Repression of Dpp Targets by Binding of Brinker to Mad sites

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SUMMARY

Signaling by Decapentaplegic (Dpp), a Drosophila member of the TGFβ superfamily of growth factors, has recently been shown to activate targets such as vestigial (vg) indirectly through negative regulation of brinker (brk). Here we show that Brk protein functions as a repressor by binding to Dpp response elements. Brk’s DNA binding activity was localized to an amino-terminal region containing a putative homeodomain. Brk bound to a Dpp response element of the Ultrabithorax (Ubx) midgut enhancer at a sequence that overlaps a binding site for the Smad protein, Mothers Against Dpp (Mad). Furthermore, Brk was able to compete with Mad for occupancy of this binding site. This recognition of overlapping binding sites provides a potential explanation for why the G/C-rich Mad binding site consensus differs the Smad3/Smad4 binding site consensus. We also found that the Dpp response element from Ubx was more sensitive than the vg quadrant enhancer to repression by Brk. This difference correlates with short-range activation of Ubx by Dpp in the visceral mesoderm, while vg exhibits a long-range response to Dpp in the wing imaginal disc, indicating that Brk binding sites may play a critical role in limiting thresholds for activation by Dpp. Finally, we provide evidence that Brk is capable of functioning as an active repressor. Thus, while Brk and Mad compete for regulation of Ubx and vg, Brk may regulate other Dpp targets without direct involvement of Mad.
INTRODUCTION

Members of the TGFβ super-family of growth factors perform a multitude of functions in regulating cell growth and differentiation(1-4). The nuclear effectors of these pathways are the Smad transcription factors(5). The activin/TGFβ pathways trigger activation of Smad2 and Smad3, while the bone morphogenetic protein (BMP) pathways utilize Smad1, Smad5 and Smad8. Each of these receptor-activated Smads is phosphorylated in response to signaling, leading to assembly of complexes with Smad4 and translocation to the nucleus, where association with cofactors results in regulation of target genes(6-9).

Signaling by the Drosophila TGFβ family member Decapentaplegic (Dpp) plays an important role in patterning of the dorsal-ventral axis, visceral mesoderm, endoderm, wing, leg and eye(10-16). An important feature of Dpp signaling is its ability to trigger the activation of target genes at different thresholds, allowing cells to interpret position along a Dpp gradient(12;17). The best understood example of this is the primoridia of the wing, where spalt (sal), optomotor blind (omb) and vestigial (vg) are expressed in progressively wider patterns in response to a postulated gradient of extracellular Dpp protein(18-22). This graded Dpp activity emanates from a stripe of Dpp-expressing cells that bisects the wing imaginal disc(23). Dpp signaling triggers phosphorylation of the founding member of the Smad family, Mothers Against Dpp (Mad)(24-27). Activated Mad associates with the Smad4 homolog, Medea(28-31), and directly regulates target genes such as vg, Ubx and tinman (tin)(32-34). Mad binds to
GC-rich sites with the consensus sequence GCCGnCGC(32), while vertebrate Smad 3 and Smad4 bind preferably to two or three tandem copies of the 4 bp Smad box sequence, GTCT(35;36). The Mad consensus bears a limited resemblance to an inverted pair of Smad boxes, but it is not clear why the two sequences differ. The Mad consensus may extend to the vertebrate BMP-responsive Smads since Smad1 acts through a Mad-like site to activate the Smad6 gene (37;38). One possibility is that the Mad consensus reflects involvement of a cofactor, consistent with the general dependence of Smads on cofactors (39;40).

An unexpected twist to the Dpp regulatory cascade came to light with the discovery of brinker (brk), a gene that is negatively regulated by Dpp and, in turn, functions as a negative regulator of Dpp targets(41-44). In the wing imaginal disc, Dpp targets such as sal, omb and vg are expressed ectopically in brk^{−} clones. Ectopic expression of these genes even occurs in cells where a brk^{−} clone overlaps a clone mutant for Mad or the Dpp receptor thickveins (tkv).

These results revealed that Dpp activates targets indirectly through repression of brk, which has recently been shown to occur in conjunction with the Mad cofactor, Schnurri(45;46). However, the Dpp response elements of vg, Ubx and tin have been shown to be dependent on Mad binding sites for the ability to direct Dpp-dependent reporter expression(32-34). Together, these observations suggest that Dpp activates targets by two distinct mechanisms: (1) direct activation by Smads, and (2) indirect activation by repression of brk. Brk protein contains a potential homeodomain and is localized to the nucleus (43), consistent
with its functioning as a repressor. Brk antagonized BMP2 signaling when expressed ectopically in Xenopus embryos, suggesting that it may be a conserved feature of Dpp/BMP pathways(41).

Here, we report that Brk protein binds and represses the Dpp response elements of vg and Ubx. We show that a previously identified Mad site is actually a composite Mad-Brk site and show that Mad and Brk are capable of competing for binding to this site. These observations suggest an explanation for why the Mad binding site consensus differs from that of Smad3 and Smad4. In addition, we found that a Dpp response element from Ubx is more sensitive to Brk repression than an element from the vg quadrant enhancer. These results correlate with the respective short- and long-range thresholds of Ubx and vg, and indicate that Brk binding sites may play an important role in setting thresholds for activation by Dpp. Our results concur with other recent reports that Brk functions as a DNA-binding repressor(47-49).

EXPERIMENTAL PROCEDURES

Plasmids

All reporter plasmids were based on the vector hsplacCasper(50). VgMD2-lacZ was a gift from A. Hudson and S. Carroll, and contained two tandem copies of the core Dpp response element,

TAGCCTGCCCCTCGCGATTCCGACAACCTTTGGGCGGACGTTGGGAGTGTG
CCATGCATGCTGATGA,

separated by a 25 bp linker and inserted between the BamH1 sites of hsplacCasper. The 2xUbx-lacZ reporter described in Figures 1
and 4 contained two tandem copies of the core Dpp response element,
AATTGGACTGGCGTCAGCGCCGGCGCTG, inserted between the EcoR1 and Kpn1 sites of hsplacCasper. The 2xUbxM11 and 2xUbxM13 plasmids described in Fig. 4 were identical to 2xUbx except for the base substitutions indicated in Fig. 4A. 4xSuh-lacZ was a gift from C. Nelson and S. Carroll and contained the synthetic sequence,
AATTGTTCACGGATCCAAAGGTTCCTACGAGATCTGTTCTCACGGATCCAAAGGTTCTCACGCGATATCC
AAGGTTCCTCACGGAATTCCGGATC, inserted between the EcoR1 and Kpn1 sites of hsplacCasper. 6xBrk4xSuh-lacZ was derived from 4xSuh-lacZ by insertion between the EcoR1 and Kpn1 sites of the synthetic sequence,
AATTAGCACCACCGCTGTACAGCGCCGGCGCTGTAATTAGCGCCGGCGCTGAC.

Effector plasmids for activated Tkv, Brk, Smad3, Smad4, activated ActR1B, Su(H) and activated Notch were based on the actin5C promoter vector pPacPL. Effector plasmids for Mad and Med were a gift from R. Padgett(27) and were based on the metallothionine promoter vector pMK33. pPac-Brk was generated by cloning a 3.4 kb Brk cDNA (gift from C. Rushlow) between the BamH1 and Not1 sites of pPacPL. In addition to the Brk coding region, the resulting plasmid contains ~500 bp of 5’ untranslated region (UTR) and ~800 bp of 3’ UTR. The pPacSu(H) and pPacN\textsuperscript{act} plasmids were a gift from T. Wittkopp and S. Carroll.

To create an MBP-Brk fusion protein, PCR was used to position an EcoR1 site 3 bp upstream of the Brk initiator ATG. The resulting clone was used to
create MBP-Brk by insertion between the EcoR1 and Hind3 sites of pMAL2C. MBP-BrkHD was generated by deleting sequences between a PstI site 517 bp downstream from the Brk ATG, and a Hind3 site at the 3’ end of the cDNA.

Transfections and Reporter Assays

Transfections of Drosophila S2 cells were carried out as described previously (36). A chemiluminescent β-galactosidase assay was performed on cell extracts using the GalactoStar assay system (Tropix, Inc.) according to supplier’s instructions. For sets of transfections involving the use of cDNAs cloned into the pMK33 metallothionine promoter vector, CuSO₄ was added to cultures 24 hrs after transfection to a final concentration of 0.3 mM. Standard deviations were calculated from the results of triplicate assays.

Purification of Fusion Proteins and Band Shift Assays

MBP-Brk fusion proteins were expressed, affinity-purified using amylose-agarose, and used in band shift assays as previously described (36) with modification of the binding buffer (3% ficoll, 20 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 0.5 mg/ml bovine serum albumin, 0.02% green food coloring), an increase in reaction volume to 50 μl (5 μl loaded) and use of smaller format (10 x 8 x 0.15 cm) 5% polyacrylamide gels. Purification of the MBP-BrkHD used for the band shift assay shown in Fig. 1C included an additional step of incubating affinity-immobilized protein with 5 μg/ml RNase A,
125 un/ml Exonuclease III and 12.5 un/ml DNase I for 1 hour at room temperature as a means of reducing contaminating nucleic acid.

RESULTS

Brinker Represses Dpp Response Elements

Dpp activates expression of vg in cells of the Drosophila wing imaginal disc through the quadrant enhancer, which is located within vg’s fourth intron(22). The quadrant enhancer contains two Mad binding sites, both of which contribute to Dpp-responsive expression(32;51). Two copies of a 60 bp fragment containing these adjacent sites (referred to as 2xMD2) is sufficient to activate a lacZ reporter in an essentially normal quadrant pattern (A. Hudson and S. Carroll, personal communication). It was previously shown that clones of homozygous mutant brk\(^{-}/brk\^-\) cells in wing imaginal discs of brk\(^{-}/+\) heterozygous larvae express vg ectopically when they occur outside the wing primordia (43), demonstrating that brk normally prevents vg expression from extending to hinge and notum regions that surround the wing proper. Limitation of 2xMD2-lacZ expression to the wing primordia suggests that brk might repress through the MD2 element, although it is also possible that control is indirect by means of an intermediate wing-specific transcription factor such as Scalloped or Drifter (51;52).

To determine whether Brk represses the MD2 element, we cotransfected Drosophila S2 cells with the 2xMD2-lacZ reporter and varying amounts of effector plasmids that express brk, Mad, Med, and an activated version of the
type I Dpp receptor, *thickveins (tkvQD)* (Fig 1A, left panel). Reporter expression was induced ~4-fold by expression of *Mad, Med*, and *tkvQD*, and this was blocked by cotransfecting 10 ng of pPac-*brk* plasmid. Levels of pPac-*brk* up to 0.4 ng had no effect. This result shows that Brk is capable of repressing *vg* through a small, Smad-responsive target element.

Expression of *vg* throughout the wing primordia at a distance from the source of Dpp may reflect a relatively low sensitivity to *brk*. In contrast, *Ubx* responds to Dpp in the visceral mesoderm only in those cells that actually express *Dpp*. We tested whether Brk contributes to this short-range Dpp-responsiveness of *Ubx* by determining whether Brk could repress the *Ubx* midgut enhancer element (53) in S2 cells. A *lacZ* reporter driven by two copies of a 30 bp fragment from the Dpp-response element of *Ubx* was co-transfected with combinations of effector plasmids expressing *Mad + Med + tkvQD*, or Brk (Fig. 1A, right panel). The 2x*Ubx-lacZ* reporter behaved much like 2xMD2-*lacZ* in response to *Mad + Med + tkvQD*, but required only 0.08 ng of pPac-*brk* to be repressed. Thus, in this transient transfection system, the *Ubx* element was about 100-fold more sensitive than *vg* MD2 to repression by Brk. This result fits with the expectation that Brk affects target gene sensitivity to Dpp.

*Brinker Binds to Dpp Response Elements*

Two bacterially-produced Brk fusion proteins were generated and tested by means of the band-shift assay for the ability to bind to DNA of the *Ubx* Dpp response element. MBP-Brk was a fusion of full-length Brk to the C-terminus of
maltose binding protein, while MBP-BrkHD contained an amino-terminal fragment harboring a putative homeodomain (Fig. 1B). Both were prepared from bacterial lysates by affinity purification with amylose beads. Despite the inclusion of a protease inhibitor cocktail during purification, fractionation on an SDS polyacrylamide gel showed that the major protein in both preparations was a ~52 Kd degradation product, with only minor amounts of the larger apparent primary products (data not shown). This 52 Kd size suggests that both fusion proteins were susceptible to cleavage just C-terminal to the putative homeodomain. Consistent with this interpretation, both preparations bound to the Ubx DNA probe (Fig. 1C; probe sequence given in Fig. 2A), while non-fused MBP lacked detectable binding activity (not shown). Approximately 4 ng of either preparation was sufficient to shift half of the Ubx probe. This corresponds to a dissociation constant of $10^{-9}$ M, a value that is in the range commonly observed for homeodomain-DNA interactions(54). Both Brk fusion protein preparations yielded multiple shifted bands, indicating that binding occurred at more than one site. In parallel, the MBP-BrkHD preparation was tested for binding to a vg probe comprising most of the MD2 element (Fig. 1C, right panel; probe sequence given in Fig. 2A). Forty nanograms of MBP-BrkHD was required to shift half of the vg probe, about ten times the amount needed to shift half the Ubx probe. As a second means of measuring the relative affinities of MBP-Brk for the Ubx and vg sequences, unlabeled preparations of the same Ubx and vg double-stranded oligos were used to compete for binding of labeled Ubx probe by a low concentration of MBP-Brk (Fig. 1D). A 50% reduction in binding of probe was
observed with a ~10-fold excess of \textit{Ubx} competitor, or a ~300-fold excess of \textit{vg} competitor. Thus, as determined by either method, the low affinity of Brk for the \textit{vg} probe relative to the \textit{Ubx} probe correlates with the lower relative sensitivity of the \textit{vg} MD2 element to repression by Brk in the transfected cell reporter assay. In contrast, a Mad fusion protein, MBP-MadNL (MH1 domain + linker), bound the \textit{Ubx} and \textit{vg} probes with approximately equal affinities (Fig. 1C, right panel). Together, these results suggest that differences in the Dpp response thresholds of \textit{Ubx} and \textit{vg} may be primarily a function of differential affinity for Brk rather than for Mad.

\textit{The Ubx element contains overlapping binding sites for Mad and Brinker}

Binding of MBP-Brk to the \textit{Ubx} and \textit{vg} probes generated multiple bands, possibly indicating that Brk bound to more than one site. The \textit{Ubx} element contains an inverted repeat of GGCGCT (solid arrows, Fig. 2A) that overlaps a previously identified Mad binding site (boxed). While the Mad site embedded in this repeat resembles the \textit{vg} Mad site, the repeat as a whole is only matched at 7 of 12 positions in \textit{vg}. We tested MBP-Brk for the ability to bind one copy of this sequence in a DNA probe that was otherwise divergent in sequence from the \textit{Ubx} element (Fig. 2A, left panel). MBP-Brk bound to the “GGCGCT” probe with affinity that was similar to its affinity for the \textit{Ubx} probe (Fig. 2A, right panel) and yielded a single major shifted band at about the same position as the lower-most band observed with the \textit{Ubx} probe. Although two weak upper bands were also
observed with the GGCGCT probe, overall, these results are consistent with high affinity interaction of MBP-Brk with just one site in the GGCGCT probe.

To investigate the specificity of Brk for the GGCGCT sequence, the effects of single base pair substitutions were determined. This was done measuring the ability of unlabeled “wildtype” (GGCGCT) and mutant DNAs to compete with the labeled GGCGCT probe. Fig. 2B shows a gel comparing the ability of GGCGCT and AGCGCT (mutant M1) probes to compete away binding of MBP-Brk to labeled GGCGCT probe. Note that M1 competitor was tested over a 10-fold higher concentration range than the GGCGCT competitor. Measurements of bound and free probe DNA from this gel and from parallel band-shift assays using eight other mutant competitors were used to generate the binding curves shown in Fig. 2C. The concentration of competitor resulting in an ordinate value of 0.5 provides a relative measure of affinity for the competitor DNA. The results are tabulated below the graph in Fig. 2C. In all, five mutants exhibited an ~20-fold reduction in the binding affinity, while the least critical position contributed as much as 3-fold to binding affinity. These results indicate that Brk makes base-specific contact across the entire GGCGCT sequence.

The GGCGCT repeat in the Ubx element overlaps a Mad binding site that can be modeled as consisting of two degenerate Smad boxes (Fig. 2A), suggesting that Brk may compete with Mad for binding. This could not be determined unequivocally using the Ubx probe because MBP-Mad and MBP-Brk complexes had nearly identical mobilities in the bandshift assay. However, the GGCGCT probe formed a complex with MBP-Mad that was easily resolved from
the main complex formed with MBP-Brk; with this probe, it was clear that formation of MBP-Brk complexes correlated with reduced binding of MBP-Mad (Fig. 3). In contrast, the same amount of MBP-Brk did not reduce binding of MBP-Mad to the M7 probe (Fig. 3), evidence that MBP-Brk reduced the level of MBP-Mad binding by competition rather than by sequence-independent inhibition.

Repression by Brinker is Disrupted by Mutation of its Binding Sites

To determine whether the Brk binding sites identified using the band shift assay are actually required for repression, the Ubx element was mutated to disrupt Brk binding. Each of three GGCGC/T sequences was changed to GTCG, or to GGCGA (Fig. 4A, top), both of which dramatically reduce Brk binding but still allow Mad to bind (Fig. 4A, bottom panels). Introduction of the same triple-substitutions into the 2xUbx-lacZ reporter resulted in an approximately 100-fold decrease in sensitivity to repression by cotransfected pPac-Brk (Fig. 4B, compare 0.08 ng pPac-Brk in top panel with 10 ng in lower two panels). These results demonstrate that Brk binding sites are required for repression and confirm that the sequence specificity characterized in band-shift experiments is also observed in cells.

Brinker is Capable of Active Repression

The overlap of Mad and Brk binding sites in the Ubx midgut element suggests that Brk might repress Dpp targets by simply competing with Mad for
occupancy of an enhancer element. However, repressors generally function by quenching the activating potential of transcription factors bound nearby or by means of long-range interfering effects on the general transcription machinery(55). To determine whether Brk is capable of functioning as an active repressor, we positioned Brk binding sites adjacent to sites for the unrelated Notch-responsive activator, Suppressor of Hairless [Su(H)], and monitored reporter expression in response to cotransfected Brk, Su(H) and activated Notch effector plasmids (Fig. 5). Brk completely prevented activation by Su(H), while a control reporter containing only Su(H) sites was repressed only 2-4-fold, an effect that may have been caused by the presence of a single Brk binding site adjacent to the hsp70 TATA box. Given this ability of Brk to function as a generic active repressor, it is reasonable to speculate that Brk might control a subset of Dpp targets without direct involvement of Mad.

DISCUSSION

We have shown that Brk acts on two Dpp targets by binding to functional Mad sites. Mutational analysis indicates that Brk and Mad compete for binding to overlapping sites, but that the sequence specificity of Brk is distinct from that of Smads. Thus we were able to identify mutations that disrupt Brk repression of Ubx with little effect on activation by Mad. This is consistent with the results of previous experiments which demonstrated that Mad sites were required for activation of several Dpp targets(32-34).
The overlap of Brk sites may explain why the Mad binding site consensus differs from the consensus identified for Smad3 and Smad4 (Fig. 6). The Mad consensus aligns perfectly with an inverted pair of GGCGCT Brk binding sites, as they occur in the Ubx element (Fig. 6). It is also apparent that the Mad consensus can be modeled as a pair of degenerate Smad boxes that align with a pair of Smad boxes arranged as AGAC GTCT, an orientation that is inverted in comparison to the Smad3-Smad4 consensus, GTCT AGAC. These alignments suggest that the Mad consensus reflects dual utilization by Brk and Mad-Med complexes. While Mad-Med complexes also recognize the Smad3/Smad4 consensus, Brk does not (unpublished results). In contrast, competition of Mad and Brk for binding to GCCGnCGC-type sites places targets under the control of both positive and negative branches of the Dpp pathway. Such dual control may be important in generating target response thresholds that span a wide range of Dpp levels. Smad1 activation of the human Smad6 promoter through a Mad-like binding site (37) suggests that a vertebrate Brk homolog might compete with Smad1, Smad5 or Smad8 for regulation certain BMP targets.

How can the requirement for Mad binding sites in certain Dpp targets be reconciled with experiments showing that Dpp targets, including vg, become independent of Dpp signaling in cells lacking functional brk? If Brk alone acts on such sites, mutations that disrupt Brk binding should result in elevated or ectopic expression rather than reduction. The conclusion that Mad acts on such Dpp targets only indirectly through Brk is based on the assumption that Dpp signaling is blocked completely in brk− cells that are also mutant for tkv7 or Mad1,2 (41-43).
However, the type I Dpp receptor encoded by *saxophone*, while normally insufficient in the absence of *tkv*, provides a low level of signaling in response to Dpp(56) that may become adequate when target response thresholds are reduced by the absence of Brk. Similarly, while the hypomorphic activity of the *Mad*<sup>1.2</sup> allele is normally inadequate, it may suffice in the absence of Brk. Thus, a low level of Dpp signaling could still be required even without Brk.

While convergent target specificity intertwines Brk and Mad function, as an active repressor Brk is also capable of functioning independently of Mad. Thus while Dpp targets are subject to competition between Mad and Brk, others may respond to Mad and Med only indirectly through their cascading affects on the expression of *brk*. The Dpp response elements of such genes may contain Brk binding sites arranged in a way that precludes binding by Mad/Med complexes, or such elements may simply lack nearby binding sites for Mad/Med cofactors.

ACKNOWLEDGEMENTS

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REFERENCES


**FIGURE LEGENDS**

Fig. 1. **Brk differentially represses and binds the Dpp response elements of vg and Ubx.** A, β-galactosidase reporter assays showing that vg-
lacZ and Ubx-lacZ reporters are repressed by Brk in Drosophila S2 cells. “+” indicates 10 ng each of effector plasmids for Mad, Med and activated Tkv. B, Diagrams of the full-length (Brk) and truncated (BrkHD) Brinker proteins showing positions of the putative homeodomain (HD), PMDLS motif, and intervals enriched in glutamine (Q), histidine (H) or alanine (A) (adapted from (42)). C, Band shift assays showing binding of MBP-Brk, MBP-BrkHD and MBP-MadNL to Ubx and vg DNA probes. Probe sequences are shown in Fig. 2A. Indicated amounts of protein preparation were included in 50 µl binding reactions together with 10⁻¹¹ M of the indicated probe. Arrows indicate bound and free DNA complexes. D, Band shift assay showing competition of unlabeled Ubx and Vg probes for binding of labeled Ubx probe to MBP-Brk. Each 50 µl binding reaction contained 3 ng of MBP-Brk protein preparation and 10⁻¹¹ M labeled Ubx probe.

Fig. 2. Brk binds to a GGCGCT motif that overlaps a Mad binding site. A, At left is shown an alignment of Mad binding sites within Dpp response elements of vg and Ubx, and the sequence of a band-shift probe containing one copy of the putative Brk binding site, GGCGCT (capitalized letters). Dotted arrows mark degenerate Smad boxes; solid arrows mark the putative Brk sites in Ubx. To the right is a band shift assay showing binding of MBP-Brk to the GGCGCT probe. The indicated amounts of protein are per 50 µl binding reaction, and probe concentration is 10⁻¹¹ M. Arrows indicate bound and free DNA complexes. B, Example of a competition band shift assay showing that GGCGCT competitor is ~ 20-fold more effective than M1 competitor (M1
sequence differs by a single G > A substitution, i.e., AGCGCT). Each 50 µl binding reaction contained the indicated concentration of unlabeled competitor DNA along with 3 ng of MBP-Brk protein preparation and 10⁻¹¹ M labeled GGCGCT probe. C, Results of competition band shift assays comparing the affinity of MBP-Brk for the GGCGCT probe (designated “wildtype”) and for a series of single base pair substitutions, M1-M9. Assays were performed in parallel as described for panel B. Ratios of bound:free probe were calculated from measurements made with a Phosphor Imager. Relative binding affinities were derived from competitor concentrations that reduce the bound:free ratio of labeled probe by 50%, i.e., an ordinate value of 0.5. The resulting values were used to calculate fold-reduction in binding affinity for each mutant competitor relative to the GGCGCT (“WT”) competitor.

Fig. 3 Brk competes with Mad for binding to the Ubx Dpp response element. Shown are two band shift assays demonstrating that 3 ng of MBP-Brk preparation competes effectively for binding of MBP-MadNL to the GGCGCT probe, but does not compete effectively for binding to the M7 probe. Probe sequences are shown in Fig. 2C. Indicated nanogram amounts of protein preparation were included in 50 µl binding reactions together with 10⁻¹¹ M of the indicated probe. Arrows indicate bound and free DNA complexes.

Fig. 4. Weakened Brk binding in vitro correlates with disrupted repression of Ubx. A, Band-shift assay showing the effects of multiple base
substitutions on Brk binding affinity. The base substitutions contained in the UbxM11 and UbxM13 probes are shown above images of band shift assays comparing these probes to the wild-type Ubx probe for binding of MBP-Brk (upper gel) or MBP-MadNL (lower gel). The indicated amounts of protein are per 50 µl binding reaction, and probe concentration is 10^{-11} M. Both mutants reduced MBP-Brk binding affinity, while M13 had no effect on MBP-MadNL binding. Compared to wild-type and M13, binding of MBP-MadNL to M11 yielded a similar bound:free ratio at 300 ng protein, but greatly reduced ratios between 10 and 100 ng. The mechanism responsible for this apparently cooperative effect remains to be determined. B, Reporter assays showing that two copies of UbxM11 and UbxM13 exhibited ~100-fold reduced sensitivity to co-expressed Brk relative to two copies of the wild-type Ubx sequence. “+” indicates 10 ng each of Mad, Med, and activated Tkv effector plasmids.

**Fig. 5 Brk can function as an active repressor.** Shown are reporter assays demonstrating that Brk can effectively repress activation by Su(H) through non-overlapping binding sites (6xBrk/4xSuh-lacZ). In comparison, the 4xSuh-lacZ control plasmid lacking Brk binding sites is less sensitive to Brk, although it still exhibits 2-4-fold repression. The basis for this “basal” response to Brk is not clear, however, we note that the hsp70 promoter sequence contained in the hsplacCasper reporter vector does have an apparent Brk site, GGCGCT, located 5 bp upstream from the TATAAA sequence.
Fig. 6  **Model for overlap of Brk and Mad binding sites.**  
A. The optimal arrangement of inverted Smad boxes, as determined for binding by Smad1, Smad3 and Smad4, is aligned with an inverted arrangement similar to the Mad binding site consensus and to the Brk sites of the *Ubx* midgut enhancer. Bases that match the Mad consensus are boxed. Arrows above the first two lines mark Smad boxes, those below the bottom line the inverted Brk binding sites.
Kirkpatrick, Fig. 1

A

![Graph showing Vg MD2-lacZ reporter activity with Mad/Med/Tkv levels ranging from 0 to 10 ng pPac-Brk.](image)

B

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<th>Brk HD</th>
<th>H A</th>
<th>Q PMDLS</th>
<th>H</th>
<th>Q</th>
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B

![Graph showing relative β-galactosidase units with Mad/Med/Tkv levels ranging from 0 to 10 ng pPac-Brk.](image)

C

![Western blot analysis with Ubx and Vg probes and MBP-Brk and MBP-BrkHD concentrations ranging from 0 to 1200 ng.](image)

D

![Western blot analysis with Ubx and Vg competitors at various concentrations ranging from 1e-11 to 1e-8.](image)
Kirkpatrick, Fig. 3

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- MBP-MadNL complex
- MBP-Brk complex
- Free probe
Kirkpatrick, Fig. 6

Smad3/Smad4

Inverted Smad boxes

Mad consensus

Inverted Brk sites
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