Inverse regulation of target genes at the brink of the BMP morphogen activity gradient

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Summary

BMP-dependent patterning in the *Drosophila melanogaster* wing imaginal disc serves as a paradigm to understand how morphogens specify cell fates. The observed profile of the transcriptional response to the graded signal of BMP relies upon two counter-active gradients of pMad and Brinker (Brk). This patterning model is inadequate to explain the expression of target genes, like *vestigial* and *spalt*, in lateral regions of the wing disc where BMP signals decline and Brk levels peak. Here, we show that in contrast to the reciprocal repressor gradient mechanism, where Brk represses BMP targets in medial regions, target expression in lateral regions is downregulated by BMP signalling and activated by Brk. Brk induces lateral expression indirectly, apparently through repression of a negative regulator. Our findings provide a model explaining how the expression of an established BMP target is differentially and inversely regulated along the anterior-posterior axis of the wing disc.

Key words: Brinker, Dpp, Morphogens, Spalt, Wing patterning

Introduction

During development of the *Drosophila* wing imaginal disc, Dpp, a member of the BMP superfamily, spreads in a graded manner from its expression domain along the A–P compartment boundary. Dpp functions together with another BMP family member, Glass bottom boat (Gbb) that is uniformly distributed across the A–P patterning field (Haerry et al., 1998). Consequently, a medial to lateral gradient of BMP signalling is generated that is ultimately responsible for proper patterning and growth of *Drosophila* adult wing (Entchev et al., 2000; Lecuit et al., 1996; Nellen et al., 1996; Schwank et al., 2008; Teleman and Cohen, 2000; Wartlick et al., 2011). The BMP activity gradient leads to differential activation of the Punt–Thickveins (Tkv) kinase receptor complex, which propagates the signal via phosphorylation of Mothers against dpp (Mad) (Kim et al., 1997; Newfeld et al., 1997). Phosphorylated Mad (pMad) along with its binding partner Medea (Med) translocates to the nucleus and regulates transcription. In fact, most BMP-induced target genes are not directly regulated by the pMad-Med complex, but are indirectly induced through repression of a transcriptional repressor Brk (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Minami et al., 1999). At the molecular level, upon BMP signalling, the pMad–Med complex binds to silencer elements in the *brk* locus and recruit the zinc-finger protein Schnurri (Shn), which confers repressive activity to the complex (Marty et al., 2000; Torres-Vazquez et al., 2000). As a result, an inverse, i.e. lateral to medial, nuclear gradient of Brk emerges, which in turn negatively regulates the expression of different BMP targets above distinct threshold concentrations (Müller et al., 2003). Brk represses BMP target genes via direct binding to the sequence GGCGYY in their regulatory regions. These targets include *spalt* (*sal*), *optomotor-blind* (*omb*) and *vestigial* (*vg*) (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Kim et al., 1997; Minami et al., 1999), and have nested expression domains centred around the high point of the BMP activity gradient at the centre of the wing disc. This nested expression pattern, which forms the basis of the threshold model of BMP/Brk target gene regulation, is explained by a differential sensitivity to Brk, with *sal* being repressed by very low levels of Brk, while repression of *vg* requiring higher levels. However, the fact that a high threshold BMP target such as *sal* is also expressed in lateral regions (Fig. 1A), where Brk is at its maximal levels, prompted us to re-examine the morphogen gradient model. Unexpectedly, our data revealed that in lateral regions of the wing disc both *sal* and *vg* are negatively regulated by BMP signalling and positively by Brk. By studying the regulation of *sal* expression in different regions of the wing disc, we identify a new mechanism where Brk induces the expression of a classic BMP target through an enhancer that contains neither Brk nor pMad-Med-Shn complex binding sites. Brk appears to induce the lateral expression of *sal* indirectly through repression of a negative regulator. Our analysis provides a working model explaining how the activities and mutual interactions of pMad, Brk and the new negative regulator of sal (NRS) differentially and inversely regulate *sal* expression along the anterior-posterior axis of the wing disc. Based on these position-specific, distinct and inverse outcomes of BMP/Brk-dependent patterning system, we subdivide the wing disc along the A–P axis into four regions (Fig. 1A).

Results

sal is negatively regulated by BMP signalling in lateral regions of the wing disc

Spreading of Dpp into both compartments establishes, with the help of uniformly expressed Gbb, a gradient of BMP signalling a

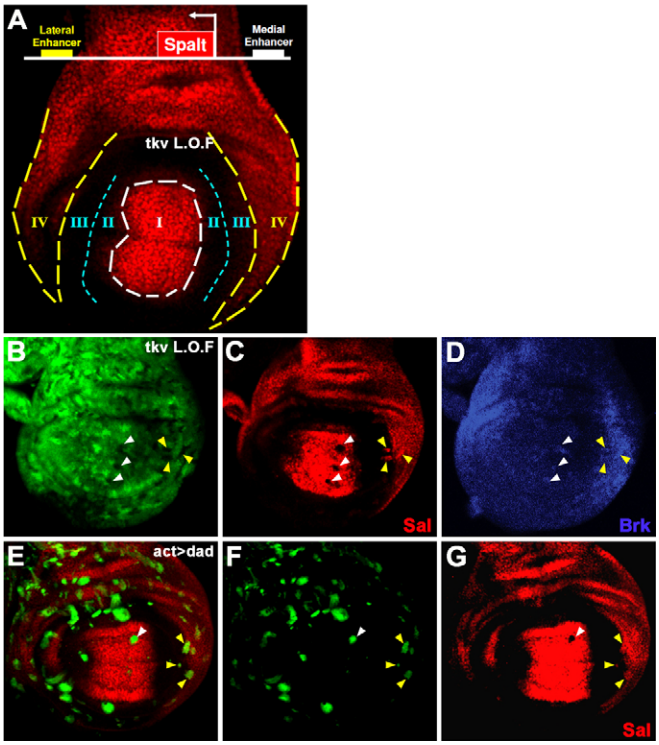

Fig. 1. Downregulation of BMP signalling has a position-specific influence on Sal expression in the wing disc. (**A**) Subdivision of the wing imaginal disc along the medial-lateral axis; region I is the centre of the presumptive wing blade (wing pouch), which coincides with high expression of Sal. The remaining area of the wing pouch was subdivided into two regions: region II, which abuts region I, and region III, which occupies the most lateral stripe of the wing pouch. Region IV is the most lateral region of the wing disc, which corresponds to the presumptive wing hinge and pleura. Region IV coincides with expression of both high Brk and lateral Sal. A simplified map of the *sal* gene and part of its regulatory regions in the wing disc is shown in the upper part of the figure. (**B–D**) *tkv* loss of function (LOF) clones (marked by loss of GFP, B) in wing imaginal disc (anterior to the left, dorsal up, in all figures), abolish Sal expression (red, C) in medial regions of the wing disc (white arrowheads) but upregulate Sal in lateral regions of the wing disc (yellow arrowheads). Note that Brk expression is elevated in clones located both in medial and lateral regions (blue, D). (**E–G**) *dad* overexpressing clones (marked by GFP, E,F) reduce Sal expression (red, E,G) in medial regions (white arrowhead), but upregulate Sal in lateral regions (yellow arrowheads).

long the A–P axis with peak levels in the centre of the wing pouch that exponentially decline towards the periphery. Consequently, the BMP activity, as reflected in the pMad concentration, continually decreases from the centre of the wing pouch establishing a parallel exponentially decaying gradient (Bollenbach et al., 2008; Entchev et al., 2000; Teleman and Cohen, 2000). It is proposed that BMP signal can attribute precise positional information only within the range of the wing pouch (Bollenbach et al., 2008). Consequently, at the periphery of the wing disc, expression of the target genes, like sal, was attributed to BMP-independent regulation (Campbell and Tomlinson, 1999; de Celis et al., 1999).

We wished to examine whether BMP activity is indeed inconsequential at the periphery as proposed. We therefore generated clones of cells mutant for the BMP type I receptor, *tkv* (which is required for signal transduction of both Dpp and Gbb) (Bangi and Wharton, 2006; Khalsa et al., 1998) and monitored Sal expression across the entire patterning field. Consistent with previous studies, we found that Sal expression was abolished in the mutant clones located in the centre of the wing pouch (region I) (Fig. 1C, white arrowheads). Strikingly, however, Sal expression was ectopically upregulated in lateral regions of the wing pouch (region III) and even in the periphery of the wing disc (region IV) (Fig. 1C, yellow arrowheads). As another read-out for BMP signalling, we monitored Brk expression in the same *tkv* mutant clones, as BMP signalling activates genes by attenuating the *brk* repressor. Although low, upregulation of Brk expression was apparent in many of the *tkv* mutant clones, located at the periphery of the wing disc (region IV) (Fig. 1D, yellow arrowheads), indicating that BMP signalling is active all across the patterning field including at the periphery.

Similar results were obtained when we overexpressed Dad (Fig. 1E–G), an inhibitory Smad that interferes with BMP signal transduction at the level of Mad (Tsuneizumi et al., 1997). These results show that BMP signalling exerts opposite influence on *sal* expression in a region-specific fashion. In the medial regions of the wing disc BMP signalling positively regulates *sal* expression, whereas in lateral regions, where the BMP activity gradient declines (regions III & IV); it appears to actively repress Sal expression apparently via pMad activity.

It is important to note that both ligands Gbb and Dpp signal via the BMP downstream machinery components including the Tkv receptor and RSMAD family of transcription factor(s) (Bangi and Wharton, 2006; Khalsa et al., 1998). Thus, our experiments cannot distinguish between the relative contributions of Gbb and Dpp to BMP signalling and hence to regulation of target genes.

sal expression is regulated not only by Brk repression but also through direct activation by pMad-Med complex (Moser and Campbell, 2005). In order to reassess the effect of activation of BMP-dependent pMAD signalling on *sal* expression, we generated clones overexpressing Tkv^{QD}, a constitutively active receptor that phosphorylates Mad in a ligand-independent manner (Lecuit et al., 1996; Nellen et al., 1996). We found, as expected, that Sal expression was ectopically upregulated all over the wing pouch (regions I, II and III; Fig. 2C,E,I). However, in most of Tkv^{QD} overexpressing clones located in the periphery of the wing disc (region IV) the normal expression of Sal was strongly repressed (Fig. 2C,E), similar to Brk expression (Fig. 2K,L). By contrast, the expression of *nab*, a newly identified positive BMP target in the wing disc (Ziv et al., 2009), was ectopically upregulated in the same lateral clones (Fig. 2F) where Sal expression was repressed (Fig. 2E). Thus, in lateral regions of the wing disc (as opposed to medial regions) the pMad-Med complex appears to actively repress *sal* expression. It should be noted, however, that at times, Sal expression was either not reduced or even upregulated (Fig. 2A–C,K) in Tkv^{QD} overexpressing clones located in region IV (see below).

Brk positively regulates *sal* expression in lateral regions of the wing disc

The unexpected observation that loss of *tkv* in lateral regions of the wing disc (region III and IV) resulted in upregulation of both *brk* and *sal* raises the possibility that in these regions *sal*, like *brk*, is directly regulated by the pMad-Med-Shn repression complex. Alternatively, *sal* expression could be positively regulated by Brk. To address this, we generated loss of function (LOF) clones of *brk* and specifically compared the accumulation of Sal in

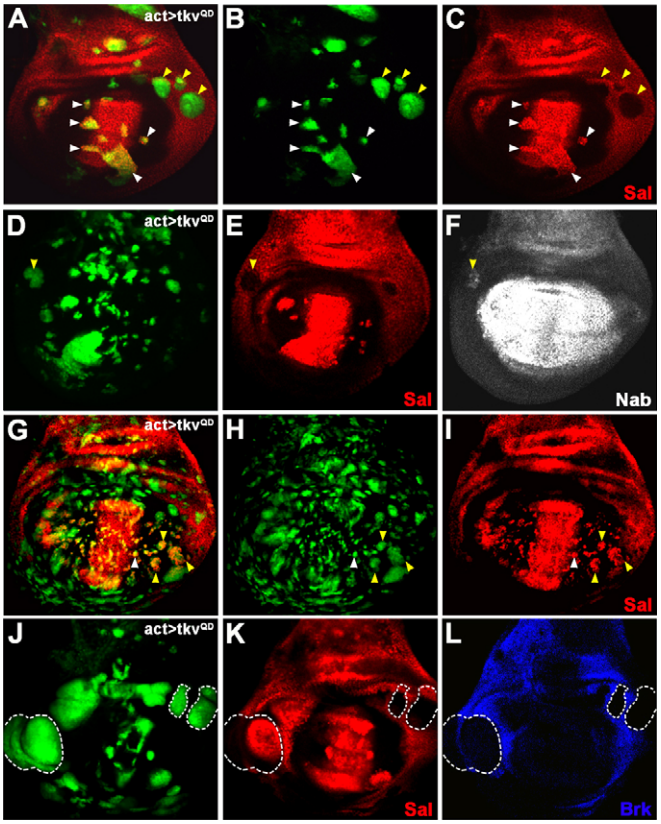

Fig. 2. Effect of activation of BMP signalling on Sal expression in the wing disc. (**A–C**) Clones overexpressing tkv^{QD} (marked by GFP, A,B) upregulate Sal expression (red, A,C) in medial regions (white arrowheads) but downregulate Sal in lateral regions (yellow arrowheads) of the wing disc. (**D–F**) tkv^{QD} overexpressing clone located in the periphery of the wing disc (marked by GFP, D, yellow arrowhead) abolishes Sal expression (red, E, yellow arrowhead), but upregulates Nab expression (grey, F, yellow arrowhead). (**G–I**) tkv^{QD} overexpressing clones (marked by GFP, G,H) upregulate Sal expression (red, G,I) across the wing pouch (regions II and III, arrowheads). (**J–L**) Brk expression (blue, L) is abolished in all tkv^{QD} overexpressing clones (marked by GFP, J). Sal expression (red, K) in lateral regions is either reduced or unaffected. The areas marked by the dashed lines represent tkv^{QD} clones in lateral regions.

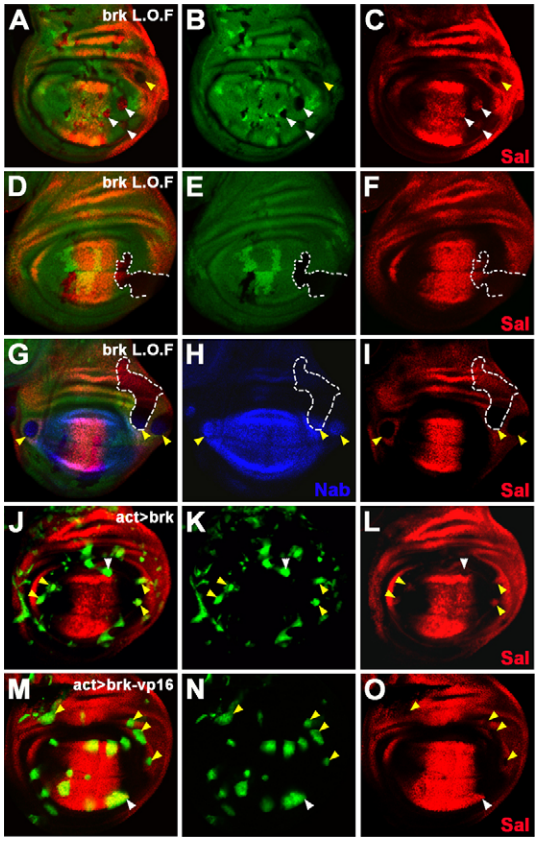

Fig. 3. Brk positively regulates Sal expression in lateral regions of the wing disc in an indirect manner. (**A–C**) *brk* LOF clones (marked by loss of GFP, A,B) upregulate Sal expression in medial regions (red, A,C, white arrowheads), but downregulate Sal in lateral regions (red, A,C, yellow arrowhead). (**D–F**) A large *brk* LOF clone (marked by loss of GFP, D-E, dashed line) upregulates Sal expression in the medial region (region II), does not affect Sal in medialateral region (region III) and downregulates Sal in the lateral region (region IV). (**G–I**) Nab expression (blue, G-H) is elevated in *brk* LOF clones (marked by loss of GFP, G), whereas Sal expression is abolished (red, G,I) in the same clones in the lateral regions (yellow arrowheads). Note that neither the expression of Sal nor Nab was affected by the removal of *brk* in the future notum region. (**J–L**) *brk* overexpressing clones (marked by GFP, J,K) downregulate Sal expression in medial regions (red, J,L, white arrowhead), but upregulate Sal in lateral regions (red, J,L, yellow arrowheads). (**M–O**) Clones overexpressing *brk-vp16* (marked by GFP, M,N) upregulate Sal expression in medial regions (red, M,O, white arrowhead), but downregulate Sal in lateral regions (red, M,O, yellow arrowheads).

clones in different regions of the wing disc. This strategy allowed us to assess the influence of Brk on *sal* expression without interfering with BMP signalling at the level of Mad phosphorylation. As expected, near the centre of the wing pouch (region II), complete removal of Brk function, resulted in ectopic expression of Sal (Fig. 3C, white arrowheads). Surprisingly, however, ectopic upregulation of Sal is not observed in *brk* clones located more laterally (region III) (Fig. 3F). Even more strikingly, Sal expression in lateral regions, where it normally coincides with high levels of Brk (region IV), was lost in *brk* mutant clones (Fig. 3C,F,I, yellow arrowheads). In contrast to *sal*, *nab* expression was ectopically upregulated even in the lateral regions upon removal of *brk* (Fig. 3H, yellow arrowheads). These results suggest that the lateral expression of *sal* is not regulated directly by the pMad-Med-Shn repression complex. More importantly, while Brk activity restricts the expression of positive BMP target genes (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Minami et al., 1999), it appears to be required for maintaining *sal* expression in lateral regions of the wing disc.

The loss of function analysis suggested that in different regions of the wing disc, Brk regulates *sal* expression in a qualitatively distinct fashion. We thus wondered if overexpression of Brk across the wing disc would also have distinct and reciprocal outcomes. To test the idea, we generated Brk overexpressing clones and specifically compared *sal* expression in clones situated in different regions of the wing disc. As previously reported, Sal expression in the centre of the wing pouch was abolished in such clones (Fig. 3L, white arrowhead). Importantly, in lateral regions of the wing pouch (region III), overexpression of Brk resulted in ectopic expression of Sal (Fig. 3L, yellow arrowheads). In summary, these results demonstrate that in lateral regions of the wing disc (regions III and IV) Brk positively regulates *sal* expression.

Brk's positive effect on *sal* regulation in lateral regions is indirect

Our results contradict the notion that Brk functions as a default repressor of BMP targets. Rather the results presented above suggest that Brk may drive lateral expression of *sal* indirectly by inhibiting a negative regulator of *sal* expression. Alternatively, Brk may act as a direct activator of *sal* in lateral regions of the wing disc. To distinguish between these two possibilities it was necessary to decipher how Brk actually regulates transcription of *sal*. To analyse this, we employed a Brk-VP16 chimeric fusion protein, in which the repression domain of the Brk repressor is replaced with the activation domain of the herpes simplex virus protein VP16 (Weiss et al., 2010). This recombinant protein is expected to display Brk's cognate DNA binding specificity. However, instead of repressing Brk target genes, it should be able to activate them. If in the lateral regions of the wing disc, endogenous Brk acts directly as an activator of *sal*, then overexpression of Brk-VP16 should also activate *sal* expression in lateral regions of the wing pouch (region III), similar to overexpression of Brk. By contrast, if in lateral regions Brk activates *sal* indirectly, via repression of another negative regulator, then Brk-VP16 will upregulate that negative regulator, ultimately leading to downregulation of *sal* expression (in region IV). As expected, in medial regions of the wing disc (region II) Brk-VP16 was able to ectopically upregulate Sal expression (Fig. 3O, white arrowhead). In lateral regions, however, overexpression of Brk-VP16 resulted in downregulation of Sal (Fig. 3O, yellow arrowheads), indicating that in order to activate *sal* expression in lateral regions of the wing disc, Brk acts as a repressor: It downregulates a (yet unidentified) negative regulator of *sal* (which we refer to as NRS hereafter), and in an indirect manner de-represses *sal* expression.

sal lateral enhancer contains neither Brk nor Mad-Med-Shn repression complex binding sites

Previous studies have identified several independent regulatory regions which are responsible for distinct spatial aspects of *sal* expression in the wing disc (de Celis et al., 1999). Characterisation of the medial enhancer that appears to control *sal* expression domain only in the centre of the wing pouch (Barrio and de Celis, 2004) (Fig. 1A) revealed that it contains Brk binding sequences. Consistent with the presence of a repressor binding sites, this particular enhancer fusion construct is not expressed in the lateral regions of the wing disc where substantial levels of Brk protein are present. Another enhancer, namely AK, recapitulates *sal* expression in the lateral regions of the wing disc when fused to a *lacZ* reporter (*AK-lacZ*) (de Celis et al., 1999) (Fig. 1A). We wished to determine using bioinformatics if this lateral enhancer of *sal* could be regulated directly by either pMad-Medea-Shn complex or Brk. We sequenced the *AK* enhancer (753 bp; supplementary material Fig. S1) and found (using fuzznuc/EMBOSS) no binding sites for pMad-Med-Shn complex, indicating that *sal* expression in lateral regions is not subjected to direct regulation by this repressive complex. Consistent with the results presented above, we found no Brk binding sites, indicating that Brk does not directly bind the regulatory region responsible for *sal* expression in lateral regions, but rather acts in an indirect manner by repressing the negative regulator of *sal* (NRS) that subsequently acts by directly binding to the *AK* enhancer. Interestingly, the AK enhancer is highly conserved in other *Drosophila* species and contains several putative binding sites for known transcriptional repressors (supplementary material Fig. S1).

The negative regulator of *sal* (NRS) is active all along the wing pouch

According to our model, the expression of the *AK-lacZ* reporter is restricted to the periphery of the wing disc presumably due to the repressing activity of the negative regulator of *sal* (NRS) in more medial regions. If this assumption is correct then increase in Brk dose will result in repression of NRS expression and subsequent activation of the *AK-lacZ* reporter in more medial regions. We tested this prediction by analysing the expression of the *AK-lacZ* reporter in Brk overexpressing clones. Indeed, we found that the *lacZ* reporter was ectopically induced in clones generated in lateral as well as medial regions of the wing pouch (Fig. 4C). This '*in vivo* activity assay' indicates that the negative regulator of *sal* (NRS), which regulates *sal* expression via the *AK* enhancer, is normally repressed by Brk in lateral regions. However, it is expressed and active all along the wing pouch where Brk levels decline (regions I, II and III). Importantly, endogenous *sal* expression was downregulated in Brk overexpressing clones located in the centre of the wing disc and upregulated in lateral

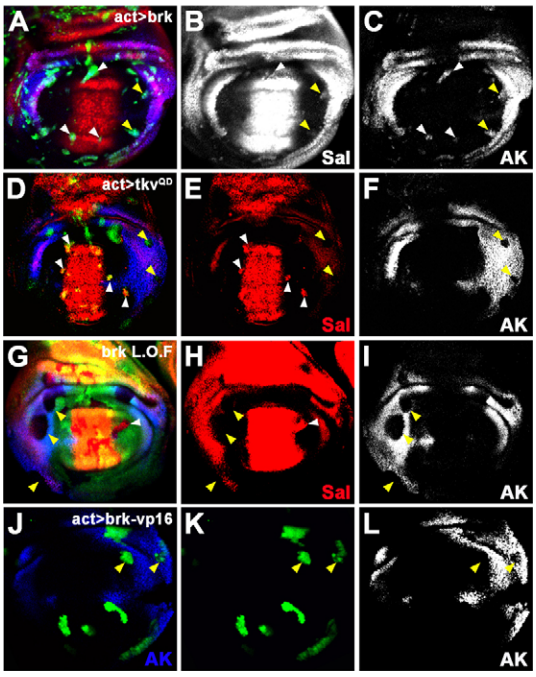

Fig. 4. Brk activates *sal*-lateral enhancer (AK) in medial and lateral regions of the wing disc. (**A–C**) Clonal overexpression of *brk* (marked by GFP, A) results in downregulation of Sal (red, A; grey, B) in the medial regions (white arrowhead), but leads to upregulation of Sal in lateral regions of the wing disc (grey, B, yellow arrowheads). AK-*lacZ* expression (blue, A; grey, C) is upregulated both in medial and lateral regions (grey, C, white and yellow arrowheads, respectively). (**D–F**) tkv^{QD} clones (marked by GFP, D) abolish Sal expression (red, D,E) in lateral regions (yellow arrowheads), but upregulate Sal in medial regions (white arrowheads). AK-*lacZ* expression (blue, D; grey, F) is downregulated (blue, D; grey, F, yellow arrowheads). (**G–I**) *brk* LOF clones (marked by loss of GFP, G) abolish Sal expression (red, G,H) in lateral regions (yellow arrowheads), but upregulate Sal in medial regions (white arrowhead). AK-*lacZ* expression (blue, G; grey, I) is downregulated (yellow arrowheads). (**J–L**) Clones overexpressing *brk-vp16* (marked by GFP, J,K) downregulate AK-*lacZ* expression (grey, J,L).

clones (region III) (Fig. 4B, white and yellow arrowheads respectively). This implies that in contrast to the isolated lateral (*AK*) enhancer, the full promoter of *sal* responds to NRS repressing activity in a position-dependent manner, presumably due to integration of additional inputs likely mediated by other regulatory regions.

Our model further predicts that activating BMP signalling or removing Brk activity in the periphery of the wing disc (region IV) should lead to upregulation of NRS and consequently to downregulation of the *AK-lacZ* reporter. Indeed, when we either generated tkv^{QD} overexpressing clones or clones mutant for *brk*, expression of the *AK-lacZ* reporter was abolished within the clones (Fig. 4F,I, respectively). Consistent with our model when we antagonized Brk-repressing activity by overexpressing Brk-VP16 in clones, we repressed the *AK-lacZ* reporter (Fig. 4L), presumably because the recombinant protein activated the expression of NRS. In summary, these results indicate that Brk drives endogenous *sal* expression in lateral regions of the wing disc (region IV) by repressing NRS, a negative regulator of *sal*, which acts through the *AK* enhancer. Moreover, the absence of *sal* expression in lateral regions of the wild type wing pouch (region III) is not a result of Brk repression but rather is engineered by NRS activity.

vestigial is positively regulated by Brk in the wing disc periphery

We wondered whether this unusual influence exerted by BMP/Brk signalling in lateral regions is unique to *sal* regulation or if it is a general mechanism that is more broadly utilised to regulate other target genes as well. In the wing disc, *vg* is a low-threshold BMP target that is expressed, like *sal*, also in lateral regions where Brk is in its maximal levels. To test if expression of *vg* in the lateral region of wing disc is also regulated by Brk, we generated *brk* LOF clones and analysed Vg expression by antibody staining. As in the case of *sal*, Vg was downregulated upon removal of *brk* in region IV (Fig. 5), suggesting that *vg* expression in the wing disc periphery is also positively regulated by Brk. Interestingly, the enhancer fragment responsible for *vg* expression in the wing pouch (vgQ) (Kim et al., 1997), is ectopically induced in the periphery of the wing disc upon removal of *brk* (Campbell and Tomlinson, 1999). Taken together with our analysis these data indicate that in the context of the full promoter Brk cannot repress *vg* expression through the vgQ enhancer in the periphery of the wing disc. Moreover, similar to *sal*, *vg* expression in the wing disc periphery is positively regulated by Brk.

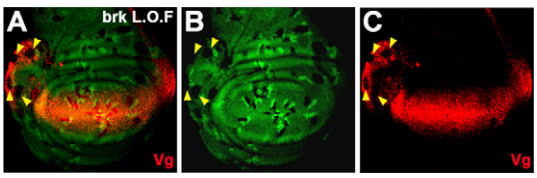

Fig. 5. Brk positively regulates Vg expression in lateral regions of the wing disc. (**A–C**) *brk* LOF clones (marked by loss of GFP, A,B) downregulate Vg expression in lateral regions of the wing disc (red, A,C, yellow arrowheads).

Discussion

Differential regulation of BMP target genes along the A/P axis of the wing disc

During *Drosophila* wing disc development the BMP morphogenetic gradient established by the collective actions of the two BMP ligands, Dpp and Gbb, patterns the cellular field by modulating gene expression in a concentration-dependent manner. How is the BMP concentration gradient translated into coordinated target gene expression? Current model relies upon two opposing activity gradients of the transcriptional regulators pMad and Brk, established in response to the BMP gradient. This model has been particularly successful in elucidating the regulatory influence exerted by BMP signalling although most of the attention has been focused on wing pouch, a region proximal to the peak of the gradient. Moreover, it is assumed that the target gene expression, in lateral regions where the BMP activity gradient decline, is independent of the signalling influence. Data presented here demonstrate that this supposition is incorrect. Alterations in pMAD signalling even in the lateral regions of the wing disc indeed lead to changes in positive target gene expression such as *vg* and *sal* albeit in an unexpected manner. In a classic 'role reversal' mode, the expression of the same targets is positively regulated by Brk and negatively by BMP signalling. Thus, while the classical morphogen model assumes that morphogens pattern a homogeneous field of responding cells, we show that in the developing wing disc interpretation and response to the BMP morphogenetic signal qualitatively differ along the anterior-posterior axis. The fact that *sal* is already expressed in both medial and lateral regions of the wing disc during early second larval stage (Grieder et al., 2009), implies that this subdivision occurs early in development.

A novel mechanism regulating *sal* expression

By comparing *sal* expression in the different regions of the wing disc, we have uncovered a novel circuitry underlying inverse regulation of an archetypal BMP target gene in distant regions where the morphogen levels decline. How is this counter regulation of *sal* achieved? Using the enhancer fragment that drives *sal* expression just in the lateral regions of the wing disc, we provide evidence that Brk induces expression of *sal* at the periphery of the wing disc indirectly through repression of a negative regulator (NRS). On one hand, NRS represses *sal* expression by binding to a cis-regulatory element that contains neither Brk nor pMad-Med-Shn complex binding sites. On the other, NRS is itself negatively regulated by Brk. The experiments described in this paper (summarised in Table 1) provide an initial working model (Fig. 6) to explain how the expression pattern of an established BMP target like *sal* is in fact differentially and inversely regulated in different regions along the anterior-posterior axis of the wing disc.

Brk represses *sal* in a position-dependent manner

Phenotypic consequences of the loss of *brk* on *sal* expression are qualitatively distinct and the effects vary in a position-dependent manner. Compromising *brk* function results in upregulation of *sal* in the central region (I and II); shows no effect towards the edge of the wing pouch (region III), and leads to a loss of *sal* expression in the periphery of the disc (region IV) (Fig. 3A–I; Table 1).

Brk is known to repress lateral expression of classic BMP responsive genes such as *omb* and *dad* through direct binding to

Table 1. Changes in the expression of Sal and AK-*lacZ* reporter in different regions in response to manipulation of *tkv* and *brk*

	Sal				AK-*lacZ*			
Genetic manipulation	I	II	III	IV	I	II	III	IV
TKV loss of function	↓	–	↑	↑*	ND	ND	ND	ND
TKV gain of function	↑	↑	–	↓	–	–	–	↓
Brk loss of function	↑	↑	–	↓	–	–	–	↓
Brk overexpression	↓	–	↑	↑*	↑	↑	↑	↑*
Brk VP16 overexpression	–	↑	–	↓	–	–	–	↓

*A low effect is seen only in some of the clones probably for two reasons. First, the clones quickly disappear (because elevation in Brk induces JNK-mediated apoptosis). Second, it is hard to see the low increase in the expression on the background of the endogenous expression.

specific sequences within their enhancers (Sivasankaran et al., 2000; Weiss et al., 2010), indicating that it is highly active in lateral regions. Since the medial enhancer of *sal* also contains two Brk consensus-binding sequences (Barrio and de Celis, 2004), this raises the question as to why the medial enhancer does not function to repress *sal* in the lateral zone? While we don't know the precise mechanism underlying this position-dependent-repression, the fact that a P-*lacZ* reporter of the *sal* medial enhancer is induced in lateral regions upon removal of Brk (Campbell and Tomlinson, 1999), indicates that in isolation the medial enhancer responds to Brk repressive activity also in the periphery of the wing disc. Similarly, Barrio and de Celis found that mutating the relevant Brk binding sites in the isolated medial enhancer of *sal* expanded the expression to lateral regions (Barrio and de Celis, 2004). Combined together, these observations suggest that in the context of the full-length endogenous promoter of *sal*, (yet unknown) trans-factors are differentially distributed along the A–P axis of the developing wing disc to prevent repression by Brk (via the medial enhancer) in lateral regions and thus to confer position-dependent expression.

NRS represses *sal* in a position-dependent manner

Our analysis implies that Brk drives endogenous *sal* expression in region IV by repressing a negative regulator of *sal* (NRS), which targets the lateral enhancer. Brk levels, which decline medially, enable NRS to be active which in turn represses *sal* expression in lateral regions of the wing pouch (region III) (Fig. 6). By manipulating Brk levels and monitoring the activity of the *AK-lacZ* reporter, we provide evidence that NRS is normally expressed and active all along the wing pouch (regions I, II and III). This raises the question as to how endogenous *sal* in the centre of wing pouch escapes repression mediated by NRS. In principle, high pMad activity in medial regions (I and II) could overcome the repressive function of NRS. While plausible, this is an unlikely scenario as in the absence of both *brk* and *mad*, *sal* is ectopically expressed in medial regions (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999). We therefore propose that in the context of the endogenous promoter, the activity of NRS is antagonized specifically in centre of the wing pouch (regions I and II) with the assistance from the localised, non-uniform distribution of trans-acting factors along the A–P axis of the wing disc to confer a position-dependent transcriptional response.

Integration of pMad and NRS antagonizing activities regulates *sal* expression in lateral regions of the wing disc

In our experiments *sal* is ectopically induced near the edge of the wing pouch (region III) in *tkv* mutant clones (Fig. 1F). Even more perplexingly tkv^{QD} overexpressing clones also behave in a similar manner (Fig. 2I). Both of these outcomes are difficult to reconcile with the current model describing how the BMP morphogen gradient is linearly interpreted as both extreme situations; either complete loss (*tkv*) or substantial gain (tkv^{QD}) of endogenous pMad activity, results in ectopic expression of *sal*. However, this conundrum can be partially resolved by taking into account the activity of the newly invoked, additional component NRS into the BMP-dependent patterning system.

In the wild-type wing disc, pMad activity in region III, although low, is still sufficient to downregulate Brk levels just enough to allow for concomitant rise in NRS levels ultimately resulting in repression of *sal* expression. Importantly, low levels of pMad in region III (acting through the medial enhancer; ME) are inadequate to antagonize the repressor activity of NRS (mediated through the lateral enhancer; LE) (Fig. 6A, region III). The absence of pMad activity in *tkv* mutant clones in region III increases the levels of Brk, which in turn represses NRS and thus de-represses *sal* expression. In the case of tkv^{QD} overexpressing clones, the substantially elevated pMad activity represses *brk* expression leading to elevated NRS activity (Fig. 6B). In region III clones (periphery of the wing pouch) the high activity of pMad (acting from the ME) overcomes the repressing activity of NRS (acting from the LE), ultimately resulting in activation of *sal*. By contrast, in the vast majority of tkv^{QD} overexpressing clones (57 out of 63 clones) located in region IV (periphery of the wing disc) endogenous expression of *sal* is either lost or diminished. Why in region IV pMad inducing activity does not have an edge over NRS repressing activity, as is the case in region III? We propose that in the context of the endogenous promoter, the activity of pMad is antagonized specifically in region IV due to the activity of unevenly distributed trans-acting factors. The rare occasions, where in the tkv^{QD} overexpressing clones located in region IV *sal* expression was upregulated could be due to a rare event leading to acquisition of wing pouch like identity by the cells at the wing disc periphery, presumably due to early exposure to high pMad activity. Nevertheless, differential transcriptional response behaviour exhibited by the cells from regions III v/s IV supports the subdivision of the developing wing disc on the basis of distinct regional competence.

Opposite regulation of target genes and the morphogen function of BMP

How an exponentially decaying morphogen gradient of BMP gives rise to computable changes in gene expression ultimately leading to discreet morphological structures is a fascinating question. A steep slope of the BMP activity gradient near the peak allows sharp expression domains of target genes to be

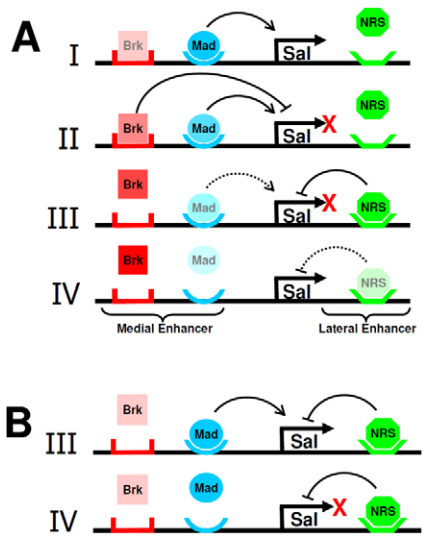

Fig. 6. Position-dependent regulation of *sal* expression along A-P axis of the wing disc. (**A**) Regions I to IV correspond to the subdivision of the wing disc shown in Fig. 1. The abundance of Brk, Mad and NRS in each region corresponds to the intensity of the colour (light, low levels; dark, high levels). When the factor is not acting directly on *sal* expression, it is depicted unbound to its DNA binding site. The medial enhancer also contains a constitutive activating enhancer (Barrio and de Celis, 2004). According to our working model, the lateral enhancer also includes a region responsible for transcriptional activation. These two enhancers are not shown in the model. Region I: In wild-type wing disc, highest pMad activity in region I on one hand represses *brk* transcription and on the other hand directly activates *sal* expression via the medial enhancer. Region II: Gradual decrease in pMad levels in region II results in parallel increase in Brk levels, which in turn represses *sal* expression via the medial enhancer. Notably, although NRS is expressed and active all over the wing pouch (regions I, II and III) it is not capable of repressing *sal* (through the lateral enhancer) in both regions I and II. Region III: pMad activity in region III, although low, is still sufficient to downregulate Brk levels just enough to allow for concomitant rise in NRS levels, ultimately resulting in repression of *sal* expression. Importantly, low levels of pMad in region III (acting through the medial enhancer) are inadequate to antagonise the repressor activity of NRS (mediated through the lateral enhancer). Region IV: Highest levels of Brk in region IV represses NRS transcription and indirectly de-represses *sal* expression. Importantly, in regions III and IV, Brk cannot directly repress *sal* expression (through the medial enhancer). (**B**) Regulation of *sal* expression in tkv^{QD} overexpressing clones located in regions III and IV. Region III: High pMad activity in region III, on one hand, represses *brk* transcription leading to a rise in NRS levels. On the other hand, it directly activates *sal* expression via the medial enhancer. In this region, high activity of pMad (acting from the ME) overcomes the repressing activity of NRS (acting from the lateral enhancer), ultimately resulting in activation of *sal*. Region IV: Similar to region III; high pMad activity in region IV represses *brk* transcription leading to a rise in NRS levels and repressing activity. However, in contrast to region III, in region IV pMad cannot directly repress *sal* expression (through the medial enhancer) and the net result is repression of *sal* expression.

defined within the wing pouch area (Bollenbach et al., 2008; Entchev et al., 2000; Teleman and Cohen, 2000; Wartlick et al., 2011). However in the lateral regions of wing disc, the activity gradient of BMP dips considerably raising the question as to how small differences in signal strength provide discrete threshold responses. Indeed, it is believed that BMP/Brk patterning system does not regulate the lateral expression of *sal* (Affolter and Basler, 2007; Campbell and Tomlinson, 1999; Jaźwińska et al., 1999). Our data argue that not only the signalling is active in the lateral regions but the inverse regulatory mode adopted by the signalling circuitry is in fact responsible for generating distinct threshold responses.

Materials and Methods

Fly strains and transgenes

Generation of Flp-out and loss-of-function clones

We generated overexpressing Flp-out clones using the *act >CD2 >Gal4* cassette, recombined to a *UAS-GFP* construct for the detection of the clones. Larvae were subjected to a 37℃ heat shock for 10 minutes. Genotypes of dissected larvae were as follows: Tkv^{Q235D}-overexpressing clones: *yw hsp70-flp; AK; act >CD2 >Gal4 UAS-GFP/UAS-*tkv^{Q235D}. Brk-overexpressing clones: *yw hsp70-flp; UAS-brk/AK; act >CD2 >Gal4 UAS-GFP*. Brk-VP16 clones: *yw hsp70-flp; UAS-brk-vp16/AK; act >CD2 >Gal4 UAS-GFP*. Dad-overexpressing clones: *yw hsp70-flp; UAS-dad; act >CD2 >Gal4 UAS-GFP*.

We generated mutant clones using Flp-mediated mitotic recombination and identified them by the loss of the GFP marker. Clones were induced by heat shock (60 minutes at 37℃). Genotypes of dissected larvae were as follows. *brk* loss-of-function clones: *Ubi-GFP FRT18A/yw* brk^{M68} *FRT 18A; hs-flp. tkv* loss-of-function clones: *yw hsp70-flp;* tkv^{a12}*FRT40/Ubi-GFP FRT40*.

Immunohistochemistry and imaging

Imaginal discs from third instar larvae were fixed and stained by standard techniques. The specific primary antibodies used were: mouse anti-β-gal (1:1000; Promega), rabbit anti-Spalt [1:1000; a gift from A. Salzberg (Halachmi et al., 2007)], rat anti-Nab [1:1000 (Suissa et al., 2011)], rat anti-Brk (1:1000; a gift from F. A. Martín and G. Morata, CDBM University Autonoma De Madrid, Madrid, Spain), rabbit anti-cleaved Caspase 3 (1:40; Cell Signaling) and Rabbit anti-Vg (1:20; a gift from Sean Carroll). Secondary antibodies used: RRX- or Cy5-conjugated (1:400; Jackson Laboratories). Images were taken on a TE2000-E confocal microscope (Nikon) using a 20× objective. Figures were edited using Adobe Photoshop 7.0.

Generating Brk-VP16 construct

DNA contains the first 173 aa of the Brk repressor, includes the DNA-binding domain (DBD) site, nuclear localisation sequence (NLS), but not the repression domain (construct A2 from (Winter and Campbell, 2004)) cloned into pUAST vector that contains VP16 activation domain (a gift from Dr Adi Zalsberg – Technion). 50 µg of Qiagen-purified DNA sent to Genetic Services Inc. (GSI) where they injected the DNA into mutant embryos lacking the gene W (for red eyes). Adult flies were single-crossed to yw, and F1 containing red eyes were selected. Transgenic flies were kept as balanced stocks.

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