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RESEARCH ARTICLE

Sequence environment of BMP-dependent activating elements controls transcriptional responses to Dpp signaling in *Drosophila*

Mrinal Chayengia[1,2,3], Ville Veikkolainen[1,3,*], Milica Jevtic[3,4,‡] and George Pyrowolakis[1,3,5,§]

ABSTRACT

Intercellular signaling pathways activate transcription factors, which, along with tissue-specific co-factors, regulate expression of target genes. Responses to TGFβ/BMP signals are mediated by Smad proteins, which form complexes and accumulate in the nucleus to directly bind and regulate enhancers of BMP targets upon signaling. In *Drosophila*, gene activation by BMP signaling often requires, in addition to direct input by Smads, the signal-dependent removal of the transcriptional repressor Brk. Previous studies on enhancers of BMP-activated genes have defined a BMP-responsive motif, the AE, which integrates activatory and repressive input by the Smad complex and Brk, respectively. Here, we address whether sequence variations within the core AE sequences might endow the motif with additional properties accounting for qualitative and quantitative differences in BMP responses, including tissue specificity of transcriptional activation and differential sensitivity to Smad and Brk inputs. By analyzing and cross-comparing three distinct BMP-responsive enhancers from the genes *wit* and *Dad* in two different epithelia, the wing imaginal disc and the follicular epithelium, we demonstrate that differences in the AEs contribute neither to the observed tissue-restriction of BMP responses nor to differences in the utilization of the Smad and Brk branches for transcriptional activation. Rather, our results suggest that the *cis*-environment of the BMP-response elements not only dictates tissue specificity but also differential sensitivity to the two BMP mediators.

KEY WORDS: Gene regulation, Tissue patterning, TGFβ signaling, wit

INTRODUCTION

Bone morphogenetic proteins (BMPs) control a vast number of developmental and homeostatic processes (Wu and Hill, 2009). In canonical BMP signaling, BMP ligands bind and activate receptor complexes at the cell membrane, which in turn phosphorylate receptor-associated Smads (R-Smads) (Shi and Massagué, 2003). Subsequently, phosphorylated R-Smads associate with the common-Smad (co-Smad, Smad4 in mammals) and the Smad complex accumulates in the nucleus to bind DNA directly and regulate transcription of target genes (Fig. 1A). In *Drosophila*, BMP-dependent gene regulation has been analyzed in multiple contexts of fly development, including cases of graded (morphogen) BMP signaling during early embryonic development and larval wing development (Affolter and Basler, 2007; Bier and De Robertis, 2015; Upadhyay et al., 2017). In both cases, a spatial gradient of the *Drosophila* BMP Decapentaplegic (Dpp) generates a gradient of phosphorylated Mad (Mad, the *Drosophila* R-Smad), which then activates target gene transcription in a threshold-dependent manner (Ashe and Briscoe, 2006; Hamaratoglu et al., 2014). Besides direct pMad input, proper activation of BMP/pMad targets requires the transcriptional repressor Brinker (Brk), which is coupled to BMP signaling through two key properties: first, Brk distributes in a pattern that is inverse to the gradient of pMad, and, second, Brk directly represses Dpp target genes (Ashe et al., 2000; Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a,b; Minami et al., 1999). Thus, Dpp-target genes integrate input from two opposing gradients: activatory input from pMad and repressive input from Brk. Their differential sensitivity to these two cues define their spatial extent of activation within the morphogen field (reviewed by Affolter and Basler, 2007; Hamaratoglu et al., 2014).

The inverse relation of the pMad and Brk distribution is not restricted to the context of graded Dpp signaling but is evident in most instances of BMP signaling during fly development. In most cases, Dpp signaling directly accounts for the distribution of Brk by negatively regulating its transcription. Dpp-dependent repression of *brk* transcription requires the repressor Schnurri (Shn) and short DNA sequences, the silencer elements (SEs), present in the regulatory region of *brk* (Charbonnier et al., 2015; Marty et al., 2000; Müller et al., 2003; Pyrowolakis et al., 2004; Torres-Vazquez et al., 2001; Yao et al., 2008). Upon signal activation, Smad trimers consisting of two pMad and one Medea molecule bind directly to the SE, which comprises three minimal Smad-binding sites (GNC; N, any nucleotide) organized in the consensus **GNCGNC**$(N)_5$**GTC**T (minimal Smad-binding sites in bold; Fig. 1B) (Gao et al., 2005; Pyrowolakis et al., 2004). Within this sequence, the two pMad molecules bind the GNC motifs of the GNCGNC box, whereas Medea binds to the GTCT motif. The SE-bound Smad complex can then recruit nuclear Shn, which mediates *brk* repression. Binding of Shn to the SE/Smad complex seems not to require direct Shn-DNA contact but rather a very specific conformation of the SE-bound Smad complex, which, in turn, depends on determinants within the SE. Specifically, Shn can only dock to the complex when the spacing between the pMad and Medea sites is precisely five nucleotides (independent of the nature of the nucleotides) and when the Medea-binding block contains a T at the last position (GTC**T**). Any deviation from these two features results in an SE that is fully able to interact with a Smad trimer but cannot recruit Shn *in vitro* and is, consequently, fully inactive in

[1]Signalling Research Centres BIOSS and CIBSS, Albert-Ludwigs-University of Freiburg, 79104 Freiburg, Germany. [2]Research Training Program GRK 1104, Albert-Ludwigs-University of Freiburg, 79104 Freiburg, Germany. [3]Institute for Biology I, Faculty of Biology, Albert-Ludwigs-University of Freiburg, Germany. [4]Spemann Graduate School of Biology and Medicine (SGBM), Albert-Ludwigs-University of Freiburg, 79104 Freiburg, Germany. [5]Center for Biological Systems Analysis, Albert-Ludwigs-University of Freiburg, Habsburgerstr. 49, 79104 Freiburg, Germany.
*Present address: Perkin Elmer, Mustionkatu 6, 20750 Turku, Finland. ‡Present address: CECAD Research Center, University Hospital, Joseph-Stelzmann-Str. 26, 50931 Cologne, Germany.

§Author for correspondence (g.pyrowolakis@biologie.uni-freiburg.de)

M.C., 0000-0003-2626-3637; V.V., 0000-0002-5925-2371; M.J., 0000-0002-8177-5515; G.P., 0000-0002-2142-5943

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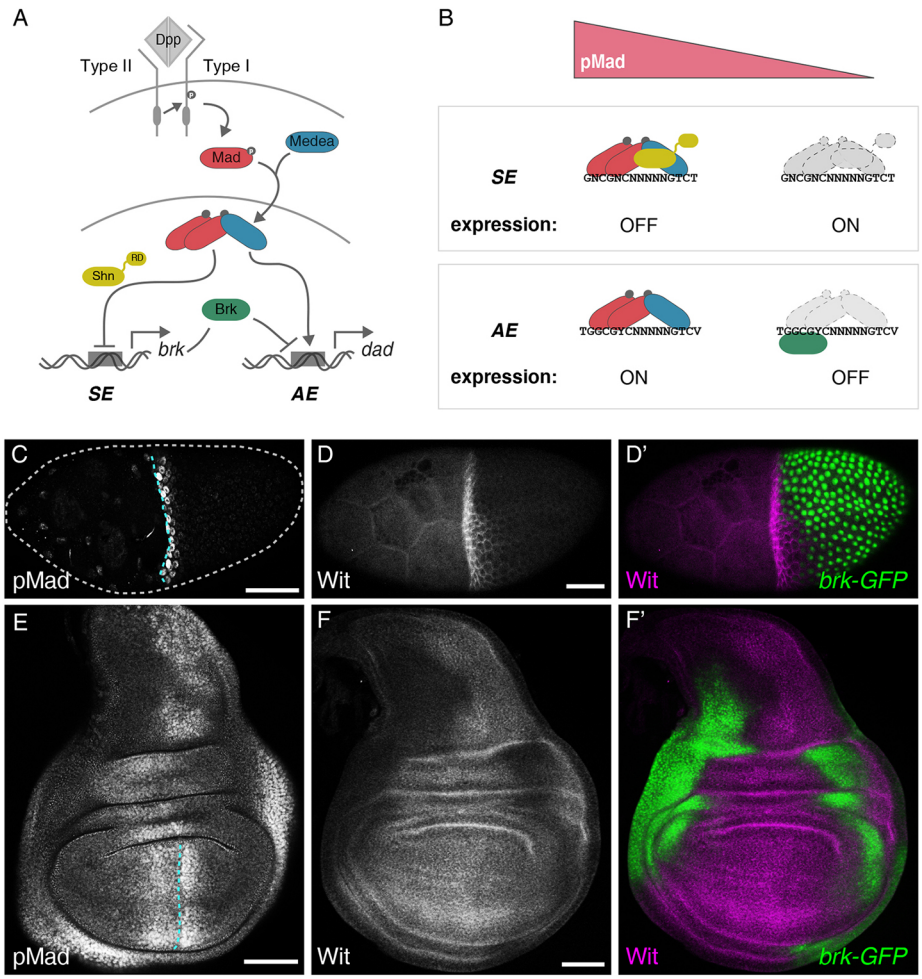

Fig. 1. Wit expression in *Drosophila* epithelia. (A) BMP signaling in *Drosophila melanogaster*. Signaling-activated Mad/Medea complexes accumulate in the nucleus and bind GC-rich motifs in target-gene enhancers. Transcriptional repression and activation of BMP target genes (exemplified by *brk* and *Dad*) are mediated by association of the Smad-Shn complex to silencer elements (SEs) and the Smad complex/Brk to activating elements (AEs), respectively. RD, repression domain. (B) Molecular events on SEs and AEs in a field of a BMP/pMad gradient. Signal-activated Smads bind to SEs to recruit Shn and repress transcription (upper box). Thus, expression of SE-regulated BMP targets is restricted to cells of low BMP/pMad levels. In reverse, AE-regulated BMP targets (lower box) are activated by Smad binding in cells of high and repressed by Brk in cells of low BMP signaling activity, respectively. (C) Activation of Mad as monitored by staining against phosphorylated Mad (pMad) is restricted to an anterior stripe of oocyte-associated follicle cells. Dashed lines mark the anterior oocyte boundary (cyan) and egg chamber outline (gray). (D,D′) Wit expression (gray in D, magenta in D′) in the follicular epithelium is restricted to cells of high BMP signaling activity as visualized by the absence of *brk-GFP* (green in D′). (E) BMP activity in a third instar wing imaginal disc. pMad is graded in both the anterior and the posterior compartment with a characteristic decrease in the cells that secrete Dpp. The dashed line indicates the anterior-posterior compartment boundary in the wing pouch. (F,F′) Wit expression (gray in F, magenta in F′) in the wing imaginal disc is restricted to cells of high BMP signaling activity as visualized by the absence of *brk-GFP* (green in F′). Scale bars: 50 µm. In this, and all subsequent images, imaginal discs and egg chambers are oriented with the anterior to the left and dorsal up. For exact genotypes and analyzed sample size for this and subsequent figures, see Table S1.

transcriptional gene repression *in vivo*. Thus a simple, yet stringent arrangement of Smad-binding sites in the SE implements BMP-dependent repression of an expanding number of BMP targets, including *brk* (Beira et al., 2014; Crocker and Erives, 2013; Esteves et al., 2014; Vuilleumier et al., 2010; Walsh and Carroll, 2007).

Gene activation by Smad signaling seems to be more complex with BMP-dependent enhancers containing a variable number of Smad- and Brk-binding sites, as well as binding sites for transcription factors that synergize with BMP signaling to achieve robust and tissue-specific target gene expression (Barrio and de Celis, 2004; Liang et al., 2012; Markstein et al., 2002; Rushlow et al., 2001; Saller and Bienz, 2001; Winter and Campbell, 2004; Zhang et al., 2001). Brk- and Smad-binding sites are either dispersed on such enhancers, or can form clusters. One extreme case of the latter is a motif termed an activating element (AE), originally identified in the regulatory region of *Daughters against Dpp* (*Dad*) but later identified in a number of BMP-responsive enhancers (Szuperák et al., 2011; Vuilleumier et al., 2018; Weiss et al., 2010). The motif, **GGCGYC**$(N)_5$**GTC**V (Smad-binding sites in bold; V: G, A or C; Fig. 1B), is very similar to the SE; however, it lacks one of the determinants for Shn recruitment (a T at the last position). Instead, the pMad-binding block (GGCGYC) corresponds also to a Brk-binding motif (GGCGYY) explaining the negative impact of Brk on *Dad* expression. Besides such core determinants for Brinker and Smad recruitment, it is not clear whether the core AE motif contains additional features. The limited available data – mostly derived from biochemical and cell culture assays – suggest that AEs may come in many variants differing from each other in the linker length and nucleotide environment of the core consensus motif (Esteves et al., 2014; Gao and Laughon, 2007). Although this might indicate a flexibility of the element towards the recruitment of the Smads and Brk, it is equally conceivable that, similar to the SE, AEs might contain sequence determinants that

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facilitate AE-bound Smads and/or Brk to recruit partners impacting on the element's output. Potential partners may include transcriptional co-activators and/or co-repressors, factors affecting the opposing Brk and Smad inputs in transcriptional output, or even proteins conveying tissue specificity to the AE.

Here, we address this question by studying the regulation of the gene *wishful thinking* (*wit*). We demonstrate that BMP signaling activates *wit* transcription in both the larval wing imaginal disc and the follicular epithelium; however, and in sharp contrast to *Dad*, BMP responsiveness in the two tissues is mediated by distinct *cis*-regulatory modules (CRMs). In addition, the two identified CRMs are differentially sensitive to Smad and Brk inputs and are equipped with AE-like motifs that differ from the prototypic AE and from each other. Using a combination of genetic mosaic analysis and reporter assays with chimeric CRMs, we demonstrate that the diversified AEs neither mediate tissue specificity nor do they account for the observed differences in the responsiveness of *wit* to Brk and Smad input in the two epithelia. Our data are consistent with the sequences of AEs being rather flexible and monotonically mediating BMP responses, with qualitative and quantitative aspects of such responses depending on BMP-independent, activatory sequences within their cognate CRMs.

RESULTS

Wishful thinking (Wit) as a transcriptional target of BMP signaling

Wit is a *Drosophila* BMP type II receptor predominantly expressed in neural cells at neuromuscular junctions to control synaptic size and function as well as in a set of neurosecretory cells to regulate expression of neuropeptide genes (Aberle et al., 2002; Allan et al., 2003; Marqués et al., 2003; Marqués et al., 2002; McCabe et al., 2003; Veverytsa and Allan, 2011; Zheng et al., 2003). Wit is also required for the formation of the anterior pMad gradient in the ovarian follicle cells (FCs; Fig. 1C) and for proper eggshell formation (Marmion et al., 2013; Pyrowolakis et al., 2017). In this context, the transcription of *wit* is activated by BMP signaling itself in an anterior, wedged-shaped stripe of oocyte-associated FCs (Fig. 1C,D,D′). Additionally, *wit* is expressed in the developing wing imaginal disc epithelium, although there is no evidence for a contribution of the receptor in transmitting BMP signals in this tissue or, generally, in wing development (Marqués et al., 2002). In a recent transcriptional profiling experiment, we have identified *wit* as a target of Dpp in the developing wing (Alexander Springhorn, M.J. and G.P., unpublished data), prompting us to re-evaluate its expression in this tissue. Wit is present in medial regions of the wing disc and absent from *brk*-expressing lateral cells, suggesting positive regulation by the BMP/pMad signaling gradient (Fig. 1E-F′). Indeed, reduction of Dpp signaling by clonal expression of Dad resulted in cell-autonomous loss of Wit in both the follicular epithelium and the wing imaginal disc (Fig. 2A-B″). In reverse, clonal activation of Dpp signaling resulted in strong, ectopic Wit expression (Fig. 2C-D″). Thus, similarly to the follicular epithelium, Wit expression is under positive control of BMP signaling in the wing epithelium. This regulatory relationship is reminiscent of *Dad*, which also encodes a pathway-inherent component and is regulated by BMP signaling in multiple tissues (Tsuneizumi et al., 1997; Weiss et al., 2010).

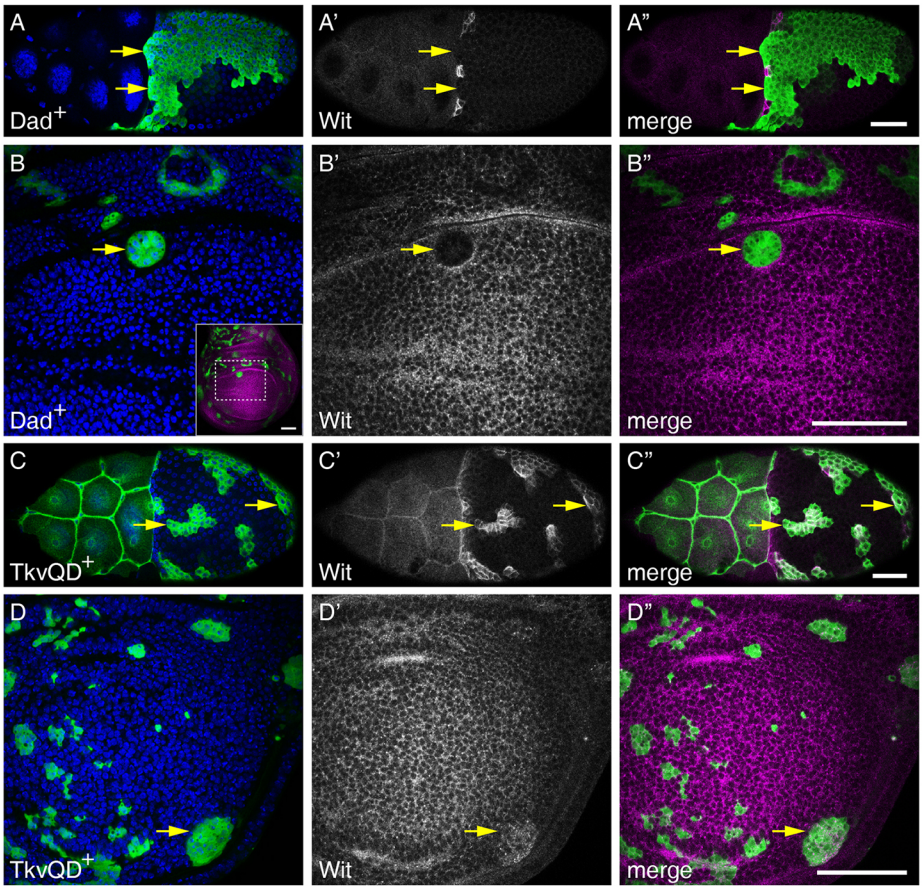

Fig. 2. Wit is a transcriptional target of BMP signaling. (A-B″) Overexpression of the inhibitory *Drosophila* Smad Dad in clones (marked by GFP; A,A″,B,B″) results in loss of Wit (gray in A′,B′) in both follicle (A) and wing disc (B) cells. The position of the magnified disc area of B is indicated in the inset. (C-D″) Clones (marked by GFP; C,C″,D,D″) expressing a constitutively active version of the Thickveins (Tkv^{QD}), cell-autonomously upregulate Wit (gray in C′,D′) in both follicle cells (C) and wing discs (D). Arrows indicate representative clones. Nuclei are stained by Hoechst (blue, A-D). Scale bars: 50 µm.

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Distinct CRMs account for BMP responsiveness of *wit* in different epithelia

Previous work on *Dad* transcription has identified a BMP-responsive CRM (*dad13*) that accounts for *Dad* expression in all tissues tested so far, including the follicular epithelium and the wing imaginal disc (Weiss et al., 2010). To understand whether the molecular principles underlying *Dad* regulation also apply for *wit*, we investigated the *cis* elements accounting for its Dpp-responsiveness in both tissues. Our previous work has identified a ∼1 kb fragment, termed *witZ*, to be expressed in a *wit*-like pattern in the follicular epithelium (Marmion et al., 2013). A sub-fragment of *witZ*, *witF* (*F* for follicle cells), comprising ∼400 bp of *witZ*, is still capable of recapitulating all aspects of *wit* expression in follicle cells and is considered here as the minimal CRM (Fig. 3A,B). However, neither w*itF* nor its parental *witZ* activate reporter expression in the wing imaginal disc (Fig. 3B′, inset), suggesting that distinct CRMs account for expression of *wit* in the wing. Testing a collection of fragments tiling the *wit* genomic locus by reporter assays in transgenic flies, revealed that the pattern of *wit* in the wing is recapitulated by a ∼600 bp long, intronic fragment hitherto referred to as *witW* (W for wing disc; Fig. 3A,C). In addition, *witW* was found to be completely inactive in the follicular epithelium (Fig. 3C′, inset). Using the same experimental set-up as for endogenous Wit, we could demonstrate that the activities of both *witF* and *witW* strictly depend on BMP input (Fig. S1). Thus, and in contrast to *Dad*, Dpp-dependent expression of *wit* in the eggshell and the wing is achieved by distinct regulatory modules.

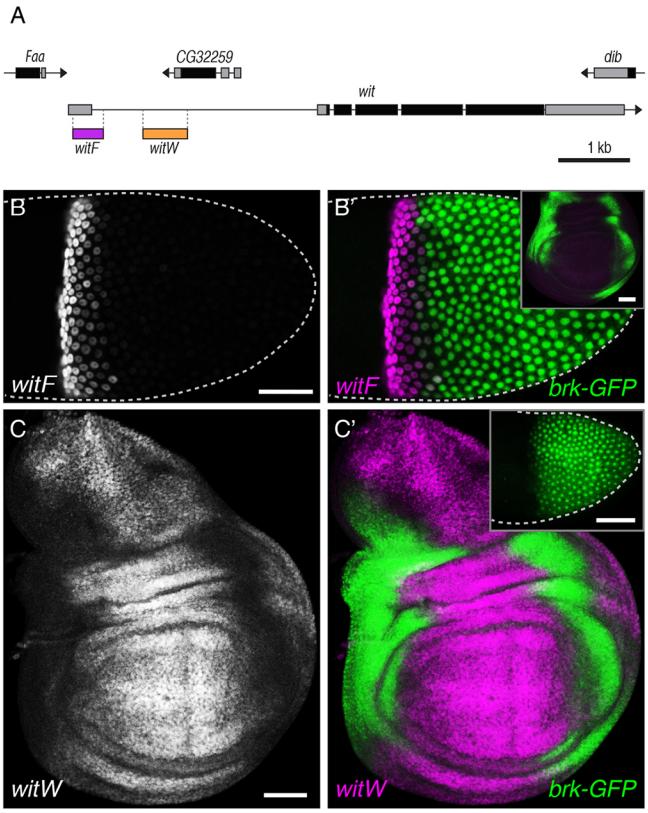

Fig. 3. Distinct enhancers of *wit* implement BMP-dependent activation in distinct tissues. (A) Schematic of the *wit* genomic locus. Protein coding sequences are shown in black, 5′ and 3′ UTR sequences in gray. *witF* and *witW* are shown in purple and orange, respectively. (B-C′) *witF* and *witW* reporter expression (gray in B,C; magenta in B′,C′) recapitulate the expression pattern of endogenous Wit in follicle cells and wing disc and do not overlap with *brk-GFP* (green in B′,C′). *witF* is inactive in the wing imaginal disc (B′, inset) and *witW* is inactive in follicle cells (C′, inset). Scale bars: 50 µm.

BMP employs different signaling branches for *wit* regulation in different epithelia

Transcriptional targets of canonical BMP signaling in *Drosophila* can be activated directly by Smads, de-repressed by Smad-dependent repression of Brk, or by a combination of both (see Introduction and Fig. 1A). To assess the relative contributions of Smads and Brk in the regulation of *wit* in the wing and follicular epithelium, we compared *wit* reporter expression in *Mad* and *brk* mutant clones. As expected, clonal loss of Mad resulted in a complete loss of reporter expression within the cells of the clone in both epithelia (Fig. 4A-B″). In the egg chamber, loss of *brk* in posterior FCs resulted in strong, cell-autonomous activation of *witF*, demonstrating a crucial contribution of the Brk-dependent branch to the BMP-mediated activation of *wit* (Fig. 4C-C″). Reporter expression levels were equally high in anterior and posterior clones and approximated the levels of the reporter at the endogenous stripe. Given the steep and restricted anterior pMad gradient in the egg chamber, it is unlikely that Smad complexes provide activatory input other than downregulating *brk* expression. Nevertheless, we directly addressed the epistatic relationship of Smads and Brk by analyzing reporter activity in clones that simultaneously lack Mad and Brk. Posterior *Mad*/*brk* double-mutant clones displayed strong upregulation of *witF*, demonstrating that Smads are not required for the ectopic reporter expression observed in *brk* mutants (Fig. 4E-E″). Importantly, *Mad*/*brk* double-mutant clones cutting across the anterior endogenous stripe of *wit* expression did not affect *witF*. Thus, the loss of *witF* activity observed in single *Mad* mutants can be completely reversed by genetic removal of *brk*, demonstrating that all effects of BMP signaling on *wit* expression in the FC epithelium can be assigned to Brk.

Applying the same experiments for the wing-specific *witW*, uncovered a different behavior. Lateral clones lacking *brk* displayed an upregulation of the *witW* reporter, albeit at levels lower than in the endogenous, medial expression domain (Fig. 4D-D″). At the same time, the reporter activity in medial *Mad* mutant clones could only be partially restored by the simultaneous removal of Brk (Fig. 4F-F″). Thus, in contrast to the *wit* regulation in the follicular epithelium but is similar to the regulation of *Dad* in the wing; activation of *witW* requires a dual input by activated Smads: de-repression (repression of Brk) and additional, potentially direct, activatory input.

Importantly, all the above regulatory interactions, deduced from mosaic analyses with *wit* reporters as a read-out, could be confirmed for endogenous Wit expression. Specifically, clonal analysis confirmed that Mad activates whereas Brk represses Wit in both the follicle cells (Fig. S2A-A″,C-C″) and the wing (Fig. S2B-B″,D-D″). As with the *witF* reporter, epistatic analyses using *Mad/brk* double-mutant clones demonstrate that the role of Mad in follicular Wit expression is limited to the repression of Brk (Fig. S2E-E″). In contrast, and consistent with the behavior of *witW*, both Brk-mediated and Brk-independent Mad input is required for Wit expression in the wing disc (Fig. S2F-F″).

AE-like elements implement BMP responsiveness of *wit*

Our data so far establish two deviations in the transcriptional regulation of *wit*. First, in striking contrast to *Dad*, which utilizes the same CRM (*dad13*) for BMP-dependent activation in multiple tissues, independent CRMs account for BMP-dependent regulation of *wit* in different tissues. Second, the two CRMs of *wit* differentially integrate the activity of the transcription factors of the pathway

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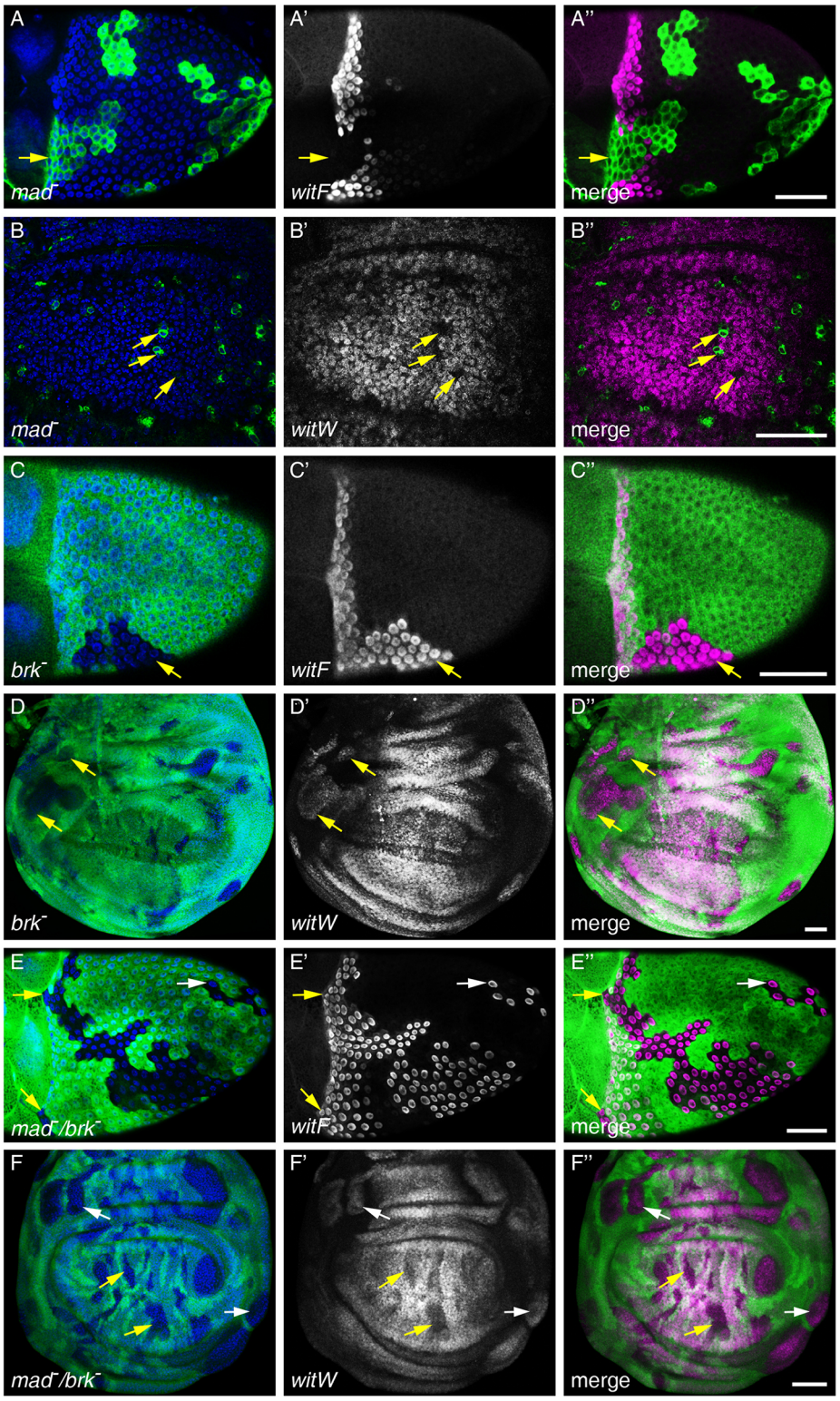

Fig. 4. Distinct *wit* enhancers utilize different branches for integrating BMP responsiveness. (A-B″) *witF* and *witW* reporter expression (gray in A′,B′; magenta in A″B″) are lost in *Mad* MARCM clones (marked by GFP) in both the follicular epithelium (A) and wing discs (B). Representative clones are indicated by arrows. (C-D″) *brk* mutant clones (marked by the absence of GFP) display ectopic reporter expression of both reporters (gray in C′,D′) in their respective tissue of expression. Representative clones are indicated by arrows. (E-E″) Posterior *Mad*/*brk* double-mutant clones (marked by the absence of GFP) result in full ectopic activation of *witF* (white arrows). Anterior mutant cells (yellow arrows) overlapping the expression stripe of *wit* do not affect *witF* activity, indicating that the effect of Mad is fully mediated by Brk. (F-F″) *Mad*/*brk* double-mutant clones (marked by the absence of GFP) located distal to the expression domain of *wit* display weak ectopic activation of *witW* (white arrows). In contrast, medial *Mad*/*brk* double-mutant clones display reduced *witW* expression. Nuclei are stained by Hoechst (blue, A-F). Scale bars: 50 µm.

(Smad and Brk). In order to understand the molecular basis for these differences, we analyzed *cis* requirements for *wit* expression. Particularly, we investigated whether the identified CRMs contain BMP-dependent response elements and whether such elements might also implement tissue specificity and/or differential sensitivity to the transcription factors of the pathway. The ‘canonical’ AE, first identified in *Dad* and subsequently shown to impose BMP responsiveness to a number of enhancers, corresponds to the consensus GGCGYCNNNNNGTCV (where N indicates any nucleotide, Y indicates C or T, and V indicates A, G or C; see Introduction). Whereas neither *witF* nor *witW* comprise such AEs, we did note that both fragments contain a highly conserved single cluster of Brk/Mad- and Med-binding sites separated by a variable number of nucleotides (Fig. 5A, Fig. S3). To test directly for a potential contribution in the expression of *wit*, we introduced deletions in *witF* and *witW* that completely remove these elements

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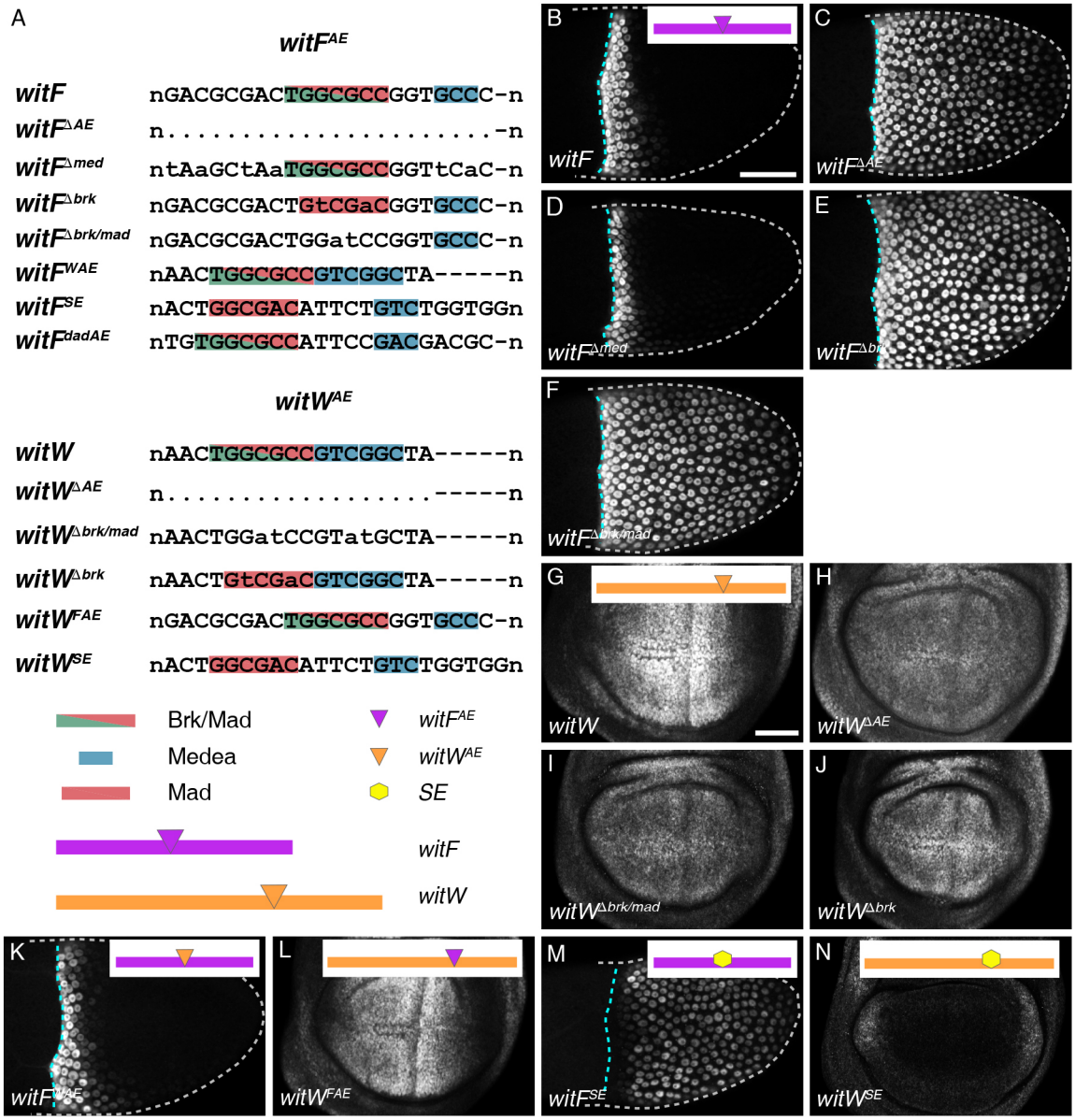

Fig. 5. *cis* analysis of *wit* enhancers. (A) Sequence of the AEs of *witF* (top) and *witW* (bottom) and sequence variants tested in the context of reporter constructs. Color coding for binding sites and reporter constructs is shown in the lower schematic. (B-F) Reporter expression of *witF* variants listed in A compared with wild-type *witF* (B) in follicle cells. Deletion of the AE (C), inactivation of the Brk-binding site alone (E), or inactivation of the Brk/Mad hybrid-binding site (F), result in uniform reporter expression throughout the epithelium. Inactivation of the putative Medea-binding site (D) has no effect on reporter expression. Dashed lines mark the anterior oocyte boundary (cyan) and egg chamber outline (gray). (G-J) Reporter expression of *witW* variants listed in A compared with wild-type *witW* (G) in wing imaginal discs. Removal of the AE (H) or inactivation of the Mad/Brk-binding sites (I) results in drastic reduction of reporter levels and expression throughout the wing disc epithelium. Targeted inactivation of the Brk-binding motif only results in expanded expression without affecting medial expression levels (J). (K,L) The AEs of *witW* and *witF* are able to fully restore wild-type expression pattern when inserted into *witF* (K) or *witW* (L) devoid of their cognate AEs, respectively. (M,N) Replacing the AE of *witF* with an SE element results in expression that is inverse to the *witF* pattern (M). Similarly, replacing the AE of *witW* with an SE results in a full pattern inversion with reporter expression being restricted to lateral-most cells of the disc (N). Scale bars: 50 µm. Egg chambers and wing imaginal discs of all panels shown have been processed and imaged in parallel and under identical conditions.

and tested for reporter activity in transgenic flies. Removing the potential AE in *witF* ($witF^{\Delta AE}$) resulted in uniform reporter expression throughout the follicular sheet (Fig. 5B,C). Similarly, the same manipulation in *witW* ($witW^{\Delta AE}$) resulted in reporter expression throughout the wing disc epithelium, but at levels that were lower compared with the parental *witW* (Fig. 5G,H). Importantly, in both cases the introduced deletions resulted in a complete loss of BMP responsiveness: neither *brk* nor *brk/Mad* mutant clones had any effect on $witF^{\Delta AE}$ or $witW^{\Delta AE}$ activity (Fig. S4). We next tested whether the differential requirement for Smads and Brk can be matched to sequence requirements within the identified motifs. Our genetic mosaic analysis predicts that only the Brk sites but not Smad-binding motifs within the AE of *witF* are relevant for reporter activity. Indeed, inactivating putative Med-binding sites ($witF^{\Delta med}$) had no effect on reporter activity (Fig. 5D). However, converting the Brk/Mad hybrid motif into a Mad-only binding site ($witF^{\Delta brk}$) resulted in the same strong upregulation of reporter expression as seen with $witF^{\Delta AE}$ (Fig. 5C,E) or with a construct lacking both Brk- and Mad-binding sites ($witF^{\Delta brk/mad}$; Fig. 5F). In the wing imaginal disc, and consistent with inputs from

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both transcription factors, inactivation of both Brk- and Mad-binding sites ($witW^{\Delta brk/mad}$) resulted in a lateral expansion of reporter activity and an overall reduction in expression levels similar to $witW^{\Delta AE}$ (Fig. 5H,I). Medial expression levels, but not the medial restriction, were restored by reinstating Smad input ($witW^{\Delta brk}$; Fig. 5J). The results of the *cis* analyses are in full agreement with the findings of the genetic mosaic analyses on the different effects of Smads and Brk on *witF* and *witW*. We conclude that BMP responsiveness of the CRMs is mediated by the identified AE-like sequences, which, however, differentially integrate the activities of the transcription factors of the pathway, the activated Smad complex and Brk.

AEs in *wit* do not contribute to tissue specificity

The sequences of the AEs of *witF* and *witW* deviate both from the original AE consensus motif and from each other. We sought to investigate whether such differences account for the observed differences in CRM behavior. Removal of the AEs exposed the existence of activators that produce spatially uniform, yet tissue-restricted, expression patterns (see above). This already indicates that sequences other than the AEs are essential for tissue specificity. Nevertheless, we directly tested for a contribution of the AEs to tissue-specific expression of their cognate CRM by generating transgenic reporters in which the two AEs where mutually exchanged ($witF^{WAE}$ and $witW^{FAE}$) (Fig. 5K,L). These reporters were found to be exclusively active in the epithelium defined by the CRM backbones and not the AEs. Thus, sequences other than the AEs dictate tissue specificity, whereas the function of the AEs is restricted to integrating BMP input. Consistent with this notion, replacing the AEs in both CRMs with an SE (see Introduction), resulted in expression patterns that are inverse to the parental reporter expression (i.e. no expression in cells with high pMad levels and high expression in *brk*-positive cells), without affecting tissue specificity (Fig. 5M,N).

AEs in *wit* do not account for differential responses to Smad and Brk

We next addressed whether sequence differences within the AEs account for the differences in Brk and Smad responsiveness of the two CRMs. In the follicle cells, the hybrid construct $witF^{WAE}$ was active in an anterior, wedge-shaped pattern, indicative of Brk-repression (see above and Fig. 5C). As expected, the expression was lost in *Mad* mutant clones (Fig. 6A-A″). Additionally, both *brk* mutant and *brk/Mad* double-mutant clones posterior to the endogenous stripe resulted in strong ectopic reporter expression (Fig. 6B-C″). At the same time, *brk/Mad* double-mutant clones overlapping the endogenous expression domain had no effect on reporter expression (Fig. 6C-C″). Thus, although the AE of *witW* integrates Brk and Smad input in the wing, it is only responsive to Brk when assayed in the context of *witF* in follicle cells. We observed a similar behavior of the *witW* AE in the context of *witF*. BMP-dependent activation of *witW* was found to be exclusively mediated by the indirect branch of the pathway (activation through repression of Brk). If sequence constrains within the AE of *witF* prohibit a direct input from the activated Smad complex, then $witW^{FAE}$ should transform into a 'Brk-only'-responsive CRM. However, when compared with the low and rather uniform expression of $witW^{\Delta AE}$, expression of $witW^{FAE}$ was not only lost in lateral cells but also increased in medial cells, consistent with both lateral repression by Brk and medial activation by Smads, respectively (see above and Fig. 5L). Indeed, $witW^{FAE}$ displayed the same responses as *witW*: although $witW^{FAE}$ was lost in *Mad* mutant clones (Fig. 6D-D″) and ectopically active in lateral *brk* mutant clones (Fig. 6E-E″), the simultaneous removal of Mad and Brk in medial clones could not reinstate peak levels of reporter activity (Fig. 6F-F″), indicating a direct role of Smads in reporter activation. Thus, the AE of *witF*, which responds only to Brk in its native context, integrates both Brk-dependent and Brk-independent Smad inputs when assayed in the *witW* environment.

The above results indicate that it is not the sequence of the AE but rather its context that dictates which branch of the BMP signaling pathway will be utilized for activation. In the simplest scenario, AEs, although tentatively able to integrate both Smad and Brk input, cannot do so in follicle cells. For instance, Smads might not be able to activate transcription directly because essential co-activators are not available in this tissue and, consequently, BMP-mediated activation of *witF* is restricted to the Brk-dependent branch. Indeed, limited co-factor availability has been elegantly demonstrated for Brk, which contains interaction motifs for multiple co-repressors allowing it to retain activity in tissues that lack its main partner Groucho (Upadhyai and Campbell, 2013). To address this possibility, we studied the activation of *Dad*, which requires direct and indirect Smad input in the wing for activation. Expression of *Dad* in the follicular epithelium has been studied using an enhancer trap and is suggested to depend on Smad activity but not on Brk removal (Chen and Schüpbach, 2006). Indeed, *Dad* reporters, including a ∼400-bp-long subfragment of *dad13*, *dad13A* (considered here as the minimal CRM of *Dad*), were found to be active in an anterior stripe that, unlike *witF*, did not appear wedge-shaped but rather coincided with the pMad stripe. In addition, clones lacking Brk had only minimal effects on reporter activity, whereas anterior clones lacking Mad or both Mad and Brk resulted in a complete or almost complete, respectively, loss of reporter expression (Fig. S5A-C″). These findings demonstrate that Smads are fully capable of activating targets independently of Brk in follicle cells and exclude the absence of the activatory branch as an explanation for the Brk-only responses of *witF* and $witF^{WAE}$. Following on from these findings, we addressed the behavior of the AE of *Dad* when the AE was replaced with *witF*. Notably, the chimeric $witF^{dadAE}$ reporter displayed the same wedge-shaped anterior expression stripe as *witF* (Fig. S5D-F) and was strictly responsive to Brk but not to Smads for activation as judged by clonal epistatic analysis (Fig. S5G-I″). Thus, the prototypic AE of *Dad*, which within *Dad* primarily integrates direct Smad activation, is converted to a 'Brk-only' element in the context of the basal *witF*. Lastly, we tested the reverse scenario, namely the behavior of the AE of *witF* when placed into the context of *dad13A* lacking its native AE. Consistent with the existence of very weak basal, uniform activity within *dad13A*, an AE-less *dad13A* construct ($dad13A^{\Delta AE}$) shows only weak and 'patchy' reporter expression throughout the follicular epithelium (Fig. S6A,B). Remarkably, inserting the AE of *witF* into this construct to generate a chimeric $dad13A^{FAE}$, fully reinstated the anterior stripe of expression, suggesting that the AE of *witF* is now able to respond to direct activatory Smad input (Fig. S6C). Furthermore, $dad13A^{FAE}$ displays features and genetic requirements that are typical for *dad13A*, rather than *witF*. First, the stripe of anterior expression is straight and not wedge-shaped along the dorsoventral axis. Second, reporter activity is only weakly sensitive to genetic removal of *brk* (Fig. S6D-D″), but almost completely lost in *Mad/brk* mutant clones (Fig. S6E-E″). Thus, the AE of *witF*, which in its native context responds solely to Brk, responds to Brk-independent Smad activatory input to boost anterior expression of an otherwise very weak basal CRM.

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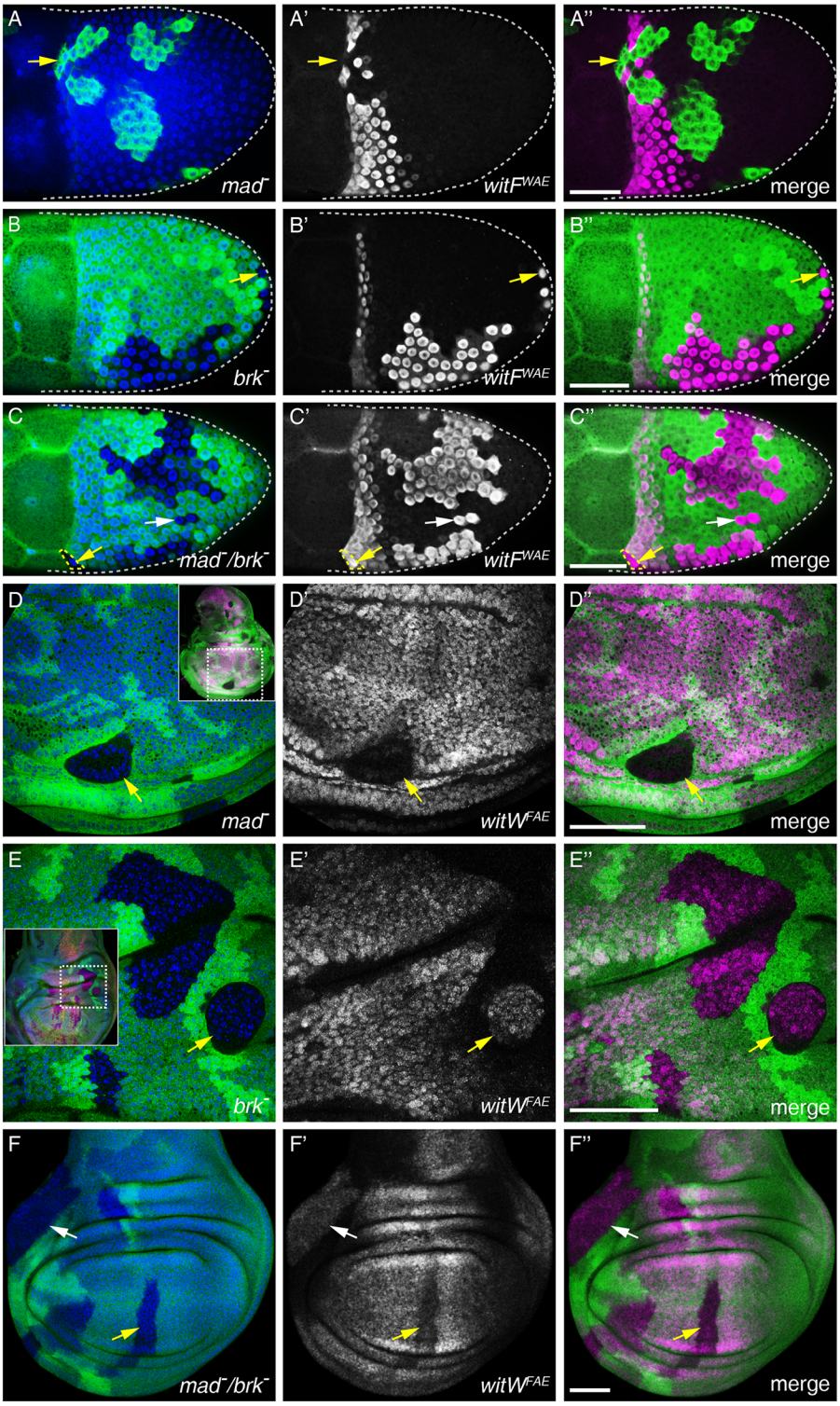

Fig. 6. The role of AEs in branch selection for BMP responsiveness. (A-C″) In follicle cell clones lacking *Mad* (MARCM clones, marked by GFP, A), *witF*^{WAE} reporter expression (gray, A′) is lost (yellow arrow). Loss of *brk* (yellow arrow in B-B″) or *Mad*/*brk* (yellow and white arrows indicate anterior and posterior clones in C-C″, respectively) in clones (marked by the absence of GFP in B,C) results in ectopic reporter expression at levels that match the endogenous expression levels at the anterior stripe (B′,C′). Thus, and similar to *witF* (compare with Fig. 4), the effects of BMP in *witF*^{WAE} are mediated by Brk, most probably by targeting the same Brk-binding site of the AE of *witW* found to be active in the wing imaginal disc. (D-F″) Medial clones lacking *Mad* (D), lack *witW*^{FAE} expression (D′,D″), and lateral clones lacking *brk* (E) display low levels of ectopic *witW*^{FAE} expression (E′,E″). Clones are indicated by yellow arrows. Medial clones double mutant for *Mad* and *brk* (F, yellow arrow) display cell-autonomous reduction, but not complete elimination of *witW*^{FAE} expression (F′,F″). Lateral clones of *mad*/*brk* double mutants (F, white arrow) display a similar weak ectopic activation of *witW*^{FAE} expression as seen in single *brk* mutants (F′,F″). Thus, similar to the parental *witW*, *witW*^{FAE} requires both indirect (Brk-dependent) and direct input from Mad, probably by utilizing the Brk- and Smad-binding sites present in the *witF* AE. Clones are marked by the absence of GFP. Nuclei are stained by Hoechst (blue, A-F). Scale bars: 50 µm.

DISCUSSION

In the present study, we establish *wit* as a transcriptional target of BMP signaling in two different epithelia, the wing imaginal disc and the follicle cells of the developing egg chamber. Given its function as a BMP receptor, *wit* adds to the small group of genes that are coordinately regulated by BMP signaling and are involved in BMP signal transmission and regulation. Such groups are often referred to as ‘synexpression groups’ to emphasize relationships in regulation and function (Karaulanov et al., 2004; Niehrs and Pollet, 1999). Accordingly, the *Drosophila* BMP synexpression group includes *Dad*, *wit*, *brk* and *pentagone* (*pent*; *magu*), which are either activated (*Dad*, *wit*) or repressed (*brk* and *pent*) by BMP in a variety of tissues and developmental stages (Hamaratoglu et al., 2014). The concept of synexpression predicts common strategies of transcriptional regulation; however, the activation of *wit* reveals substantial differences in comparison to *Dad*. This might mirror differences in expression and, potentially, requirement of Dad and Wit during fly development. *Dad* is activated by BMP signaling in

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all tissues and developmental stages analyzed so far – hence its widespread use as a reliable marker for BMP pathway activation. Moreover, expression of *Dad* is mediated by a single CRM (*dad13*), which seems to respond to BMP signaling in all contexts, including the wing imaginal disc and the follicle cells. In contrast, and despite its BMP-dependent activation in wing discs and follicle cells studied here, expression of *wit* is not always connected to BMP signaling. In contrast to *Dad*, *wit* is not expressed in all cells with active BMP signaling; for example, *wit* is not a BMP target in the early embryonic epidermis or in germline stem cells. In addition, there are instances in which *wit* transcription is independent of BMP signaling. The prominent neuronal expression of *wit*, for example, seems not to be induced by BMP signaling (Robin Vuilleumier and Douglas Allan, personal communication). This versatility in *wit* expression might explain the lack of a 'universal' (*Dad*-like) BMP-dependent CRM and instead necessitates distinct, tissue-restricted CRMs. This might also explain the pronounced differences in BMP input in the regulation of *wit* and *Dad* in follicle cells. Whereas *Dad* is directly activated by Smads in the follicular epithelium, activation of *wit* in the same tissue is delegated to tissue-specific factors, and the BMP input is exclusively mediated by Brk repression (see model in Fig. 7). In addition, the observed differences might complement quantitative constrains. In this case, the tissue-specific, uniform input would be strong enough to activate *wit* transcription through *witF* in follicle cells and BMP's role is de-repression (Brk repression) without providing direct activatory input. In contrast, BMP-independent inputs for *Dad* activation in the same tissue are extremely weak; hence, BMP signaling is primarily required for direct activation rather than alleviating Brk repression. Between these two extreme scenarios, it is conceivable that cues that direct low level basal activation (probably in *witF* and *Dad* in the wing) require both activatory Smad input as well as repressive Brk input to boost expression at regions of high pMad levels and erase expression at the low end of the gradient, respectively (Fig. 7).

At the molecular level, both enhancers of *wit* comprise similar, yet not identical, AE-like elements that fully account for BMP responsiveness to BMP signaling. Importantly, the sequence requirements within the AEs are fully consistent with our genetic analyses. Specifically, our finding that *wit* and *witF* are exclusively regulated by the indirect, Brk-dependent branch of the BMP pathway in follicle cells is fully supported by the mutational analysis, which identifies a clear requirement for the Brk-binding site – but not for the Smad sites – within the AE of *witF*. Similarly, transcription of *wit*/*witW* in the wing integrates both direct and indirect BMP inputs and, indeed, mutations that inactivate either the Brk- or the Smad-binding sites of the AE predictably affect the activity of *witW*.

The sequences of the identified AEs in *witW* and *witF* deviate from the prototypic AE of *Dad* and from each other; however, these differences, despite being evolutionarily conserved, do not seem to have functional consequences and do not account for the different behavior of the cognate CRMs. Consequently, *witF* and *witW* respond to Brk alone or to Brk/Smad inputs, respectively, even when their AEs are swapped. An extreme demonstration of the latter phenomenon is exemplified by the *Dad* and *witF* chimeric constructs in the follicular epithelium. The original AE of *Dad* strongly responds to Smad input in the context of the *Dad* CRMs; however, placing this element into the *witF* backbone fully overrides its ability to respond to Smads and converts it into a Brk-only response element. In reverse, the AE of *witF*, which responds exclusively to Brk in its native context, responds to Brk-independent Smad activation in the context of the *Dad* CRM. Notably, our results are in agreement with a recent study focusing on

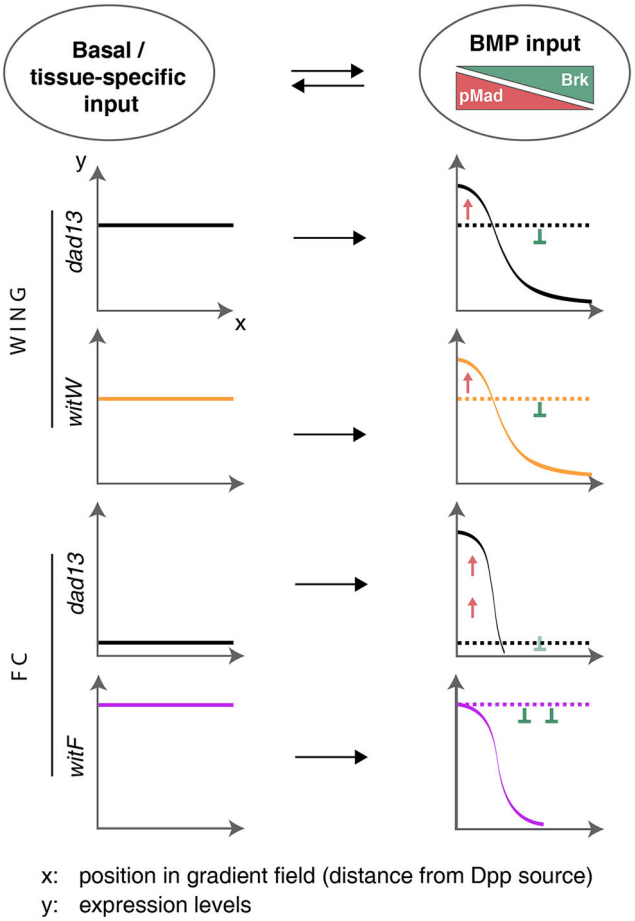

Fig. 7. The role of AEs in BMP-dependent gene activation. Summary of BMP-dependent regulation of *Dad* and *wit* in the wing and follicular (FC) epithelium. In both tissues, the primary extracellular BMP gradient generates gradients of nuclear pMad and Brk that are in inverse relation to each other. In the wing disc, tissue-specific factors activate uniform expression of *Dad* (*dad13*, black) and *wit* (*witW*, orange), which is shaped by inputs of both nuclear gradients: Basal CRM activity is increased by pMad in cells near the morphogen source (medial cells, only half of the bilateral gradient is schematically depicted here) and counteracted by increasing Brk levels in more distal cells. In the follicle cells (FCs), the basal activity of *dad13* is marginal and expression is strongly activated by pMad. In contrast to the wing, Brk plays only a subordinate role in shaping *Dad* expression in this tissue. At the same time, BMP-dependent activation of *wit* in FCs is mediated by a distinct CRM, *witF* (purple). *witF* receives strong and uniform basal activatory input and relies solely on Brk-repression to integrate BMP signaling activity.

evolutionary diversification of *wit* expression between *Drosophila* species as illustrated by differences in the width of the anterior *wit* stripe in *D. melanogaster* and *D. virilis* egg chambers (Marmion and Yakoby, 2018). Marmion and Yakoby independently identify Brk and the Brinker-binding site as the mediators of BMP-dependent *wit* expression. At the same time, they demonstrate that differences in the sequences immediately flanking an otherwise identical Brk-binding motif do not account for the observed differences in the expression patterns between the two *Drosophila* species. The findings cumulatively suggest that the CRM environment, rather than the BMP-response element itself, dictates how the latter will respond to BMP signaling. Such CRM activatory input(s), although able to impact on and equalize the output of any AE variant, have limitations as they cannot depolarize the activity of the SEs. The nature of these activatory elements, which obviously also implement tissue specificity, as well as their integration with the

BMP-response elements, need to be elucidated in future studies. It is unclear whether the underlying mechanisms affect binding of Brk and Smads to their cognate sites or whether the decision is made after their binding to the AE. In any case, our data clearly highlight an unexpected flexibility in the structure of the AE, which needs to be considered when employing such elements for *in silico* detection of BMP target CRMs and genes.

MATERIALS AND METHODS

Fly stocks and mosaic mutant analysis

brkB-GFP and *brkB14-GFP* were used to visualize *brk* expression in follicle cells and wing disc, respectively (Charbonnier et al., 2015). The *dad13-lacZ* fly line has been previously described (Weiss et al., 2010). The following fly lines and chromosomes were used for mosaic analyses: $brk^{M68}FRT18A$, $mad^{12}FRT40A$, and corresponding *FRT* chromosomes carrying *ubiGFP* constructs or mosaic analysis with a repressible cell marker (MARCM) components. *Mad/brk* double-mutant clones were generated using $[brk^{BAC}]ubiGFPFRT40A$ (gift from K. Basler, Institute of Molecular Life Sciences, University of Zurich, Switzerland) in a *brk* mutant background; $[brk^{BAC}]$ is a genomic rescue construct of *brk* inserted on 2L22A (Charbonnier et al., 2015; Schwank et al., 2008). FRT-mediated FLP-out clones were generated using (*ywhsFLP; Sp/CyO; act>CD2>Gal4, UASGFP*) and Dpp signaling was altered by utilizing *UAS-dad* (Tsuneizumi et al., 1997) and $UAS\text{-}tkv^{QD}$ (Nellen et al., 1996). Larvae (72-96 h after egg laying) or female flies (3- to 5-days old) were subjected to a 37°C heat shock for 1 h or 7-10 min for the generation of mitotic mutant clones or flip-out clones, respectively. Wing discs and ovaries were dissected 48 h after heat shock treatment. A detailed list of fly stocks used in each panel of this study is provided in Table S1.

Reporter constructs and fly transgenesis

PCR was used to amplify genomic sequences from *wit* and *Dad* loci including introduction of deletions or point mutations. All reporter fragments were subcloned into the *placZattB* reporter vector and verified by sequencing. A detailed list of primers used to generate the reporter fragments is provided in Table S2. All constructs were inserted by PhiC31/attB-mediated integration into chromosomal position Chr3L, 68A4 (attP2) (Bischof et al., 2007; Groth, 2004).

Immunohistochemistry and microscopy

Drosophila female ovaries and third instar larvae were dissected and fixed in 4% paraformaldehyde/Schneider's S2 medium for 10 min. After multiple washes with PBSTx (1× PBS and 0.1% Triton X-100), samples were incubated with primary antibodies overnight at 4°C. After washing, secondary antibodies were incubated for 2 h at room temperature. The following primary antibodies were used: mouse anti-Wit (1:10; 23C7, Developmental Studies Hybridoma Bank, DSHB) (Aberle et al., 2002), mouse anti-β-Gal (1:500; Z3781, Promega), rabbit anti-β-Gal (1:500; 55976, MP Biomedicals) and chicken anti-GFP (1:1000; ab13970, Abcam). Alexa fluorophore-conjugated secondary antibodies (1:500; A11031, A11039 and A11036, Molecular Probes) and Hoechst 33342 (1:5000; H3570, Invitrogen) were used. Images were obtained using a Nikon C2 confocal microscope and processed with ImageJ and Adobe Photoshop. All images of larval wing discs and egg chambers are positioned posterior to the right and dorsal up.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.C., V.V., M.J., G.P.; Investigation: M.C., V.V., M.J., G.P.; Data curation: M.C., V.V., M.J., G.P.; Writing - original draft: G.P.; Writing - review & editing: M.C., G.P.; Visualization: M.C., V.V., M.J., G.P.; Supervision: G.P.; Project administration: G.P.; Funding acquisition: G.P.

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Supplementary information

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