr* border ventrally. Snail (Sna) is thought to repress *pnr* in the mesoderm, as *pnr* expands into the entire ventral region in the absence of both Brk and Sna (Jaźwińska et al., 1999a).

The *pnr* expression domain comprises two patterns

To study the cis-regulation of *pnr*, we isolated a 2.3 kb fragment from its first intron that recapitulated the wild-type blastoderm *pnr* pattern in transgenic reporter assays (Fig. 2A,C). We dissected the 2.3 kb enhancer into four overlapping subfragments termed P1-P4 (summarized in Fig. 2A), and two of these, P3 and P4, were able to drive blastoderm *lacZ* reporter expression (Fig. 2D,E), but gave different patterns. P3 gave rise to a dorsal expression domain similar to the wild-type *pnr* domain, and two posteriorly located stripes. P4 drove four anterior stripes that could be clearly distinguished. These results indicated that: (1) the stripe pattern can be separated from the broad dorsal pattern, which we now refer to as the dorsal patch; and (2) the stripes can be separated from each other, suggesting that they are regulated by discrete enhancers, as in the case of pair-rule genes such as *hairy* (*h*) and *even skipped* (*eve*) (Howard et al., 1988; Harding et al., 1989), although the *pnr* stripes are more closely spaced than pair-rule stripes.

The observation that the *pnr* pattern is a composite of two patterns was further substantiated by assaying the reporter genes in *dpp* and *brk* mutant backgrounds (Fig. 2F-I). In *dpp* mutants, the P3-*lacZ* dorsal patch was abolished, but the posterior stripes remained (Fig. 2F). P4-*lacZ* was unaffected (data not shown), indicating that only the dorsal patch in the P3 enhancer is Dpp responsive, whereas the stripes are regulated by other factors. In *brk* mutants, the pattern expanded ventrally (Fig. 2G-I) in a similar manner to the expansion of endogenous *pnr* in *brk* mutants (Fig. 1D), but the patch expanded to a lesser degree than the stripes to only ~40% of the circumference, whereas the stripes expanded through the ventral neuroectoderm (Fig. 2H,I). This explains why, in the *brk* mutant, the expanded *pnr* stripes become more evident. These results indicate that the *pnr* pattern comprises two subpatterns of a dorsal patch and six stripes that are regulated by different factors. Dpp activates the patch, whereas AP factors are likely to direct the precise location of the stripes (see below). Interestingly, Brk represses both patterns in the ventrolateral region.

Smad sites are crucial for *pnr* patch expression

In order to identify Smad binding sites in the P3 and P4 enhancers, we performed DNA binding assays (gel shifts) with five overlapping DNA fragments (I-V; Fig. 2B) and recombinant GST-fused Mad (GST-Mad) and GST-fused Medea (GST-Medea) proteins. Two fragments (II and III) spanning 350 bp in the P3 region were able to bind Mad and Medea, whereas the fragments in P4 showed no binding (data not shown). This is consistent with the genetic analysis of P3 and P4 showing that only P3 is Dpp responsive. To search for specific sequences responsible for Smad binding, we performed gel shift assays with ten overlapping oligonucleotides (a-j) covering most of fragments II and III within P3 (Fig. 3A; binding summarized in Fig. 2B as blue ovals). Strong Mad binding was observed with oligonucleotide f (Fig. 3A), which contains the sequence GGCGCC (Fig. 3B, labeled as M3), resembling the GC-rich *Drosophila* Mad consensus sequence (DSC in Fig. 3B) GCCGnCG (Kim et al., 1997; Xu et al., 1998). Strong

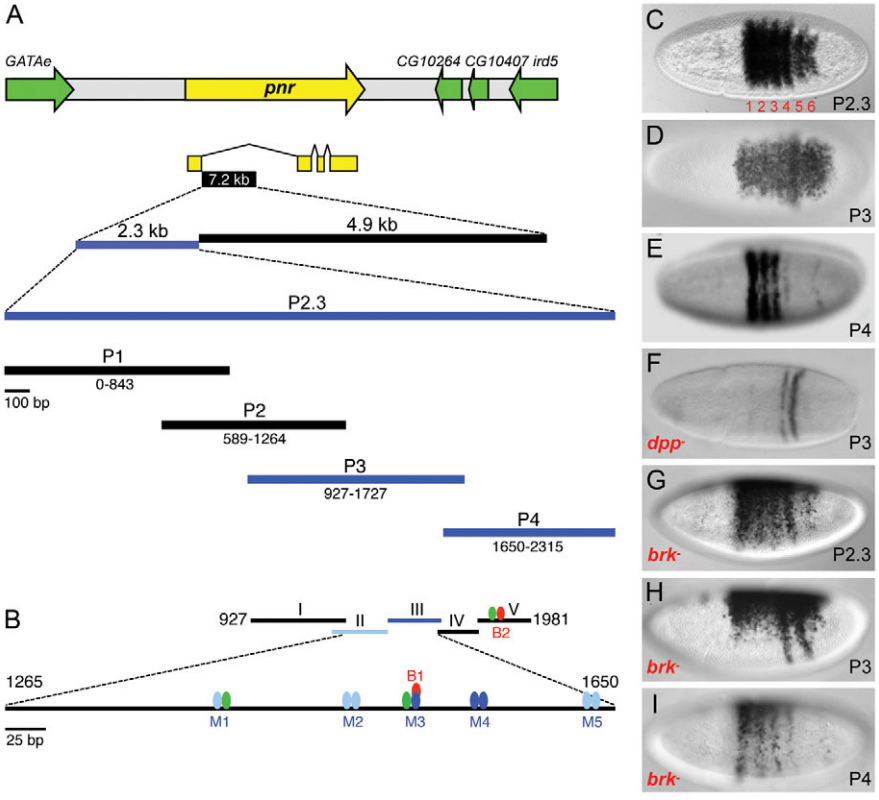

Fig. 2. Dissection of the *pnr* enhancer reveals a composite expression pattern comprising a dorsal patch and six AP stripes. (**A**) A segment of *Drosophila* chromosome 3R including *pnr* (yellow) and surrounding genes (green). DNA fragments mapping to the first intron of the *pnr*-α transcript were tested in transgenic reporter (*lacZ*) assays. Fragments that drove blastoderm-specific expression are represented in blue. Numbering is with respect to nucleotide position within the 2.3 kb fragment. (**B**) Map of *pnr* fragments (I-V) used in DNA binding assays. Blue ovals and lines denote Smad binding sites (strong, dark blue; weak, light blue); red ovals, Brk binding sites; green ovals, Zen binding sites. (**C-I**) Dorsal (C-F) or lateral (G-I) views of wild-type (C-E) and mutant (F-I) blastoderm embryos hybridized with *lacZ* probe. The 2.3 kb fragment (P2.3; genome coordinates 11852900-11855219, dm3) was capable of activating *lacZ* expression in a pattern identical to the endogenous *pnr* pattern in blastoderm embryos (C). Fragments P3 (D) and P4 (E) drove subsets of the *pnr* pattern. Expression driven by P3 is Dpp dependent (F). The stripes (G-I) and dorsal patch (G,H) expand ventrally in *brk*$^{-}$.

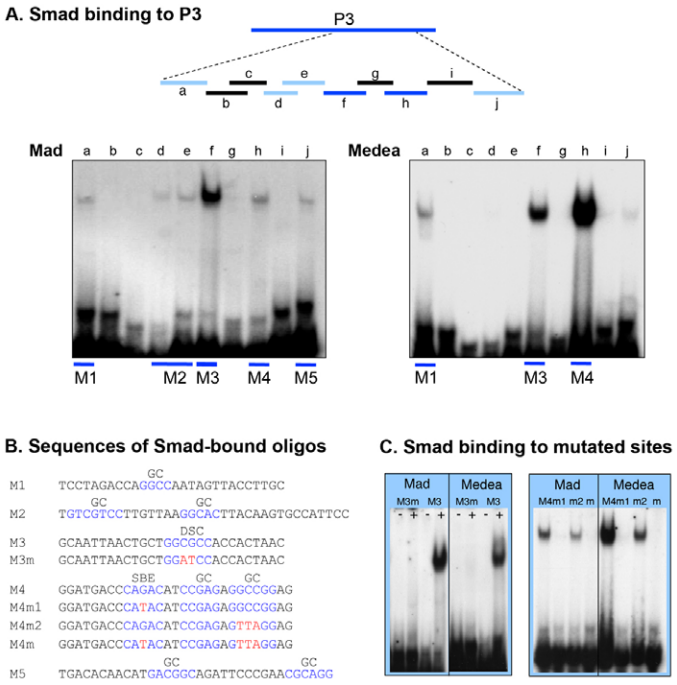

Fig. 3. Smads bind to the *pnr* enhancer. (**A**) Oligonucleotides (a-j) comprising the P3 fragments II and III were tested for binding with GST-Mad and GST-Medea in gel shift assays. Bound oligonucleotides were further designated M1-M5. (**B**) DNA sequence of M1-M5 with putative Smad binding sites labeled in blue. DSC, *Drosophila* Smad consensus site; SBE, Smad binding element; GC, GC-rich. Oligonucleotides with nucleotide changes (red) in the strongest Smad binding sites (M3m, M4m1, M4m2 and M4m) were tested in gel shift assays. (**C**) M3m and M4m were not capable of binding Smads. +, with protein as indicated; –, no-protein control.

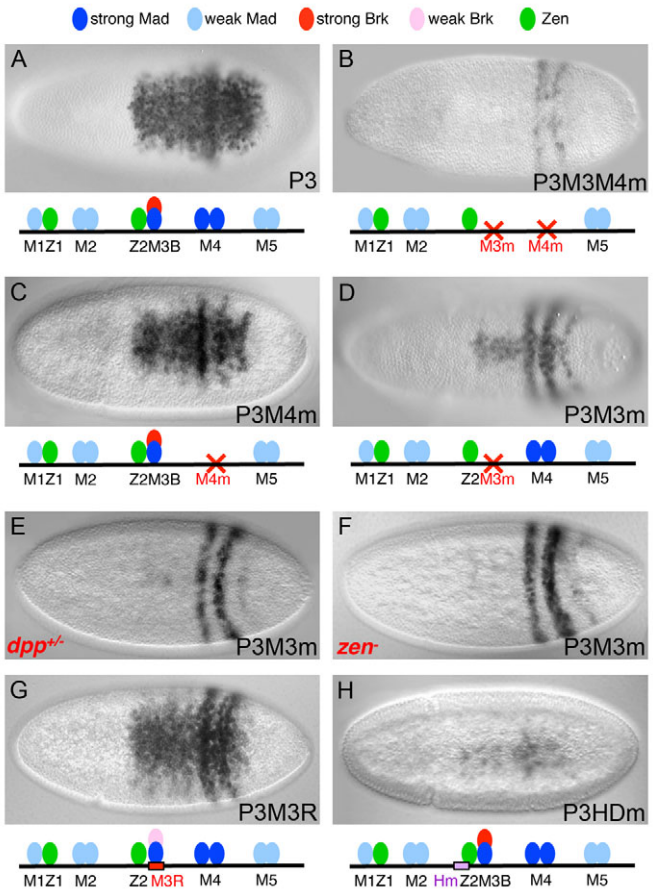

Fig. 4. Two Smad sites and an adjacent HD site are crucial for *pnr* patch expression. Dorsal views of transgenic *Drosophila* embryos hybridized with *lacZ* probes carrying wild-type (A) and altered (B-H) P3-*lacZ* transgenes. Summaries of binding site alterations are given beneath (sequences as in Fig. 3B). (**A**) The wild-type P3 enhancer drove a *lacZ* expression domain that was 30-35 cells wide. (**B**) Mutating both the M3 and M4 Smad binding sites abolished expression. (**C**,**D**) Mutation of the M3 site caused a dramatic narrowing of the patch domain width to 5-10 cells (D), whereas mutation of M4 caused a moderate narrowing to 25 cells (C). The stripes expanded only when the M3 site was altered (B,D), as M3 contains a Brk site, which was also altered. (**E**,**F**) Expression of P3M3 disappeared in dpp^{H46} heterozygous embryos (E) and zen^{w36} embryos (F) indicating that P3 without the M3 site behaves like a high-level Dpp target. (**G**,**H**) Replacement of the M3 site with a Smad site from the *Race* enhancer had little effect on the *lacZ* pattern (G, see sequence in Fig. 5B), whereas mutation of the HD site that lies adjacent to the M3 Smad site caused a severe reduction and narrowing of *lacZ* expression (H, see sequence in Fig. 5G).

Medea binding was observed with oligonucleotide h, which contains a sequence matching the Smad binding element (SBE in Fig. 3B) CAGAC (Zawel et al., 1998), as well as a GC-rich sequence (GC in Fig. 3B).

Two Brk binding sites, B1 and B2, located in the P3 and P4 enhancers, respectively, were identified by DNase I protection (footprinting) assays (supplementary material Fig. S1; summarized in Fig. 2B as red ovals) and confirmed by gel shift (see Fig. 5F). Both sites contain the Brk recognition core GGCGC (Kirkpatrick et al., 2001; Rushlow et al., 2001; Zhang et al., 2001). B1 is a stronger Brk site than B2, probably because it contains two overlapping Brk core sites in opposite orientation (GGCGCC). Importantly, the B1 site overlaps with the Smad M3 site (Fig. 3B), suggesting that Mad-*P* emanating from the dorsal side and Brk from the ventral region (Jaźwińska et al., 1999a) set the border of the *pnr* patch by competing for binding to the *pnr* enhancer, as has been shown for other Dpp targets (Sivasankaran et al., 2000; Kirkpatrick et al., 2001; Rushlow et al., 2001; Zhang et al., 2001).

To determine whether the Smad binding sites are important for *pnr* activation, mutations were introduced into the two strongest Smad sites M3 and M4. We first tested oligonucleotides containing mutated Smad sites in gel shift assays. Mutation of M3 (Fig. 3B, M3m) abolished Smad binding (Fig. 3C). For M4, we tested oligonucleotides that altered the SBE site (M4m1), the GC-rich site (M4m2) or both (M4m). M4m1 greatly reduced Smad binding, whereas M4m2 has only a slight effect; mutating both sites (M4m) abolished all in vitro Smad binding (Fig. 3C).

Since only the patch is Dpp responsive, we tested the in vivo relevance of the Smad site mutations in the context of the P3-*lacZ* reporter gene. Surprisingly, eliminating the two strong Smad sites M3 and M4 caused complete loss of the patch pattern (Fig. 4B), similar to what happens to P3 in dpp^{-} (Fig. 3F), but the two posterior stripes remained, further verifying that the stripes are regulated by different cues. The ventral expansion of these two stripes can be explained by a lack of direct Brk repression, as mutation of the M3 site also abolished Brk binding.

Mutation of the M3 and M4 sites individually caused different effects on the patch pattern, particularly on its DV width. Compared with the wild-type P3 pattern (Fig. 4A), the M4m mutation resulted

in a slightly narrower patch (Fig. 4C), whereas the M3m mutation resulted in a very narrow patch domain (Fig. 4D) that resembled the pattern of *Race*. Hence, by abolishing a single Mad site, we converted a low-level target into a high-level target. We further examined this idea by assaying P3M3m-*lacZ* in different mutant backgrounds. Expression of the patch pattern was absent in dpp^{H46} heterozygotes (Fig. 4E) and zen^{w36} homozygotes (Fig. 4F), indicating that P3M3m-*lacZ* responds only to high levels of Dpp [*Race* expression disappears in such mutants (Xu et al., 2005)]. These results suggested that Smad sites collectively contribute to the broad domain of *pnr*, but that the M3 site is crucial.

The structure of the *pnr* enhancer resembles that of the high-level Dpp target *Race*

The dramatic effect of mutating the M3 site suggested that a single Smad site might mediate the response to low levels of Dpp. One possibility is that M3 is a high-affinity Smad site that is capable of binding Smads when present in low concentrations. To address this, we first compared the enhancer structure of *pnr* with those of *Race* and *C15*, all of which give different expression boundaries. To our surprise, the *Race* and *pnr* enhancers had a similar number of Smad sites (Fig. 5A,B), whereas the *C15* enhancer contained many more Smad sites spread over a broad region (Fig. 5A). We next performed gel shift assays with DNA fragments of the *Race*, *C15* and *pnr* enhancers (Fig. 5C) and oligonucleotides containing individual Smad sites (Fig. 5D). Unexpectedly, the strongest Smad sites in the *pnr* enhancer, M3 and M4, showed a similar degree of binding to Smads as sites in the *Race* enhancer (Fig. 5D).

To further compare Smad sites, we replaced the Smad site in the M3 oligonucleotide with that from the *Race* enhancer (M3R; sequences shown in Fig. 5B). Competitive gel shift analysis showed that M3 and M3R had similar Smad binding affinities (Fig. 5E). Importantly, swapping the M3R site for M3 in the P3-*lacZ* transgene had little effect on patch expression (Fig. 4G); however, the posterior stripes expanded, a likely result of the observed decreased binding of Brk to M3R (Fig. 5F). Based on these results, the affinity threshold model is not sufficient to explain the broad expression domain of *pnr*, as there is no correlation between Smad binding affinity and the extent of the *Race* and *pnr* expression domains.

A conserved DNA sequence is required for broad *pnr* expression

The fact that responsiveness to the Dpp gradient does not depend on differential Smad binding raised the possibility that other factors might be involved; for instance, a co-factor might interact with Mad to enhance Smad binding to M3 in vivo. A study of the BMP-responsive enhancer of *Msx2*, a mammalian homolog of *tinman*, revealed that the sequence AGCAATTAA, which includes a consensus site (AATTAA) for Antennapedia (Antp) homeodomain (HD) factors, lies adjacent to a Smad site (CGGCTCCGGC), and

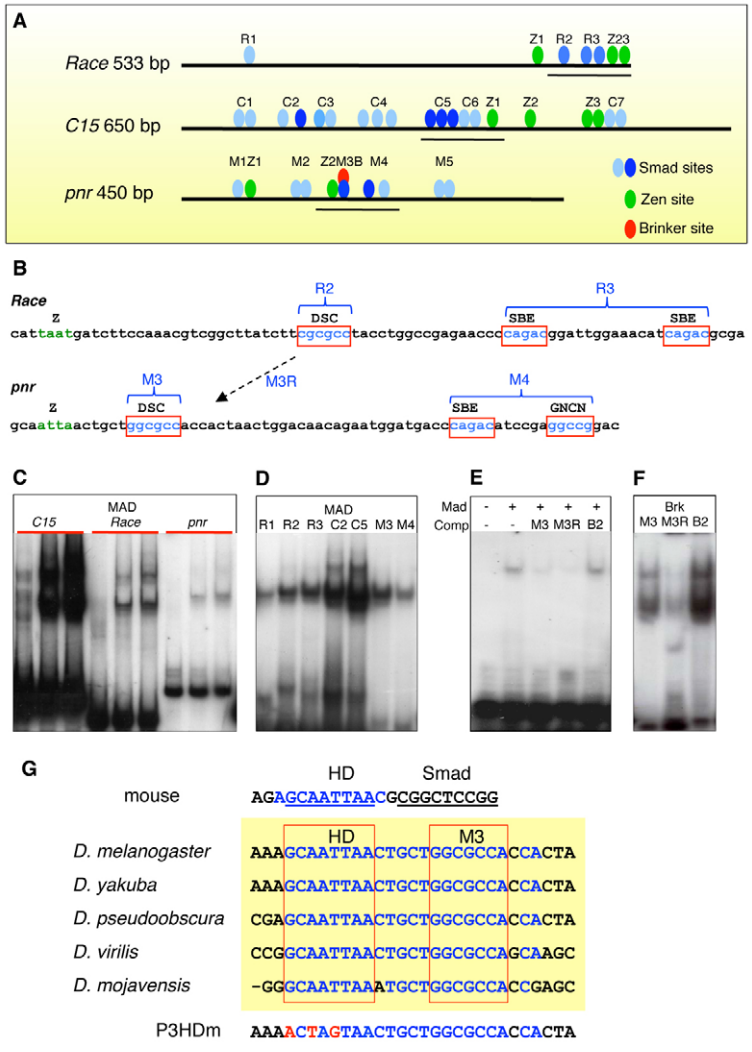

Fig. 5. The *pnr* enhancer structure is similar to that of *Race*. (**A**) Summary of Smad and Zen sites in the *Race* [R1 (Wharton et al., 2004); R2 and R3 (Xu et al., 2005)], *C15* (Lin et al., 2006) and *pnr* enhancers. The bar indicates the 100 bp fragment used in gel shift assays in C. (**B**) Comparison of DNA sequences and organization of functional Smad sites in the *Race* and *pnr* enhancers. Arrow designates the site from *Race* that was swapped into P3 (P3M3R). (**C-F**) Gel shift analysis of 100 bp fragments (C) and oligonucleotides (D-F) containing Smad sites from the different enhancers as indicated. (C,D) The Smad binding affinities of the *pnr* and *Race* fragments are similar. (E) Unlabeled M3 or M3R can compete with labeled M3 for Mad binding, suggesting that M3R has the same binding capability as M3. B2, an oligonucleotide that contains the Brk site from the P4 enhancer, was used as a negative control and was unable to compete with M3. (F) Brk binding to M3R was weaker than to M3. (**G**) The HD site, which has been shown to be essential for the BMP responsiveness of the mouse *Msx2* enhancer, is conserved between mouse and *Drosophila* species.

that both parts of this 'bipartite' motif are required for BMP responsiveness in mouse embryos (Brugger et al., 2004). Intriguingly, the exact same sequence AGCAATTAA lies adjacent to the M3 site (Fig. 5G). Comparison of this bipartite motif in the *pnr* gene among *Drosophila* species showed that these nine nucleotides are highly conserved (Fig. 5G) and, importantly, are not present in the enhancers of *Race* or *C15* (Fig. 5B; data not shown).

Introducing a mutation into the bipartite site (Fig. 5G, P3HDm) in the P3-*lacZ* reporter gene resulted in a dramatically altered expression pattern that was sporadic and narrow (Fig. 4H). Thus, as in the case of the mouse *Msx2* enhancer, both the Smad site and the HD site of the *pnr* bipartite motif are essential for proper response to Dpp.

AP factors regulate the *pnr* stripe pattern

The six stripes of *pnr* comprise 20-60% egg length (Fig. 1C) (Winick et al., 1993). The anterior-most stripe overlaps with *eve* stripe 2 and the posterior-most stripe abuts *fushi tarazu* (*ftz*) stripe 7; thus, *pnr* extends from the labial head segment through the entire abdomen (Herranz and Morata, 2001). Since pair-rule stripe borders are established by gap repressor proteins, which are localized in specific domains across the AP axis, we analyzed *pnr* expression in *bicoid* (*bcd*) and gap gene mutants (Fig. 6). The *pnr* pattern was affected in all of the mutants that we tested [*bcd*, *hunchback* (*hb*), *knirps* (*kni*), *Kruppel* (*Kr*), *giant* (*gt*) and *tailless* (*tll*)] and the changes correlated with what is known about the function of these genes and their cross-regulatory interactions (Fig. 6H). More specifically, *pnr* expression expanded anteriorly in *bcd*, *hb* and *gt* mutants (Fig. 6B-D), which was likely to be due to a lack of anterior Hb and Gt repression (Clyde et al., 2003). Likewise, *pnr* expanded posteriorly in *tll*$^{-}$ (Fig. 6G) due to a lack of posterior Tll repressor activity (Moran and Jimenez, 2006). Note that weak derepression of *pnr* also occurs in the head region in *tll*$^{-}$. The lack of mid-body region repressors in the *Kr*$^{-}$ and *kni*$^{-}$ mutants caused shifting and/or expansion of gap domains (Kosman and Small, 1997); thus, both direct and indirect effects alter the middle *pnr* stripes in these mutants (Fig. 6E,F).

Results from these genetic experiments, together with genome-wide binding data in which all AP factors that we tested except Bcd were found to bind to the *pnr* enhancer (Li et al., 2008; MacArthur et al., 2009), and with binding site predictions using ClusterDrawII (Papatsenko, 2007) across P3 and P4 (not shown), strongly suggest that the gap genes establish the AP limits of the *pnr* domain and the stripes within the domain.

DISCUSSION

Our studies on the low-level Dpp target gene *pnr* revealed that: (1) the *pnr* expression pattern is a composite of a dorsal patch and six AP stripes; (2) the *pnr* patch depends on Dpp, although we predict that additional co-factors that bind adjacent to Smads enable the *pnr* enhancer to respond to low levels of Dpp, leading to the unique broad domain of *pnr*; (3) Brk repression establishes the ventral border of both the patch and stripe expression domains and thus Brk shapes the *pnr* expression domain on the DV axis; (4) *pnr* expression is precisely positioned along the AP axis by the segmentation gap genes.

The *pnr* enhancer integrates spatial cues from diverse patterning systems

Most blastoderm genes are regulated primarily on either the DV or AP axis. For example, the gap genes are expressed in one or two domains of expression along the AP axis and, although some of them may exhibit regulation along the DV axis, they are nonetheless considered AP genes. *pnr* represents an interesting case because although it was originally reported as a DV gene (Jaźwińska et al., 1999a; Ashe et al., 2000), closer inspection of its expression pattern in wild-type and mutant embryos and detailed dissection of its cis-regulatory enhancers revealed that *pnr* is highly regulated by both AP and DV genes (summarized in Fig. 7). Its pattern is a composite of two superimposed patterns that each exhibit AP and DV spatial regulation: a dorsal patch and six AP

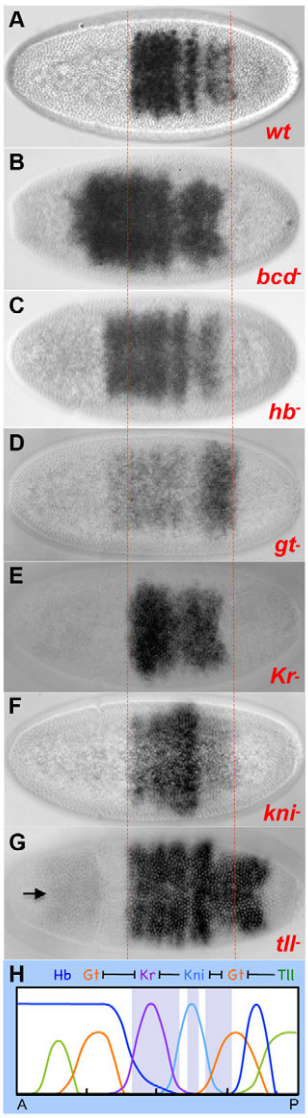

Fig. 6. Gap genes regulate the expression pattern of *pnr*. Dorsal view of embryos in wild-type (A) and different mutant (B-G) backgrounds hybridized with *pnr* probe. Red dotted lines delimit the anterior (left) and posterior (right) borders of the *pnr* expression domain. (**A-D**) Compared with wild type (A), in *bcd*, *hb* and *gt* mutants the anterior border extends anteriorly. (**E**) In the *Kr* mutant, *pnr* was slightly compressed in the DV axis and became less stripey, probably owing to expansion of Gt and Kni in the *Kr* mutant. (**F**) In the *kni* mutant, stripes 3 and/or 4 were expanded. (**G**) In the *tll* mutant, the posterior domain of *pnr* extended toward the posterior end. The arrow indicates anterior *pnr* expression. (**H**) Representation of the expression of gap genes along the AP axis. Purple rectangles represent the *pnr* stripe domains.

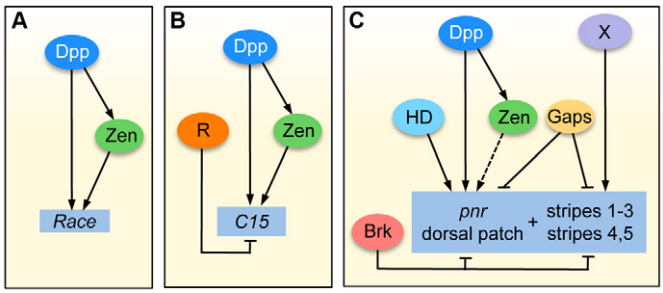

Fig. 7. Comparison of the gene networks that regulate the Dpp target genes *Race*, *C15* and *pnr*. (**A**,**B**) Dpp first activates *zen* expression, then Smads and Zen bind directly to the *Race* (A) and *C15* (B) enhancers to activate their transcription in regions of high-level and intermediate-level Dpp, respectively. A repressor (R) is required to set the *C15* border of expression (B). (**C**) Low levels of Dpp in the dorsolateral region function with other activators, including a putative homeodomain factor, to activate *pnr* in a patch domain, while Brk repression establishes the ventral border of *pnr* expression. The *pnr* stripes are also repressed ventrally by Brk, but the activators (X) remain unknown. Gap genes (Gaps) establish the *pnr* patch and stripe domains along the AP axis. Dashed line indicates possible activation by Zen.

stripes, which are limited to the dorsal 30% of the embryo (Fig. 1A). The patch domain, but not the stripes, disappeared in *dpp* mutants (Fig. 1B), whereas both the patch and stripes expand ventrally in the absence of Brk (Fig. 1D). The stripes are more sensitive to Brk repression because activation of the patch domain is limited to the region where Dpp is present dorsally, whereas the stripes can be activated along the entire DV axis. Brk in the ventrolateral region and Sna in the ventral-most region repress stripe expression (Fig. 1F) (Jaźwińska et al., 1999a). Since *pnr* specifies dorsomedial fates, restricting its expression to the dorsal 30% of the circumference is crucial. Ectopic expression of *pnr* ventrally causes transformation of ventral epidermis into dorsomedial epidermis (Herranz and Morata, 2001).

Competition between Brk and Smads for binding to overlapping DNA sequences is likely to set the border of the patch domain. Two Smad sites are particularly important for patch expression (Fig. 4B), and one of these, the M3 site, is a composite site that binds both Brk and Smads (see Fig. 2B and supplementary material Fig. S1), raising the possibility that the patch border is established by competition between activating inputs from Smads in the dorsal region and repressive inputs from Brk emanating from the ventral region. Competition between Brk and Smads for overlapping binding sites has been observed for several Dpp target enhancers (Sivasankaran et al., 2000; Kirkpatrick et al., 2001; Rushlow et al., 2001; Zhang et al., 2001).

Repression of the AP stripes ventrally requires both Brk sites B1 and B2 (Fig. 2B, red ovals). The two posterior stripes driven by P3 expand to a lesser degree (Fig. 2D) than the four anterior stripes driven by P4 (Fig. 2E). This can be explained by the fact that P4 lacks Brk site B1, which is a stronger Brk site. Loss of both Brk sites would likely result in expansion to the edge of the mesoderm, as seen in embryos that lack Brk protein (Fig. 1D; Fig. 2G-I). Repression by Sna is likely to involve the Sna binding sites in the *pnr* enhancer, as genome-wide binding studies have shown that the *pnr* enhancer is bound by Sna (summarized in Fig. 6G) (Zeitlinger et al., 2007).

The positioning of the stripes, as well as of the patch, along the AP axis is regulated by the gap genes. Our results suggest that Hb, Gt and Tll set the anterior edge of the *pnr* domain, whereas Tll sets the posterior, and that direct and indirect interactions among the gap proteins establish the stripe borders relative to one another, as has been observed for *eve* (Clyde et al., 2003). For example, the broad central stripe seen in *kni*$^{-}$ (Fig. 6F) could be explained by the lack of direct Kni repression. However, owing to the complex cross-regulatory interactions among the gap genes, it is difficult to predict which gap proteins regulate the *pnr* stripes directly, although genome-wide binding data of the gap factors (Li et al., 2008; MacArthur et al., 2009) support their direct binding to the *pnr* enhancer. Although Bcd does not appear to bind directly to the *pnr* enhancer, its effects are mediated through its targets Gt and Hb.

Dpp target genes utilize multiple mechanisms to set their domains of expression

In depth studies of three genes with different boundary positions in the dorsal region, *Race* (Xu et al., 2005), *C15* (Lin et al., 2006) and *pnr* (this study), indicate that complex combinatorial mechanisms are employed to establish their expression domains, with each gene having a unique regulatory network of its own. Although they all respond to Dpp signaling, their borders of expression are not set by a simple threshold response to the Dpp gradient that depends on differential binding site affinity.

The feature that has been shown to be important for high-level Dpp target expression is the feed-forward motif involving Dpp and Zen (Xu et al., 2005). High levels of Dpp/Smads first activate *zen* expression in the dorsal-most region, the presumptive amnioserosa, and then both Zen and Smads bind and activate the *Race* enhancer (Fig. 7) (Xu et al., 2005). The intermediate-level target *C15* has a different enhancer structure than high-level targets, containing many Smad sites that act in a cumulative manner to drive expression in regions of intermediate Dpp levels. Mutation analysis has shown that the number of intact Smad binding sites, rather than their affinity, is important for the *C15* response (Lin et al., 2006). Nevertheless, the enhancer structure of *C15* might promote high levels of Smad binding in vivo, and this may increase the response to Dpp. Do all intermediate-level Dpp targets have a similar enhancer structure? We examined the enhancer that drives expression of the intermediate-level Dpp target gene *tup* (Zeitlinger et al., 2007) for putative Smad binding sites (SBEs and GC-rich regions), and observed multiple Smad sites across the enhancer (data not shown), similar to that seen with *C15*. Thus, the multiple Smad site signature might be necessary for response to lower than peak levels of Dpp. In addition, intermediate-level targets may utilize repression mechanisms to help establish their borders of expression, as was shown for *C15* (Fig. 7) (Lin et al., 2006).

Our studies here revealed that the *pnr* enhancer resembles that of a high-level target in Smad site organization and Smad binding site affinity (Fig. 5A,B). In fact, it was surprisingly easy to convert the low-level target enhancer into a high-level target by mutating a single Smad site (Fig. 4D). This result could be easily explained if the M3 site had a higher affinity for Smads than those in *Race*; however, comparison of the binding sites by gel shift showed they have similar affinities (Fig. 5D). Furthermore, replacing the M3 Smad site with a *Race* Smad site had little effect on the expression pattern (Fig. 4G). These results suggest that activation of *pnr* in its broad domain has little to do with Smad binding affinity. How then does *pnr* respond to low levels of Dpp? One possible mechanism involves the highly conserved AGCAATTAA site that lies adjacent to the Smad sites (Fig. 5G). In the absence of this site, the P3 enhancer could not respond to low-level Dpp (Fig. 4H). It is possible that this site, when bound, leads to greater Smad binding, which would then promote *pnr* activation.

What factor(s) might bind to the AGCAATTAA site? ATTA is the core binding site for Antp class HD proteins. Although Zen binds to the ATTA site in vitro (supplementary material Fig. S1), neither the endogenous *pnr* pattern (Fig. 1C) nor P3-*lacZ* expression (data not shown) is significantly affected in *zen* mutants. To identify candidate factors, we used the TOMTOM tool (Gupta et al., 2007) at FlyFactor Survey (Zhu et al., 2011), and the best match was to the HD protein Hmx, which binds CAATTAA. However, *Drosophila* Hmx is expressed only in an anterior region that does not overlap with *pnr* (see FlyBase). Likewise, although several Antp class HD proteins were predicted to bind to the ATTA core sequence, their timing or domains of expression do not overlap ideally with those of *pnr*.

Brugger et al. (Brugger et al., 2004) proposed that the AGCAATTAA site in the *Msx2* enhancer might bind a factor in addition to an HD protein via the 5′ half of the site, perhaps a transcriptional partner such as FAST1, which was previously shown to function with Smads (Chen et al., 1996). Although our search did not reveal any candidates, if this is the case for *pnr* then the bipartite motif could potentially bind four proteins: Smads, Brk, HD and 'partner X'. The combination of these proteins in a given cell along the DV axis would determine *pnr* transcriptional activity. The fact that the bipartite motif is not present in the enhancers of *Race* or *C15*, or in the other *pnr* enhancers we identified, demonstrates the versatility of how Dpp uses different partners to establish multiple target gene domains.

Lessons from studying Dpp target gene regulation

Is the structure of the *pnr* enhancer typical for low-level Dpp targets? This is difficult to address owing to the lack of candidate low-level Dpp targets. Brk is considered a low-level Dpp target in imaginal disc development; however, Dpp represses *brk*, giving rise to a reciprocal gradient of the Brk repressor (Jaźwińska et al., 1999b; Pyrowolakis et al., 2004). Target gene borders are thus established by competition between Smad and Brk for overlapping binding sites, as mentioned above for *pnr*. The *brk* enhancer contains multiple enhancer/silencer modules consisting of activator and repressor (Mad/Medea/Schnurri) binding sites, which contribute to threshold responses to the Dpp gradient (Yao et al., 2008), and thus it does not resemble the *pnr* enhancer. Although we have made good progress in understanding how *pnr* is expressed in regions with low levels of Dpp, learning the general rules that control broad dorsal patterns will require the analysis of more enhancer elements.

What rules do target genes for other morphogens follow? Long before the 'feed-forward' term was coined (Shen-Orr et al., 2002; Lee et al., 2002) it was shown that both the Dl and Bcd morphogens interact with their high-level targets, Twi and Hb, respectively, to activate downstream targets (Jiang et al., 1991; Ip et al., 1992; Small et al., 1992); thus, combinatorial motifs are generally utilized. Moreover, as more target genes of Dl and Bcd were identified and studied, it became apparent that the affinity threshold model could not explain all cases of differential response to the gradient. For example, analysis of several enhancers that drive Bcd-dependent expression in anterior regions of the embryo revealed a poor correlation between Bcd binding site affinity and the AP limits of the pattern (Ochoa-Espinosa et al., 2005). Also, although Dl targets remain archetypal examples of genes that utilize the affinity threshold mechanism, it was found that genes expressed in the lateral region also require input from the Zelda (Vielfaltig – FlyBase) transcription factor for expression in regions of low-level Dl (Nien et al., 2011). Zelda binding sites are present in target enhancers, and it was proposed that Zelda boosts Dl binding to help activate the neuroectodermal genes (Nien et al., 2011).

Downstream target gene interactions also shape domains of expression, in particular cross-repression among the targets. In both the *Drosophila* neuroectoderm and the vertebrate neural tube, morphogen targets are expressed in discrete domains rather than nested overlapping domains due to the repression of one target by another (reviewed by Dessaud et al., 2008; Ashe and Briscoe, 2006). This mechanism establishes sharp boundaries among the target genes.

Thus, it is clear that additional factors help morphogens set threshold responses. Given that the *pnr* enhancer could potentially interact with four different factors along the DV axis and at least four factors along the AP axis, several combinations of inputs could regulate other Dpp target genes. More generally, depending on the number of different factors that interact with the cis-regulatory regions of target genes, morphogen gradients could elicit multiple threshold responses, as has been seen for morphogens such as Dl in *Drosophila*, Activin in the *Xenopus* blastula (Green et al., 1992) and Shh in the vertebrate neural tube (Ericson et al., 1997), where up to seven threshold responses have been described. Only by dissecting enhancers can we fully understand how target genes integrate diverse inputs.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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