Brinker Autoregulation and Gradient Formation in the *Drosophila* Wing

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Establishing patterns of differentiation is an important theme in developmental biology. A key mechanism involved in creating these patterns of differentiation is the establishment and interpretation of transcription factor gradients. The *Drosophila* transcriptional repressor Brinker (Brk) is expressed in lateral-to-medial gradients across the anterioposterior axis of the wing imaginal disc where it negatively regulates the spatial patterns of expression of genes including *spalt* (*sal*) and *optomotor-blind* (*omb*); the precise pattern of expression of these targets is determined by their sensitivity to repression by Brk so that the *sal* domain is narrower than that of *omb* largely because it is repressed by lower levels of Brk than *omb*. The *brk* gradient is established by an inverse gradient of BMP signaling through the secreted BMP homolog Decapentaplegic (Dpp): the intracellular Smad effectors of Dpp signaling, pMad and Medea, bind together with the repressor protein Schnurri (Shn) to silencer elements at the *brk* locus and repress the activity of a constitutive enhancer. My studies have revealed that the generation of the *brk* gradient is not simply a precise negative read-out of the *dpp* gradient; Brk must also negatively autoregulate its own expression by interacting with the pMad/Medea/Shn repressor complex. Additionally, I have demonstrated that this Brk/pMad/Medea/Shn repressor complex alone cannot establish the graded profile of *brk* but that an additional positive *cis*-regulatory element that is activated by pMad is required. This may provide the first example of a transcription factor both activating and repressing the same gene, *brk*, in the same cells at the same time via two different response elements. In conclusion, generating the *brk* gradient

requires at least two positive and two negative inputs: constitutive activation by an activator, Brk negative autoregulation, and both activation and repression by pMad. Generating a stable expression gradient appears to be much more complex than previously thought and may reflect the importance of multiple inputs in generating intermediate levels of gene expression rather than a simple on/off threshold response.

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PREFACE

I would like to first thank my advisor, Dr. Gerard Campbell. Gerard is a dedicated scientist whom I would like to thank for granting me the ability to work independently and to create my own successes through the guidance and training he provided me. I would also like to thank him for his support in my pursuit of my love of teaching. I also thank the past and present members of the Campbell lab for their help throughout my time in the lab, especially Dr. Stephanie Winter for recruiting me to the department and the lab, teaching me how to push flies, and for being one of the best friends I've ever had.

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encouragement in life as well as in my graduate studies. You've brought happiness to my life; without you, I truly wouldn't be where I am today. We will share together in our love for the rest of our lives.

1.0 INTRODUCTION

Developmental biologists have long asked the question: How does a single, undifferentiated cell, the fertilized egg, undergo changes to produce millions of cells that give rise to various tissues, organs, appendages, and ultimately an entire organism? What must become different between these cells, allowing them to differentiate and become specialized, carry out different functions, and interact with other cells to form complex patterns within tissues? To understand development, we must understand the steps that lead to the correct development of tissues and higher order structures that create a properly formed organism. I am interested in how spatial patterns of gene expression are established and how these establish patterns of differentiation in developing organisms.

1.1 DEVELOPMENT IN EUKARYOTES IS REGULATED BY DIFFERENTIAL GENE EXPRESSION

An important feature of all eukaryotic cells is their ability to transcribe only a portion of the genes present in their genomes. This differential gene expression is what allows cells to become different from each other and is controlled by the spatial and temporal cues cells receive from their environment. The cues in the environment are predominantly different types of secreted signaling polypeptides, which control gene expression through signaling pathways that regulate

the activity of transcription factors. Thus, transcription factors ultimately regulate cell differentiation.

1.2 REGULATION OF EUKARYOTIC GENE EXPRESSION

The regulation of transcription is essential for the establishment of proper patterns of gene expression. The transcription of eukaryotic protein-coding genes is dependent upon the positioning of RNA polymerase II at the correct initiation sites. One of the factors involved in this proper placement is the core promoter. Core promoters are found at the transcription start site and position the *trans*-acting basal transcriptional machinery to initiate transcription (Smale and Kadonaga, 2003). These regions typically include DNA elements that can extend about 35 basepairs upstream and/or downstream of the transcription initiation site (Juven-Gershon et al., 2006).

Spatial and temporal patterns of gene expression are controlled by a variety of *cis*-acting DNA elements at response elements (also known as proximal promoters). While core promoter elements are largely the same for all genes, response elements are unique for practically every gene. Because RNA polymerase II is incapable of promoter recognition and accurate transcription initiation on its own, its interactions with basal transcription factors at the core promoter as well as the interactions of sequence specific transcription factors at response elements are all required for proper gene expression.

1.2.1 Core promoter elements

RNA polymerase II interacts at the core promoter with basal transcription factors. Core promoter elements include the TATA box, TFIIB recognition element (BRE), initiator (Inr), downstream promoter element (DPE), and a more recently identified Motif Ten Element (MTE) (reviewed by Smale and Kadonaga, 2003). The TATA box was the first eukaryotic core promoter motif to be identified and is recognized by the TATA-binding protein (TBP) subunit of the TFIID complex (Smale and Kadonaga, 2003). Putative TATA box motifs are A/T rich, located about 30 nucleotides upstream of the transcription start site, and are found in ~43% of *Drosophila* core promoters and ~32% of human core promoters (Kutach and Kadonaga, 2000; Suzuki et al., 2001). The BRE is found upstream of about 12% of TATA box containing promoters where it serves to augment the binding of TFIIB to the core promoter (Lagrange et al., 1998).

TATA boxes are not the only core promoter elements; TFIID interacts at the Inr element, a discrete core promoter element with the ability to function in *Drosophila* independently of the TATA box often by functioning together with the DPE. The DPE is conserved from *Drosophila* through vertebrates and is found approximately 30 nucleotides downstream of the transcription start site where it associates with the TBP-associated factor (TAF) subunits of TFIID (Smale and Kadonaga, 2003). DPE-containing promoters often have both Inr and DPE motifs but lack a TATA box motif; in *Drosophila*, the DPE-driven promoter is utilized as frequently as the TATA-driven promoter (Willy et al., 2000). DNaseI footprinting studies revealed that TFIID binds cooperatively to the Inr and DPE motifs with a precise spacing required for maximal transcriptional stimulation by the Inr and DPE elements (Burke and Kadonaga, 1996; Smale and Kadonaga, 2003). In *Drosophila*, computer predictions suggest that approximately 14% of

promoters contain both TATA and DPE elements while approximately 31% do not appear to contain either motif suggesting that other motifs may remain to be identified (Kutach and Kadonaga, 2000).

The MTE is a relatively newly identified element found in promoters that, for the most part, do not contain a TATA box. The MTE promotes transcription by RNA polymerase II in conjunction with the Inr and synergistically with the DPE by contributing to the binding of TFIID to the core promoter (Lim et al., 2004). The MTE was demonstrated to be functional *in vitro* in human promoters, indicating that all of these core promoter elements function not only in *Drosophila* transcription but are conserved all the way to humans (Lim et al., 2004).

1.2.2 Response elements

Response elements (also known generically as cis-regulatory elements) function to regulate levels of gene activity through their contained binding sites for sequence-specific transcriptional activators and repressors (Arnosti, 2003). Response elements can be utilized to drive reporter gene expression in patterns identical to those of the gene they regulate. The decision to transcriptionally activate or repress a specific promoter is dependent on the particular regulatory transcription factors that bind in a sequence-specific manner to the *cis*-regulatory response elements associated with each specific gene. Once bound to these elements, regulatory transcription factors function specifically to either activate/enhance or repress/silence transcription from a given promoter.

Most response elements are characterized by one or more enhancer and silencer elements, though the distinction between the silencer and enhancer portions of the response element is often not possible as many response elements do not have clearly separable enhancer and

silencer elements. Activator proteins bind to enhancer elements to activate transcription whereas transcriptional repressors bind to silencer elements to direct transcriptional repression. Both positively and negatively acting transcription factors often bind to overlapping binding sites contained within the same response elements and the sites are often redundant as most regulatory mutations involve the removal of multiple, not individual, transcription factor binding sites (Arnosti, 2003).

1.2.2.1 Response element function

Response elements have been suggested to function by several different pathways that are differentiated by the transcription factors recruited. First, enzymatic modifications of histones modulate repression by nucleosomes to either enhance or interfere with the binding of the basal transcriptional machinery or activators. In addition to the basal transcriptional machinery, response elements are necessary for the transcription of genes because the eukaryotic genome is packaged into chromatin. Covalent modifications of histones include post-translational modifications such as methylation, acetylation, ubiquitination, and sumoylation; these modifications affect higher-order complex assembly as well as the binding of other factors (Bhaumik et al., 2007). Gene activation is thus thought of as relief of repression by nucleosomes, often through the histone acetyltransferase activities of coactivators whereas corepressors often utilize deacetylation to re-establish repression (Kornberg, 1999). This notion was supported by the observation that in the absence of nucleosomes, transcriptional activators are not necessary for gene activation and that nucleosome reassembly is required for gene repression following active transcription (Adkins and Tyler, 2006).

Second, response elements can function through direct interactions with the general transcription machinery (reviewed by Blackwood and Kadonaga, 1998). Some coactivators

function at response elements to bind transcription factors, recruit RNA polymerase II, and interact with the general transcriptional apparatus in order to regulate levels of transcription (Spiegelman and Heinrich, 2004).

Third, response elements can function through the recruitment of chromatin remodeling proteins. Chromatin remodeling is ATP-dependent and disrupts the histone-DNA contacts thus altering the position of histone octamers on DNA to create either a dense cluster of nucleosomes or a region of DNA containing no nucleosomes (Kokavec et al., 2008). All of the mechanisms described above function *in vivo* and can regulate levels of transcription or switch gene expression on or off (Arnosti, 2003).

1.3 APPROACHES TO IDENTIFYING REGULATORY ELEMENTS

Regulatory elements were initially identified through genetic approaches; a good example includes the mutations that altered expression of HOX genes in the *bithorax* complex (reviewed by Lewis, 1998). Transgenic technology in model organisms like *Drosophila* has allowed promoter/response element testing experiments to identify regulatory elements through functional studies (Arnosti, 2003). Specifically, portions of regulatory elements or putative candidate sequences thought to make up response elements are cloned into P-element transformation vectors that use reporter genes like *lacZ* or GFP to monitor gene expression driven by the regulatory element (Barolo et al., 2000). Transgenic animals are created and reporter gene expression is analyzed in multiple transgenic lines. This allows transcriptional output to be assessed based on the level as well as the spatial and temporal pattern of reporter gene expression.

Bioinformatic approaches are often used to identify evolutionarily conserved clusters of transcription factor binding sites that may constitute putative response elements (Arnosti, 2003). Binding sites contained within regulatory elements can be altered to analyze the difference in reporter gene expression compared to normal expression domain of the element thus determining the importance of these sites for regulation of gene expression.

The "minimal" enhancer or response element is the smallest portion of a gene's regulatory region which, when used to drive a reporter gene, is sufficient to exhibit expression in a pattern that is a reasonable approximation to the endogenous gene. It is important to consider that in order to truly understand how gene expression is being controlled, all of the response elements necessary for its function must be analyzed. Often, single response elements or regulatory regions are tested in reporter constructs; these often do not exactly recapitulate gene expression because proper gene expression often requires multiple response elements that are not always near one another in the genomic context; this is often overlooked deliberately in order to characterize a new element or perhaps to make studies of a particular regulatory element more manageable.

1.4 MORPHOGENS AS SECRECTED SIGNALING MOLECULES

Secreted signaling pathways control gene expression via a limited number of signaling pathway molecules or proteins that regulate the activity of transcription factors, ultimately directing a cell in a developing organism to become different from other nearby cells. Because secreted signaling molecule pathways control most gene expression during development through a limited number of signaling proteins, their observed complexity in output must be built by alternative

mechanisms, for example, combinatorially, through differences in timing of expression, or through quantitative outputs that depend on the level of activity of the pathway (Ashe and Briscoe, 2006). Some secreted signaling molecules function as morphogens in order to generate these quantitative outputs. Morphogens, originally characterized as "form-giving molecules", are signaling molecules secreted by a group of cells; the morphogen moves away from the cells in which it was originally expressed resulting in the formation of a gradient of the secreted signaling molecule (Vincent and Perrimon, 2001). Cells can detect their position in the gradient with respect to the source of the morphogen because the activity of the morphogen is a function of its concentration and distance from the source of secretion (Vincent and Briscoe, 2001). Pattern formation primarily depends on gradients of morphogens and their subsequent organization in discrete locations in developing tissues (Lawrence and Struhl, 1996).

The key feature of morphogen gradients is the ability of cells to respond to the morphogen in a concentration dependent manner, detecting threshold levels of the morphogen and responding in different ways to high, intermediate, and low levels (Fig. 1-1 A-D) (Gurdon and Bourillot, 2001; Wolpert, 1969). These responses include the transcriptional activation or repression of gene expression based on threshold levels of morphogen signaling detected. This is important and will be discussed in more detail in Chapter 4. Genes responsive to low levels of morphogen signaling are expressed by cells at a greater distance from the source of the secreted polypeptide than those requiring higher levels of activity, which are expressed more closely to the cells secreting the signal (Strigini and Cohen, 1999). Through this mechanism, the intracellular signal transduction pathway receiving the signal identifies the concentration of the morphogen it is receiving and subsequently activates target genes only above the appropriate

threshold level allowing cells to take on different fates depending on their position in the cellular field (Ashe and Briscoe, 2006).

1.5 TRANSCRIPTIONAL REGULATORS

The regulation of transcription of specific nuclear target genes in response to the binding of a secreted signaling ligand to the pathway's receptor and the subsequent stimulation of an intracellular signal transduction cascade is a conserved method of function for all major facets of developmental biology. The transcriptional control exhibited by the pathway is facilitated by one or more transcription factors that interact at specific response elements in the upstream regulatory regions of target genes (Barolo and Posakony, 2002).

Regulatory transcription factors interact at response elements that, as described above, contain binding sites for these regulatory proteins; transcription of a gene can then be activated or repressed by these transcription factors and their associated co-factors (Hanna-Rose and Hansen, 1996; Johnson, 1995). Eukaryotic genes contain very complex response elements that are capable of binding different regulatory transcription factors thus allowing for differential gene expression. Regulatory transcription factors can act in two different manners: to activate or repress transcription.

1.5.1 Transcriptional activation

Transcriptional activator proteins facilitate transcriptional activation; they are modular and contain DNA-binding (DBD) domains to recognize regulatory sequences as well as activation

domains to interact with additional transcription factors or transcriptional machinery to initiate transcription (Kornberg, 1999). Activator proteins typically interact with another group of proteins known as co-activators; these proteins interact with transcription factors and often contain (or recruit other secondary co-activator proteins that contain) the enzymatic ability to modify chromatin allowing effective transcription to take place (Spiegelman and Heinrich, 2004). Mediator is a multiprotein complex that acts as a transcriptional co-activator and is required for the successful transcription of almost all protein-coding eukaryotic genes (Kornberg, 2005). It functions by binding the C-terminal domain of RNA polymerase II and acts as a bridge between this enzyme and other transcription factors thus linking transcriptional control at response elements with the promoter (Kornberg, 2005). Additionally, many other co-activators exist including TBP-associated factors (TAFs) that associate with TATA-box-binding protein (TBP) to form the TFIID portion of the RNA polymerase core promoter where they direct transcriptional activation (Burley and Roeder, 1996).

1.5.2 Transcriptional repression

Transcription factors that function as repressors bind to specific sequences in the regulatory regions of genes thereby preventing the initiation of transcription by RNA polymerase II (Barolo and Posakony, 2002). There are several ways in which the cell-specific regulation of gene expression might occur. One way would be for the organism to control where the activators were located. In this situation, transcriptional repressors would never be necessary. A second mechanism would be for activators to be widely expressed while the expression of repressors would be tightly controlled such that cell-specific gene expression would be regulated largely by

where the repressors were located. In many, if not most systems, it is the second mechanism that is important for spatial cues to control different gene expression.

In much the same manner as transcriptional activators associate with co-activators, transcriptional repressors often recruit co-repressors that assist in the inhibition of transcription at a given locus (Gray and Levine, 1996). The methods of repression utilized by transcriptional repressors include passive and active mechanisms as well as long and short-range repression.

1.5.2.1 Passive and active repression

Passive repression is not often used in eukaryotes, but can result from competition between a repressor and an activator for a DNA binding site or other site necessary for transcriptional initiation. In situations where the repressor is found at higher concentrations than the activator, the repressor will either compete away the activator or bind the target DNA binding site with a higher affinity than the activator (Cowell, 1994; Johnson, 1995).

Active repression is a characteristic of repressors that contain particular protein domains or motifs in addition to a DNA-binding domain (Hanna-Rose and Hansen, 1996). These domains or motifs can either function alone or can recruit additional co-repressors to mediate repression, often by altering chromatin structure, blocking the activity of an activator, or by interfering with activity of the basal transcriptional machinery at the promoter (Hanna-Rose and Hansen, 1996; Johnson, 1995). Active repression utilizing co-repressors can act over both long and short ranges.

1.5.2.2 Long and short range repression

Long-range repressor proteins often inactivate entire chromosomal loci by making a response element resistant to activation from all enhancers including those found thousands of basepairs

away from the repressor binding site (Courey and Jia, 2001). A familiar example of long-range repression is the co-repressor Groucho (Gro), a protein that mediates repression over as much as 1 kb of DNA in *Drosophila* and functions with repressors to silence transcription of promoters in a global manner (Chen and Courey, 2000). Groucho orthologs are found in all metazoan organisms and are often recruited to the WRPW tetrapeptide motif of the DNA-binding transcriptional repressor proteins to which they are recruited (Courey and Jia, 2001). Groucho protein molecules appear to oligomerize with each other creating higher order, condensed complexes that organize chromosomal domains into transcriptionally silent states through interactions with co-repressors that possess histone deacetyltransferase activity as well as by causing inhibitory interactions with the basal transcriptional machinery and/or preventing activator binding (Courey and Jia, 2001; Sekiya and Zaret, 2007).

Short-range repressors, on the other hand, do not interfere with all transcription but instead inhibit the activity of only DNA-bound activators located close to a particular locus (Courey and Jia, 2001). A popular example of a short-range repressor is C-terminal Binding Protein (CtBP), a co-repressor found in *Drosophila* as well as vertebrates including humans that is recruited to DNA by a repressor containing the PXDLS motif and mediates repression over a distance not exceeding 150 basepairs (Chinnadurai, 2007; Gray and Levine, 1996). Studies suggest that CtBP may function by interacting with a nearby DNA-bound activator protein to obstruct interactions of the activator and the general transcriptional machinery (Courey and Jia, 2001). Additionally, the CtBP co-repressor complex contains enzymatic constituents that mediate histone modification by deacetylation as well as other mechanisms (Chinnadurai, 2007; Courey and Jia, 2001; Shi et al., 2003). In *Drosophila*, several short range repressors such as

Snail, Knirps, and Kruppel exert their transcriptional activity during embryonic development primarily by recruiting CtBP (Chinnadurai, 2007).

1.5.3 Proteins can function as transcriptional activators or repressors

Notable, studies of gene repression originally performed in bacteria revealed that a single protein can function as either a transcriptional repressor or as an activator protein depending on the arrangement of its binding sites with respect to the promoter and the other gene regulatory proteins with which it is in complex (Johnson, 1995). In some cases, a repressor may bind cooperatively with an activator while in other situations the repressor instead recognizes other DNA-bound proteins. Smads, intracellular signal-transducers in the TGF-ß signaling pathway, target specific genes for either transcriptional induction or inhibition by directly recruiting co-activators or co-repressors to Smad-targeted promoters (Massague et al., 2005). Suppressor of Hairless, Su(H), is a transcription factor that functions as an activator of gene expression during Notch pathway signaling; however, in the absence of signaling, it represses target genes through its interactions with Hairless (H), a *Drosophila* protein that antagonizes Notch signaling by inhibiting DNA binding by Su(H), and through the recruitment of two co-repressor proteins, CtBP and Gro (Barolo et al., 2002). Another example conserved from *Drosophila* to mammals includes the Tcf/Lef family of transcription factors important for transcriptional activation upon association with ß-catenin in the nucleus of cells signaled by the Wnt/Wingless signaling cascade (Llimargas, 2000). The Tcf/Lef molecules can also function as transcriptional repressors in the absence of a Wnt/Wingless signal by binding with different transcriptional co-repressors including CtBP (*Drosophila*) or Gro (vertebrates) (Brantjes et al., 2001). In both of the

situations described above, the same response element sequence is bound by Su(H) or Tcf/Lef when these transcription factors function to both activate and repress transcription.

1.5.4 Transcription factor gradients

A common feature of many morphogen gradients is their ability to establish transcription factor gradients; these gradients can specify positional information by either transcriptionally activating or repressing target genes that possess differential sensitivities to the signaling protein of the morphogen gradient (Hewitt et al., 1999). An inverse gradient of a transcriptional repressor that is reciprocal to the transcriptional effector or activator protein that has been activated by the morphogen signal is a common phenomenon of morphogen gradients (Fig. 1-1 E-F) (Ashe and Briscoe, 2006). Different target genes of secreted signaling molecules are differentially expressed because they respond in a concentration dependent manner to the gradients of transcription factors set up by the signaling pathway (Ashe and Briscoe, 2006).

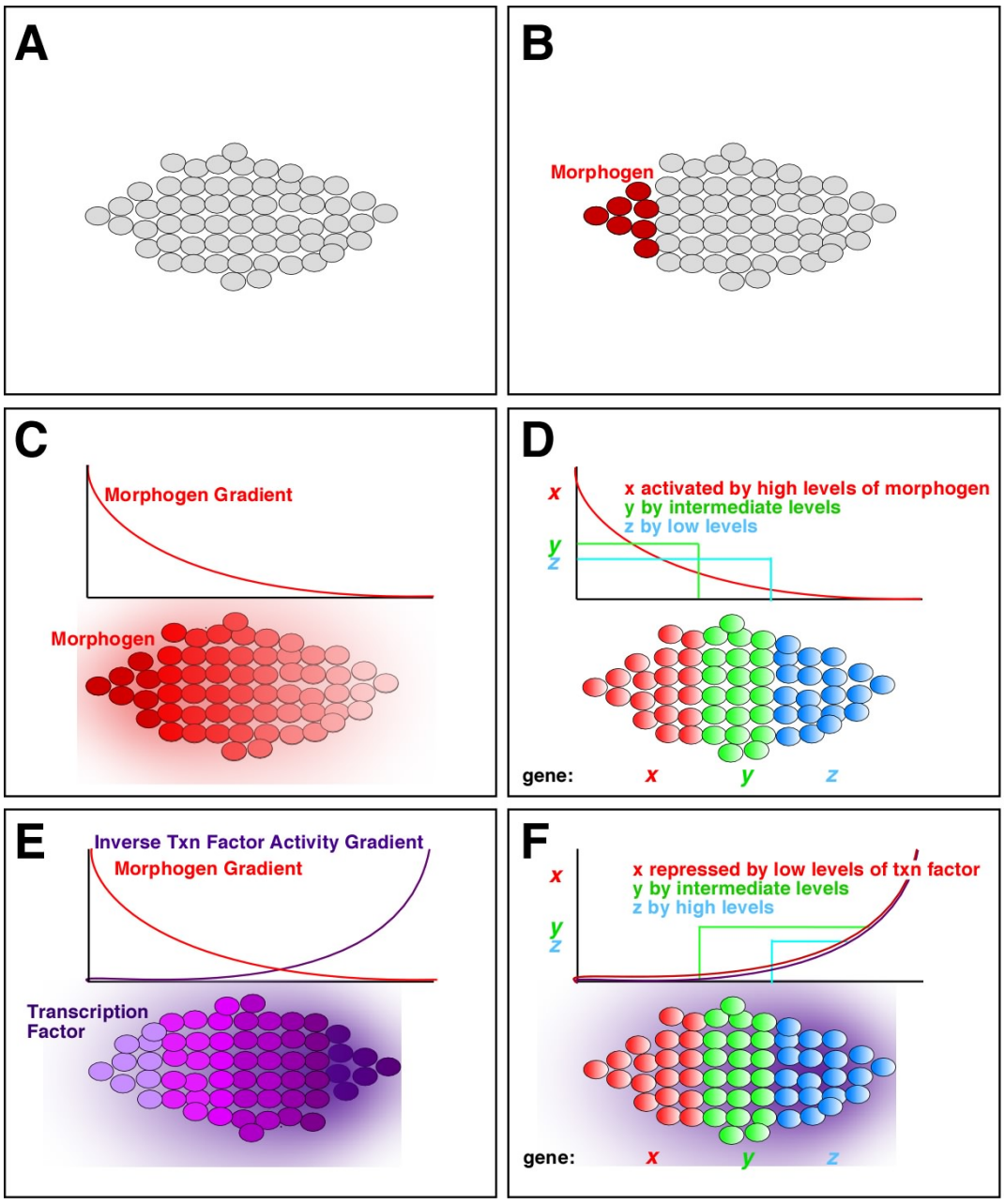

Figure 1-1: Morphogen and transcription factor gradient model.

(A) A cellular field. (B) The morphogen is a localized source of a signaling molecule with high levels near the source (left side of page), shown in red. (C) Following secretion and diffusion, the morphogen is present in a gradient with highest levels near the source (red cells) and lowest levels farthest from the source (pink cells). (D) Target genes are activated above distinct threshold levels of morphogen to which they are exposed, with gene X being activated by the highest levels, gene Y by intermediate levels, and gene Z by the lowest levels. (E) Morphogen gradients (as shown in panel C) can induce inverse/complimentary transcription factor activity gradients where the highest levels of transcription factor may be found where the concentration of the morphogen is lowest (dark purple cells) and lowest concentrations where the amount of morphogen is the greatest (light violet circles). (F) Target genes can be repressed differentially by the concentration of the transcriptional repressor protein present where gene X is repressed by low levels of the transcriptional repressor, gene Y by intermediate levels, and gene Z by high levels.

1.6 *DROSOPHILA MELANOGASTER* AS A MODEL SYSTEM IN DEVELOPMENTAL BIOLOGY

Drosophila is often considered the experimental model organism of choice for examining a number of important developmental biological processes (Roberts, 1998). Gradients of transcription factors can be generated by diffusion from localized sources of transcription or maternal message found within the early embryo because it develops as a syncytium. The role of these gradients in patterning the early embryo is described below. Later in embryonic development, organized pockets of tissue called imaginal discs are set-aside to later become adult structures including the appendages; this tissue is largely patterned during larval life before the adult structures develop during the pupal period.

I am interested in understanding the regulation of pattern formation during animal development and how these patterns of differentiated tissue create functional structures like appendages. The developing *Drosophila* provides many opportunities to study pattern formation due to the vast knowledge of the regulation of the function of many genes as well as the numerous molecular and genetic techniques available to researchers.

1.7 PATTERNING THE DROSOPHILA EMBRYO BY TRANSCRIPTION FACTOR GRADIENTS

In the early *Drosophila* embryo, gradients of transcription factors are established along the A/P axis from a localized source of RNA because the embryo develops as a syncytium. The factors diffuse easily through the cytoplasm allowing the formation of transcription factor concentration

gradients forms along axes where target genes are differentially sensitive to the levels of the graded transcription factor. This single factor can provide the information necessary to establish spatial patterns of gene expression along much of a given body axis. In the *Drosophila* egg, the activity of the Bicoid (Bcd) transcription factor was the first identified example of how quantitative information contained in a morphogen gradient is transformed into precise patterns of target gene expression. The *bcd* mRNA is produced during oogenesis and deposited into the anterior pole of the embryo; following translation, the Bcd protein, a homeodomain-containing transcription factor, can diffuse along the A/P axis giving rise to a concentration gradient with its highest point at the anterior pole (Dreiver, 1988) (Fig. 1-2). High levels of Bcd pattern the anterior most region of the embryo and promote the expression of genes including *buttonhead* whereas lower levels of protein expression in the posterior of the embryo regulate the expression of *hunchback* (Burz et al., 1998; Crauk and Dostatni, 2005).

In other situations (aside from syncitially developing *Drosophila* embryos), gradients of activated transcription factors are also established along developing axes, as demonstrated for Dorsal (Dl), which is the second critical transcription factor gradient responsible for patterning the major body plan axes of the *Drosophila* embryo. However, this the gradient of activated Dl cannot be established in the same manner as described for the A/P axis in the embryo as there are membranes between the cells, so gradients, like that of activated Dl, are typically established by a gradient of an extracellular signal. The Dl protein is uniformly distributed in the egg and initially restricted to the cytoplasm. Dorsoventral (D/V) patterning by Dl occurs because a Dl activity gradient is produced by a processed form of the Spatzle protein, which is present in the perivitelline space surrounding the embryo but is processed in the ventral region resulting in a ventral to lateral Dl gradient (Papatsenko and Levine, 2005). The graded nuclear localization of

Dl is promoted by the activation of the Toll receptor by Spatzle (Papatsenko and Levine, 2005). This nuclear Dl concentration gradient is responsible for early patterning of the D/V axis and specifies three basic regions/germ layers: the mesoderm, neuroectoderm, and dorsal ectoderm (Fig. 1-2) (Jiang et al., 1992; St Johnston, 1992). The expression patterns of genes along the D/V axis such as *decapentaplegic* and *twist* are determined by their sensitivity to either activation (*twist*) or repression (*decapentaplegic*) by the Dl protein (Papatsenko and Levine, 2005; Stathopoulos and Levine, 2005).

In the developing *Drosophila* wing, gradients of extracellular signaling proteins result in a similar gradient of activated transcription factor. The signaling protein Decapentaplegic (Dpp) is a TGF-β homolog whose gradient controls patterning and development along the A/P axis of the wing.

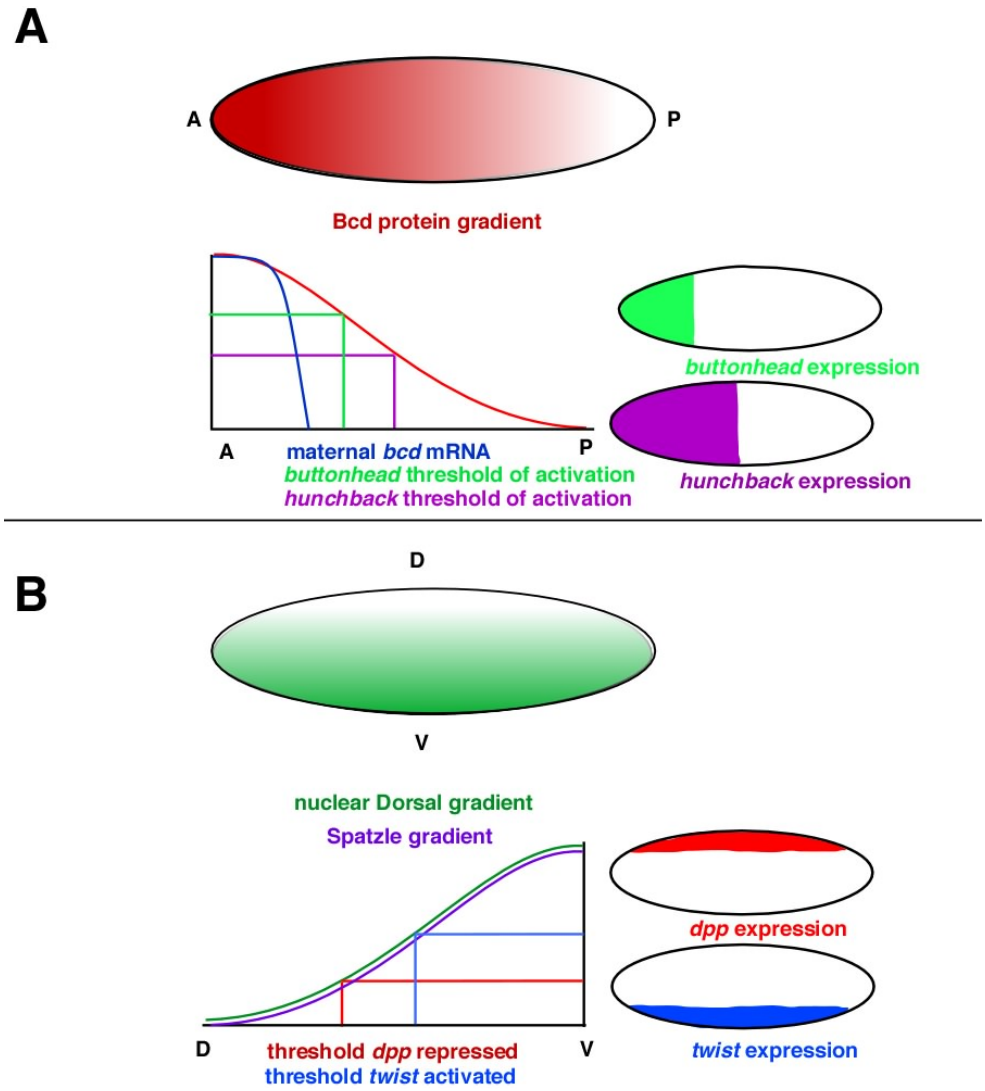

Figure 1-2: Gradients of transcription factors pattern the *Drosophila* embryo.

(A) The *bcd* mRNA is produced during oogenesis and deposited into the anterior pole of the embryo. Following translation and diffusion, the Bcd protein gradient is established with highest concentrations of Bcd in the anterior and lowest levels in the posterior. High levels of Bcd promote the expression of genes like *buttonhead* while lower levels allow the expression of genes like *hunchback* in the posterior. (B) Spatzle protein is processed in the ventral region of the embryo producing a ventral to lateral cytoplasmic Dorsal protein gradient. Activation of the Toll receptor by Spatzle promotes the nuclear accumulation of Dorsal establishing a nuclear Dorsal protein gradient with peak levels ventrally and progressively lower levels laterally and dorsally. Target genes like *dpp* are repressed by lower levels of nuclear Dorsal protein while others like *twist* are activated by higher levels of nuclear Dorsal protein.

1.8 IMAGINAL DISCS DEVELOP INTO ADULT WINGS

In *Drosophila*, the appendages of the adult fly develop from larval imaginal discs (Blair, 1995). Discs are set aside during embryonic development as small clusters of 10-40 cells that divide during larval life to form large epithelial sacs of tissue (Blair, 1995). The developing *Drosophila* embryo contains parasegments that ultimately give rise to the segments of the larva and adult (Wolpert, 2002). Primordial wing imaginal disc cells originate in response to a positional cue from the Wingless protein in the embryonic ectoderm on opposite sides of the parasegment boundary (Brook et al., 1996). Because of this, founder cells that comprise the disc primordium are made up of cells from two adjacent parasegments; those in the posterior compartment inherit *engrailed* expression from the embryonic segment, which is important later for patterning the appendage along the A/P axis (Brook et al., 1996).

Extensive studies on the transplantation of imaginal disc fragments has demonstrated that the majority of the patterning information necessary for the generation of adult appendages is not present in early larval stages, but becomes defined in the discs by the end of the third larval instar (Cohen, 1993). During metamorphosis, various regions of the wing disc epithelium differentiate into the epidermal tissues of the wing to form two fused sheets of cuticle secreted by epithelial cells as well as characteristic patterns of bristles and veins (Fig. 1-3 A-B) (Blair, 1995).

1.9 WING DEVELOPMENT IS REGULATED BY DPP

Decapentaplegic (Dpp) is a protein in the TGF-β family that regulates both growth and patterning across the A/P axis of wing imaginal discs through its activity as a morphogen. In the

wing disc, the identity of cells in the posterior compartment is dictated by the expression of the *engrailed* gene, as described above. As a result, cells in the posterior secrete Hedgehog (Hh), which acts as a morphogen signaling to anterior compartment cells and patterns the central region of the wing blade primordium while also inducing *dpp* mRNA expression in a stripe of cells just anterior to the A/P compartment boundary (Zecca et al., 1995). Following protein synthesis and secretion, the Dpp protein is located in a symmetrical medial-to-lateral gradient in the anterior and posterior compartments (Fig. 1-3 C-D) (Teleman and Cohen, 2000).

Dpp is essential for the growth of wing cells and is responsible for pattering the wing via a concentration-dependent mechanism utilized to induce the expression of target genes such as *spalt* (*sal*) and *optomotorblind* (*omb*) at varied distances from the A/P compartment border (Fig. 1-4) (Nellen et al., 1996; Tabata, 2001). Characteristic nested patterns of target gene expression are generated by Dpp where genes requiring high levels of Dpp for activation are expressed closest to the source of Dpp whereas genes requiring lower amounts of activity are transcribed in more broad expression domains (Zecca et al., 1995). For example, *sal* and *omb* are regulated by different levels of Dpp signaling: the *sal* expression domain is more narrow than that of *omb* indicating that *sal* is transcribed in regions of the disc where Dpp levels are high while *omb* is transcribed in a wider domain corresponding to the notion that lower levels of Dpp are necessary for the activation of *omb* expression (Fig. 1-3 C-D).

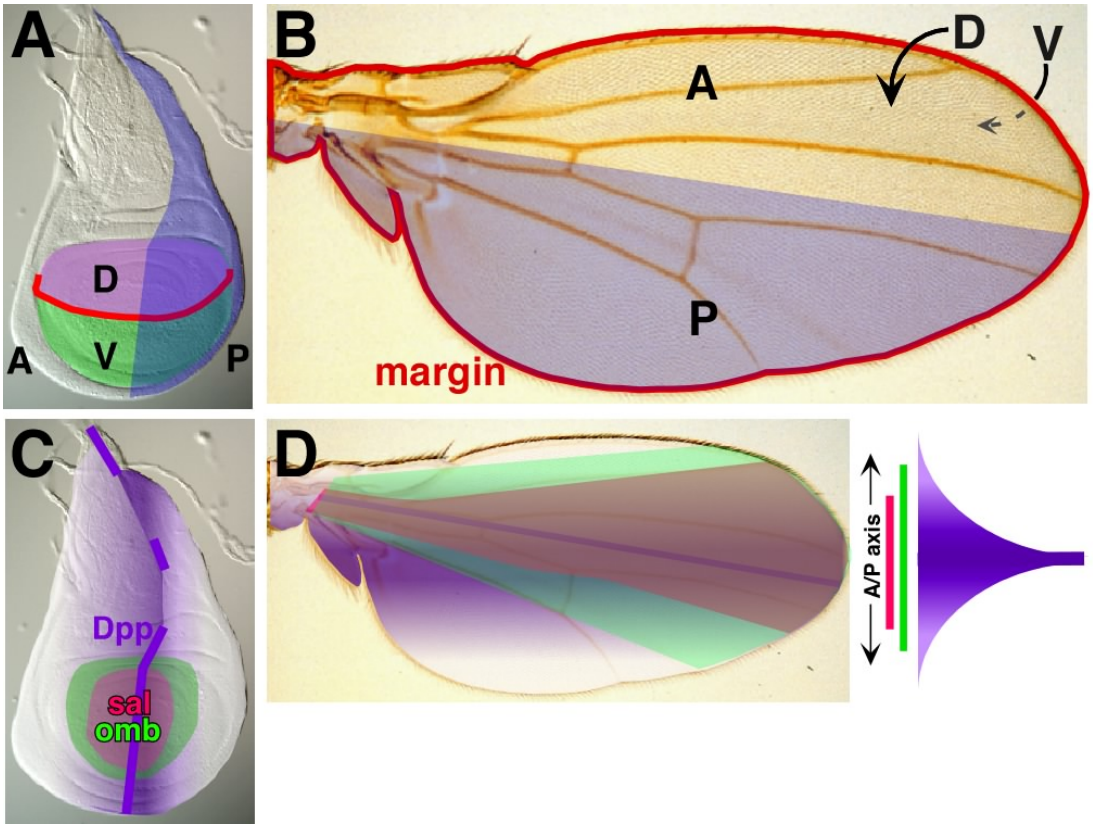

Figure 1-3: Fate map and gene expression in wing imaginal disc projected onto adult wing.
(A) Wing imaginal disc illustrating regions that correspond to adult wing. The wing disc is divided into anterior (A), posterior (P), dorsal (D), and ventral (V) compartments. (B) The adult wing is comprised of dorsal and ventral sheets of epithelial tissue fused at the margin (red). (C) Wing imaginal disc illustrating stripe of Dpp mRNA expression (purple line) as well as the diffused Dpp morphogen gradient (purple gradient). Regions corresponding to target gene expression are shown for *sal* (pink) and *omb* (green). (D) Projection of regions of the adult wing patterned by Dpp targets *sal* and *omb* (colors as in C).

1.9.1 Transforming growth factor-beta signaling pathway

The transforming growth factor-β (TGF-β) family members include TGF-βs, activins, and bone morphogenetic proteins (BMPs); this morphogen superfamily is utilized throughout animal development to control the transcription of target genes involved in the regulation of many different biological processes including the control of cell growth and proliferation (both positively and negatively), fate determination, cell motility, cell adhesion, cell death, and cell

cycle regulation as well as a number of other developmental processes (Gelbart, 1989; Padgett, 1998; Yamamoto and Oelgeschlager, 2004). More than 20 members of the TGF-β signaling family have been studied in a variety of species ranging from corals to mammals (Yamamoto and Oelgeschlager, 2004). The ineffective regulation of TGF-β family signaling has been linked to human diseases including cancer, nonfamilial pulmonary hypertension, fibrosis, and autoimmune diseases indicating that the proper regulation of this pathway is vital for development (ten Dijke et al., 2000; Yamamoto and Oelgeschlager, 2004).

1.9.2 Receptor complexes

Both biochemical and genetic analysis has demonstrated that two classes of membrane associated molecules are the primary effectors of TGF-β signaling: the type I and II receptors. Type I receptors are transmembrane serine-threonine kinases that contain a glycine-serine (GS domain) rich region; signaling of these receptors requires the phosphorylation of the GS domain (Padgett, 1998). Type II receptors are constitutively active kinases that activate type I receptors by directly phosphorylating them (Padgett, 1998). Following BMP ligand binding to the type II receptor, the type I and II receptors dimerize forming a heteromeric complex where the type II receptor phosphorylates the type I receptor on its GS domain thus allowing the type I receptor to relay the signal to downstream effectors (Smad proteins) via phosphorylation events dictated by its kinase domain (Padgett, 1998; ten Dijke et al., 2000).

1.9.3 Intracellular signal transducers

Smad proteins are the nuclear effectors/intracellular signal transducers of the TGF-β superfamily that function to transmit ligand signaling into the nucleus of cells where they interact with other proteins to direct transcriptional responses such as activation or repression of target gene expression (ten Dijke et al., 2000). Smad proteins are conserved between *Drosophila* and vertebrates and can be classified into three groups: receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads).

R-Smads are differentially phosphorylated by activated type I receptor kinases and can form heteromeric complexes with Co-Smads (Padgett, 1998; ten Dijke et al., 2000). Upon complex formation, the R-Smad/Co-Smad complexes move into the nucleus where they bind DNA sequences and are involved in the transcriptional regulation of target genes (Padgett, 1998; ten Dijke et al., 2000).

R-Smads and Co-Smads contain two domains of high sequence similarity at their N- and C-termini, called the Mad-homology domains, or MH1 and MH2, respectively (ten Dijke et al., 2000). While the MH1 domain has intrinsic DNA-binding activity, the MH2 domain is where phosphorylation of the C-terminal serine residues of R-Smads occurs and is also important for homo- and heteromeric complex formation between multiple R- and Co-Smads (ten Dijke et al., 2000). The MH1 and MH2 domains are separated by a proline-rich linker region that has the ability to associate with other transcription factors (Massague et al., 2005; ten Dijke et al., 2000).

The I-Smads act antagonistically to BMP signaling in *Drosophila* by acting as negative regulators of R-Smad signaling via interactions with activated type I receptors to block the phosphorylation/activation of R-Smad signaling (Schmierer and Hill, 2007; ten Dijke et al., 2000).

1.10 BMPs REGULATE DEVELOPMENT VIA ACTIVITY GRADIENTS

The establishment of a BMP activity gradient is important not only in *Drosophila* but also in vertebrate systems where it controls multiple developmental processes including skeletal development, stem cell regulation, neurogenesis, and embryonic D/V axis patterning (Gazzerro and Canalis, 2006; Yanagita, 2005). Due to the multitude of dose-dependent processes regulated by BMP signaling, a requirement exists for the tight regulation of BMP activity. BMP signaling is regulated via several mechanisms including: (1) tissue-specific BMP expression, (2) the tissue-specific localization of cell-surface receptors, and (3) antagonists (Yanagita, 2005). While the tissue-specific localization of BMPs and their receptors can provide the primary mechanism to regulate activity of the signaling pathway, this is not always the case and the specific control of activity is often achieved through the use of extracellular BMP antagonists whose expression is precisely regulated, as discussed in the next section (Yanagita, 2005).

1.10.1 Extracellular antagonists of BMP activity gradients

A number of differentially expressed, extracellular, secreted proteins known as antagonists have been identified that negatively regulate the interaction of BMPs with their receptors by binding BMPs in the extracellular space thus inhibiting downstream signaling (Gazzerro and Canalis, 2006; Yamamoto and Oelgeschlager, 2004). Based on sequence alignment, vertebrate antagonists have been categorized into four subgroups including: noggin, the chordin family, twisted gastrulation, and the differential screening-selected gene aberrative in Neuroblastoma (DAN) family. BMPs are widely expressed during *Xenopus* embryogenesis, but a gradient of BMP signaling activity important for patterning the presumptive D/V axis is achieved by the

graded distribution of extracellular antagonists including Chordin and Noggin, which are largely redundant and expressed in the Spemann's organizer. Among other roles in skeletal development, Noggin is a secreted by the Spemann's organizer in *Xenopus* embryos where it antagonizes BMPs in order to induce neuronal tissue by binding BMP-2, 4, and 7 (Yanagita, 2005). Chordin binds to BMP-2 and 4 in order to prevent the interactions of these signaling molecules with the receptors in its functions at the Spemann's organizer (Yamamoto and Oelgeschlager, 2004). Twisted gastrulation (Tsg) is another glycoprotein antagonist that forms stable complexes with Chordin and BMPs; it is thought that Tsg acts as a BMP-4 antagonist during the formation of the vertebrate skeleton and T-cells (Yanagita, 2005). DAN is a family of secreted antagonists of BMP signaling; most members are expressed during embryonic development where they antagonize BMP signaling, particularly in the regulation of skeletal formation (Gazzerro and Canalis, 2006). It appears that the primary mechanism utilized to control BMP activity gradients in vertebrates is through negative regulation by secreted antagonists of BMP signaling.

1.10.1.1 Antagonists of BMP activity in *Drosophila*

Two of the above described secreted antagonists that function to regulate BMP signaling throughout vertebrate development also have *Drosophila* homologs. Short gastrulation (Sog) is the *Drosophila* Chordin homolog that antagonizes Dpp signaling in embryonic D/V axis development (Carneiro et al., 2006). *Drosophila* also have their own Tsg protein BMP antagonist also important for D/V axis formation (Gazzerro and Canalis, 2006). An extracellular antagonist functioning to regulate BMP signaling during larval wing imaginal disc development has not been identified leading to the possibility that BMP activity is regulated via a mechanism other than secreted antagonists to Dpp signaling.

1.11 DECAPENTAPLEGIC SIGNALING PATHWAY

Dpp acts as a morphogen by providing positional information required to pattern *Drosophila* tissues including the embryonic ectoderm and the wing imaginal disc (Gelbart, 1989; Padgett, 1998). The pathway that transduces the Dpp signal utilizes combination of type I and II receptors where Thickveins (Tkv) and Saxophone (Sax) are the type I receptors and Punt (Put) is the type II receptor (Tabata, 2001). Tkv is crucial for wing development as its constitutively active form, when ectopically expressed, is capable of inducing the expression of the target genes *sal* and *omb* (Tabata, 2001). Put phosphorylates Tkv, which in turn recruits and subsequently phosphorylates the founding R-Smad member of the Smad family, Mothers against Dpp (Mad) (Sekelsky et al., 1995). Phosphorylated Mad (pMad) then interacts with its Co-Smad, Medea (Med); this heteromeric complex translocates from the cytoplasm to the nucleus where it binds the regulatory elements of target genes and directs transcriptional activation or repression (Xu et al., 1998). A Dpp target, Daughters against Dpp (Dad), has been shown to regulate the Dpp activity gradient. Dad, an I-Smad, competes with Mad for binding to the Tkv receptor thereby antagonizing the phosphorylation of Mad to create a negative-feedback loop that limits the domain of pMad (Tabata, 2001).

Evidence demonstrating that Dpp acts in a concentration-dependent manner and directly on cells, rather than through a signal relay mechanism, came from experiments that used a constitutively active form of the Dpp receptor, Tkv (Lecuit et al., 1996; Nellen et al., 1996). Ectopically expressed constitutively active Tkv induces the expression of the Dpp target genes *sal* and *omb* (Lecuit et al., 1996). The key to demonstrating that Dpp acts directly on cells was based on the determination of whether or not the effect of expressing activated Tkv was cell-autonomous—if Dpp functions as a morphogen, the effects of activated Tkv would be cell-

autonomous because a second signal would not be secreted from the constitutively active Tkv-expressing cells. The cell-autonomous effects observed for the induction of *sal* and *omb* by activated Tkv demonstrated that Dpp functions directly on target cells as a morphogen (Lecuit et al., 1996; Nellen et al., 1996).

The Dpp signaling system becomes more complicated when the contributions of Glass bottom boat (Gbb), the other BMP ligand responsible for patterning the wing imaginal disc along its A/P axis, are considered. *gbb* is expressed in a considerably broader domain than *dpp*; while Dpp primarily signals to cells relatively close to its expression domain, Gbb acts over a longer distance in order to establish boundaries of target gene expression at the low points of the BMP signaling gradient in both the anterior and posterior compartments of wing discs (Bangi and Wharton, 2006). Therefore, the wing patterning BMP activity gradient is not comprised of a single graded molecule but rather by the contributions of two BMPs that exhibit different effective ranges in the wing imaginal disc. Because both ligands signal through the Tkv type I receptor in the activation of the same downstream effector, Mad, the difference in their effective ranges is probably based on how the ligands interact with other molecules (Bangi and Wharton, 2006). While I refer to the *Drosophila* TGF-ß signaling pathway as primarily functioning through the Dpp ligand, it is important to keep in mind that Gbb also appears to play a role laterally in the patterning of the A/P axis of the wing imaginal disc.

1.11.1 Dpp can direct both transcriptional activation and repression

Dpp is a signaling system with the ability to either activate or repress transcription upon pathway activation. As members of the Dpp signaling pathways, the R-Smad (Mad) and Co-Smad (Med) bind to the *cis*-regulatory elements of Dpp target genes in a variety of tissues throughout

Drosophila development in order to alter the transcription of target genes. Smad complexes target specific genes for transcriptional activation or repression via their direct recruitment of transcriptional co-activators or co-repressors to the regulatory regions of target genes.

Smads activate transcription often through the recruitment of general co-activators thought to be important for chromatin remodeling (Massague et al., 2005). One instance where Smads activate gene expression is in *Drosophila* embryonic development where they are critical for mesodermal *tinman* induction. A second example includes the wing where Mad binds to and is required for the activation of an enhancer within the *vestigal* gene used for pattern generation across the entire developing wing blade (Kim et al., 1997; Xu et al., 1998).

Due to the fact that DNA binding site context can discriminate between transcriptional induction or inhibition, Mad and Med have also been shown to transcriptionally repress Dpp target gene expression in *Drosophila* through their recruitment of the zinc finger-containing repressor protein Schnurri (Pyrowolakis et al., 2004). The determination of whether Mad and Med activate or repress transcription is based on the sequence and spacing between their binding sites, as discussed below. One such target repressed by Dpp signaling in the developing wing imaginal disc is *brinker* (*brk*).

1.12 DPP SIGNALING REGULATES BRINKER

The *brk* gene was independently characterized as a modifier of Dpp signaling in three laboratories where it was found to function as a regulator of Dpp target genes (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999). In the *Drosophila* wing

imaginal disc, the medial-to-lateral Dpp gradient present along the A/P axis was found to be complemented by a lateral-to-medial gradient of Brk (Campbell and Tomlinson, 1999).

Several studies confirmed that Dpp signaling regulates *brk*. High levels of *brk* are expressed by clones of mutant cells lacking the Dpp receptor Tkv; this is not dependent upon their location along the A/P axis indicating that in the absence of a Dpp expression gradient, *brk* expression would occur ubiquitously throughout the wing pouch (Jazwinska et al., 1999a). Consistent with the notion that *brk* is negatively regulated by Dpp, expression of an enhancer trap near the *brk* gene (with a reporter gene expression pattern identical to *brk*) was lost in wing discs ubiquitously expressing Dpp while *dpp* mutant discs demonstrated ubiquitous expression of the reporter (Campbell and Tomlinson, 1999).

1.13 DPP REGULATES TARGETS INDIRECTLY THROUGH BRK REPRESSION

In the *Drosophila* wing, Brk functions to repress the expression of Dpp target genes including *sal* and *omb*. In *brk* mutants, the *sal* and *omb* expression domains are expanded laterally (Fig. 1-4). Ectopic expression of *brk* in the central region of the wing disc (where it is not normally expressed) results in a loss of both *sal* and *omb* expression (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Muller et al., 2003). These results indicate that the Brk protein must be either absent or expressed only at reduced levels for the expression of *sal* and *omb*. This is indeed the situation in the middle of the wing disc where high levels of Dpp repress *brk*. Furthermore, the ectopic expression of Dpp target genes was induced when a *brk* mutation was combined with mutations that prevented the transduction of Dpp signaling suggesting that Brk

functions to inhibit the Dpp target genes directly (Bray, 1999; Campbell and Tomlinson, 1999; Jazwinska et al., 1999a).

While Dpp seems to act via an indirect mechanism of target gene regulation (repressing *brk* that in turn represses targets), studies have also indicated that Dpp directly regulates (through Mad) some of its targets. The *sal* gene encodes a zinc-finger protein that is involved in vein patterning and the growth of the adult wing (Kuhnlein et al., 1997). While Dpp signaling appears to not be required for *sal* expression, *brk* mutant clones in lateral regions of wing discs exhibit lower levels of *sal* expression than those in the central region and a loss of both *brk* and *mad* in the central region results in a reduction in the expression level of *sal* (Campbell and Tomlinson, 1999). Thus, maximal *sal* expression levels may require Dpp signaling. Another target of Dpp signaling is the *omb* gene, which encodes a member of the T-box family of transcription factors and is repressed by Brk binding within its regulatory regions (Sivasankaran et al., 2000). However, *omb* is also most likely directly activated by pMad, a component of the Dpp signaling pathway; upregulation of pMad expression antagonizes the ability of ectopic Brk to repress *omb*, lending support to the notion that pMad may activate *omb* while Brk is at the same time repressing it (Moser and Campbell, 2005).

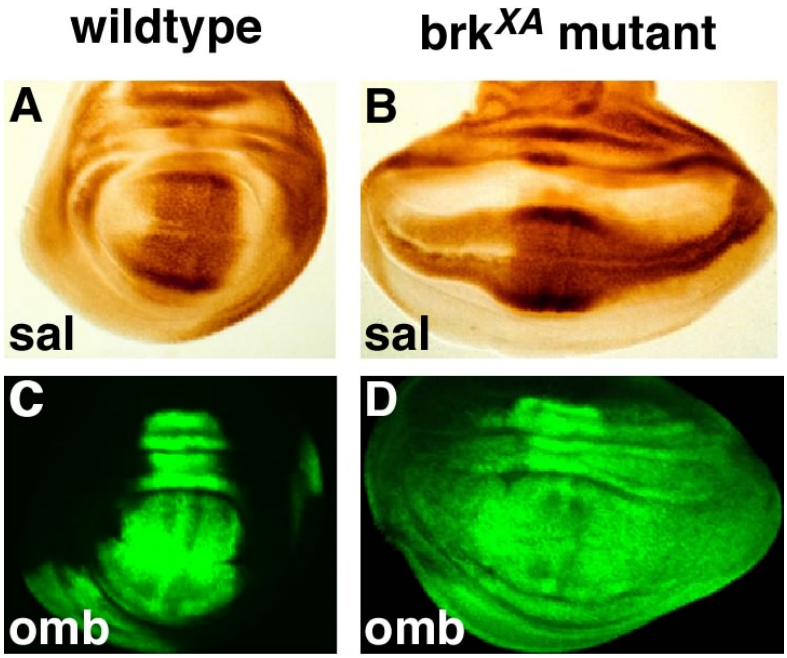

Figure 1-4: Dpp target gene expression in wildtype and *brk* mutant discs.
Figure adapted from Campbell and Tomlinson (1999). Wildtype wing discs compared to those of a brk^{XA} mutant. Note that brk^{XA} mutant discs demonstrate an overgrowth phenotype along the A/P axis associated with the de-regulation of Dpp target gene expression. (A) *sal* expression (anti-sal) in a wildtype disc is confined to the medial portion of the disc. (B) The *sal* expression domain is widened into the expanding wing pouch in the brk^{XA} mutant disc. (C) *omb* expression (UAS-GFP; omb-Gal4) in a wildtype disc is confined to the medial portion of the disc. (D) The *omb* expression domain is widened into the expanding wing pouch in the brk^{XA} mutant disc.

1.14 SENSITIVITY OF DPP TARGETS TO BRK

While Dpp signaling induces the expression of its targets *sal* and *omb*, the *brk* expression gradient is necessary to organize the nested patterns of gene expression of these targets. The *sal* expression domain is narrower than that of *omb*. It appears that *sal* has a lower threshold than *omb* to repression by Brk leading to the differences in widths of the genes' expression domains along the A/P axis of the wing imaginal disc (Fig. 1-5) (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999).

Some mis-expression studies have supported the notion that *sal* and *omb* are differentially sensitive to Brk in that different levels of Brk can elicit distinct outputs of Dpp target gene expression (Moser and Campbell, 2005; Muller et al., 2003). Some of the most likely mechanisms to explain differences in sensitivity of threshold responses to a transcription factor gradient relate to the number and affinity of binding sites in the response elements of target genes as well as the position of binding sites relative to a promoter (Hewitt et al., 1999; Ochoa-Espinosa et al., 2005; Papatsenko and Levine, 2005).

It is important to remember that *brk* is not the only gene or gene product that controls the expression of Dpp targets; they are themselves often activated or influenced by Dpp signaling through Smad complexes, as described in detail above. Therefore, Brk is not the only protein involved in their expression, although it appears to be the primary factor responsible for properly positioning their expression domains.

The establishment of a *brk* expression gradient is significant in that Brk is required to repress Dpp target genes above distinct threshold levels of signaling. Without exact gradient placement, *sal* and *omb* would not be expressed in their characteristic nested patterns of gene expression where more Brk is required to repress *omb* than is required to repress *sal*. Without the proper regulation of expression domain placement of *sal* and *omb*, wing veins II and V are not properly developed thereby making the regulation of the *brk* expression gradient crucial for the establishment of target gene expression and thus adult wing structure and function (Campbell and Tomlinson, 1999). While no *brk* gene has been identified in animals other than arthropods, there also does not appear to be an extracellular antagonist functioning to regulate BMP signaling during larval wing imaginal disc development in *Drosophila*. These observations raise the possibility that *brk* may be important for wing patterning because it functions to regulate Dpp

signaling in the absence of a secreted antagonist. The study of the *brk* expression gradient is significant in terms of all developmental biology in that it provides a model for the understanding of the establishment and usage of transcription factor gradients and BMP signaling to regulate spatial patterns of gene expression.

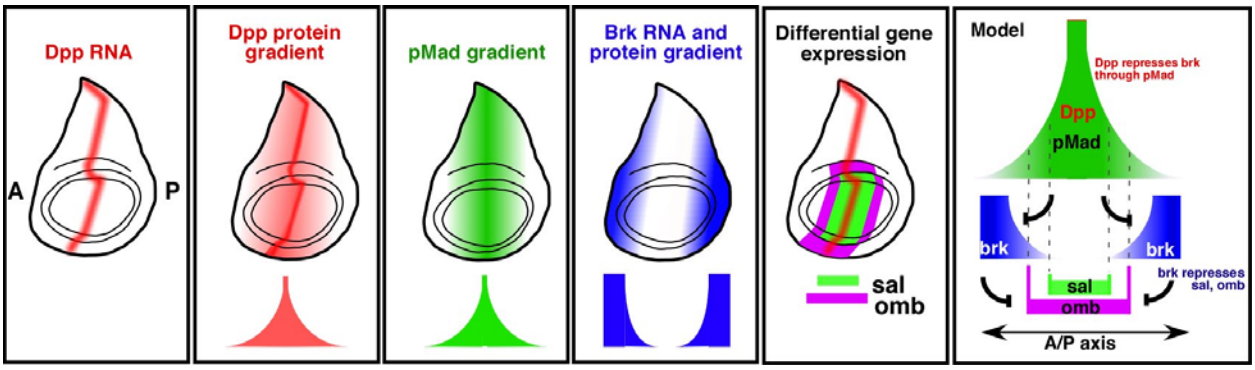

Figure 1-5: Differential gene expression in the *Drosophila* wing by Dpp and Brk.
dpp RNA is expressed in the center of the A/P axis of the wing disc. Following secretion and diffusion of the protein, Dpp becomes distributed in a mediolateral gradient. pMad is expressed in a complementary gradient. Brk is expressed at high levels in the lateral-most regions of the disc and shows graded expression toward the center of the disc. Dpp directly regulates this expression through pMad. The Dpp target genes *sal* and *omb* are differentially expressed in a nested pattern where *sal* requires higher levels of Dpp than *omb*. This model suggests that *sal* and *omb* are repressed by Brk and are differentially sensitive to it such that *sal* is repressed by lower levels of Brk than *omb*.

1.15 MECHANISMS OF BRK REPRESSION

The *brk* gene encodes a nuclear, 704 amino acid protein with a sequence-specific N-terminal helix-turn-helix (HTH) DNA binding motif (Fig. 1-6) (Campbell and Tomlinson, 1999). It uses this domain to interact directly with the *cis*-regulatory elements of a number of Dpp target genes in order to repress their expression (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and

Bienz, 2001; Sivasankaran et al., 2000; Zhang et al., 2001). DNA binding site selection and footprinting studies identified that Brk binds to multiple Dpp target genes via the sequence GGCGYY and helps to repress them in the absence of Dpp signaling, thus acting as a default repressor (Sivasankaran et al., 2000; Zhang et al., 2001).

Target gene repression by Brk requires its DNA-binding domain (DBD) as well as a repression domain/motif that is responsible for recruiting co-repressor proteins (Winter and Campbell, 2004). Different Brk target genes may be transcriptionally repressed by different mechanisms. The *cis*-regulatory elements of the embryonic Brk targets *zen* and *UbxB* contain Brk binding sites that overlap with sites where Mad binds and functions to activate transcription (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001; Winter and Campbell, 2004). While *in vitro* binding experiments demonstrated that Mad and Brk can compete for binding to those overlapping sequences, recent studies in the Campbell lab have indicated that Brk proteins containing only the DBD are insufficient for the repression of targets containing overlapping Brk and Mad binding sites (Winter and Campbell, 2004).

There are at least three non-equivalent repression domains/motifs in Brk through which it exerts transcriptional repression: the Gro interaction motif (GiM), CtBP interaction motif (CiM), and 3R (Fig. 1-6) (Winter and Campbell, 2004). The CiM and GiM recruit the corepressor proteins CtBP and Gro; loss of the CiM or GiM results in derepression of some Brk targets but not others, indicating that Brk might use different mechanisms and different domains/motifs to repress a variety of target genes (Hasson et al., 2001). Repressive activity in the absence of CtBP and Gro is provided by 3R, a domain of the Brk protein located near the DBD (Winter and Campbell, 2004). Experimental evidence suggests that all the domains/motifs contained within Brk are not equivalent. For example, the cofactors CtBP and Gro are differentially required by

Brk to repress a subset of targets including *sal* while they are dispensable for the regulation of other genes like *omb* as indicated by the observation that 3R is sufficient for the repression of *omb* but not *sal* (Hasson et al., 2001; Winter and Campbell, 2004). While it still remains unclear if Gro alone is sufficient for the repression of target genes by Brk, Brk may contain multiple domains/motifs so that it can repress different Dpp-responsive genes based on their response element context for both qualitative and quantitative purposes (Winter and Campbell, 2004).

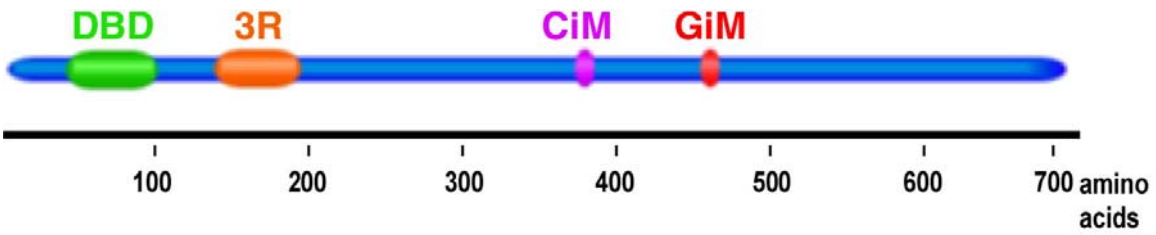

Figure 1-6: Schematic of the domains/motifs of the Brk protein.
Figure adapted from Winter and Campbell (2004). Domains/motifs in the 704 amino acid Brk protein included the DNA-binding domain (DBD) (green), the 3R repression motif (3R) (orange), the CtBP interaction motif (CiM) (purple), and the Groucho interaction motif (GiM) (red).

1.16 THE BRK REGULATORY REGION HAS SEPARABLE ENHANCER AND REPRESSOR ACTIVITIES

To understand the role played by Dpp in the generation of the *brk* expression gradient, the regulatory elements of the *brk* gene important for the regulation of its expression were analyzed by the Basler lab. Putative *brk* regulatory fragments from genomic *lambda* phages were cloned into a *lacZ* reporter P-element vector and transgenic flies were evaluated to determine which regulatory regions were responsible for *brk* expression. It was reported that a fragment, B14,

drives reporter gene expression in a pattern that recapitulates all aspects of late embryonic and larval *brk* expression (Muller et al., 2003). B14 is a 4.7 kb response element fragment located about 5 kb upstream of the start of *brk* transcription and was found to be divisible into two separable elements: a ubiquitously active, constitutive enhancer portion and a regulated repression element (Muller et al., 2003). Creating an extensive collection of truncated reporter constructs narrowed down both the repressive and activating properties of the B14 response element. The constitutive enhancer portion (for which the identity of the activator is not yet known) drives reporter gene expression ubiquitously in the wing disc while systematic point mutations throughout the repression element identified the basepairs essential for repressive activity leading to the conclusion that the repressive region, termed the *brk* silencer (S) element, is the minimal fragment necessary to direct repressive activity (Muller et al., 2003).

The *brk* S can operate independently of the *brk* enhancer in that it can impose repression on heterologous enhancers active in wing discs; this activity is strictly dependent upon Dpp signaling as demonstrated in *tkv* mutant cells which autonomously lose repression activity and by the ability of the S to regulate embryonic enhancers as well as ß-gal reporter assays in *Drosophila* S2 cells (Muller et al., 2003; Pyrowolakis et al., 2004). It was also demonstrated that *brk* expression could be altered at any level of Dpp signaling by altering the copy number of silencers and enhancers; increasing the number of silencers results in a lateral shift of reporter gene expression while adding multiple copies of the constitutively active *brk* enhancer leads to more medial reporter gene expression (Muller et al., 2003).

1.17 A REPRESSIVE PROTEIN COMPLEX IS ASSEMBLED ON THE *BRK* SILENCER

In *Drosophila*, Schnurri (Shn) is a large zinc-finger containing protein that interacts with pMad and Med in response to Dpp signaling (Muller et al., 2003; Pyrowolakis et al., 2004; Yao et al., 2006). The *brk* S assembles a pMad/Med/Shn (MMS) complex, where pMad and Med bind directly to the silencer element and then recruit Shn; this complex functions *in vivo* in a single copy (Fig. 1-7) (Muller et al., 2003; Pyrowolakis et al., 2004). Evidence for this observation consisted of electrophoretic mobility shift assays (EMSAs) of the *brk* S DNA using S2 cells lysates transfected with Mad, Med, and/or Shn containing plasmids. Tkv^{QD}, a constitutively active receptor in the Dpp signaling pathway, was also expressed in the cells in order to activate the Dpp signaling pathway (Raftery and Sutherland, 1999). A protein/DNA complex was formed upon transfection of Mad alone, however, a more prominent complex was observed upon co-transfection of Mad and Med (Muller et al., 2003). When Shn was transfected alone or with only Mad or Med, no protein/DNA complex was formed; however, when Shn was transfected with Mad and Med, a protein/DNA complex of slower mobility than either Mad or Med alone was established indicating that Mad and Med recruited Shn to the *brk* S (Muller et al., 2003). The molecular composition of the complexes was confirmed by supershift analysis with antibodies directed against the tags on the co-transfected MMS complex members.

The DNA sequence and spacing between binding sites necessary for the establishment of the MMS complex on the *brk* S has been well characterized through EMSA probe sequence mutation and subsequent binding studies with MMS complex members to be GRCGNCN(5)GTCTG (Gao and Laughon, 2006; Pyrowolakis et al., 2004). The *brk* S is bound by a heterotrimeric complex containing two Mad subunits (which bind to the GRCGNC

sequence) and one Med subunit (which binds to the GTCTG sequence) where all three subunits contributed directly to sequence-specific DNA contact through their MH1 domains (Fig 1-8) (Gao et al., 2005). Strikingly, Shn was found to need any five basepairs of sequence between the Mad and Med binding sites; any alteration (i.e. addition or removal of a base) results in a loss of Shn recruitment to the complex and a loss of repression when transgenes are analyzed in wing discs (Fig. 1-8) (Muller et al., 2003; Pyrowolakis et al., 2004). This indicates that the spacing between the Mad and Med sites is crucial for Shn recruitment and subsequent repression by the MMS complex as was confirmed by experiments that demonstrated minor alterations in sequence or spacing of binding sites can result in Dpp-responsive transcriptional activation instead of repression due to the failure of Shn recruitment to the MMS complex (Gao et al., 2005).

Shn confers transcriptional repressive activity to the MMS complex by acting as a co-repressor mediating Dpp-dependent repression of *brk* (Gao et al., 2005; Muller et al., 2003). The MMS complex is established in genes repressed, not activated, by Dpp signaling because Shn, which appears to act as a co-repressor, is only recruited to silencers in the presence of pMad/Med. *brk* is not repressed when the *brk* S is missing or mutated, Dpp input is prevented, or when the C-terminal zinc fingers of Shn are deleted/mutated; similarly, the formation of the MMS complex is dependent upon these same requirements (Pyrowolakis et al., 2004). It is believed that the *brk* S controls the establishment of the *brk* expression gradient by assembling the MMS multiprotein complex; however, it is unclear if the MMS complex is the only input necessary for the establishment of the Brk expression gradient; this will be a focus of my work in this dissertation.

In contrast to *brk*, *shn*-related genes have been identified in other animals and serve important roles in cell regulation; both *Drosophila* Shn and hShn1 from humans mediate Dpp-dependent repression of *Drosophila* Dpp-responsive silencers (Yao et al., 2006). In vertebrate cells, however, a BMP-responsive element is acted on in a positive manner by both Shn and hShn1 (Yao et al., 2006). These results seem to indicate that complexes formed between Shn and Smads may function differently in vertebrates and *Drosophila*, perhaps in context-dependent manners through the recruitment of positively or negatively acting co-factors.

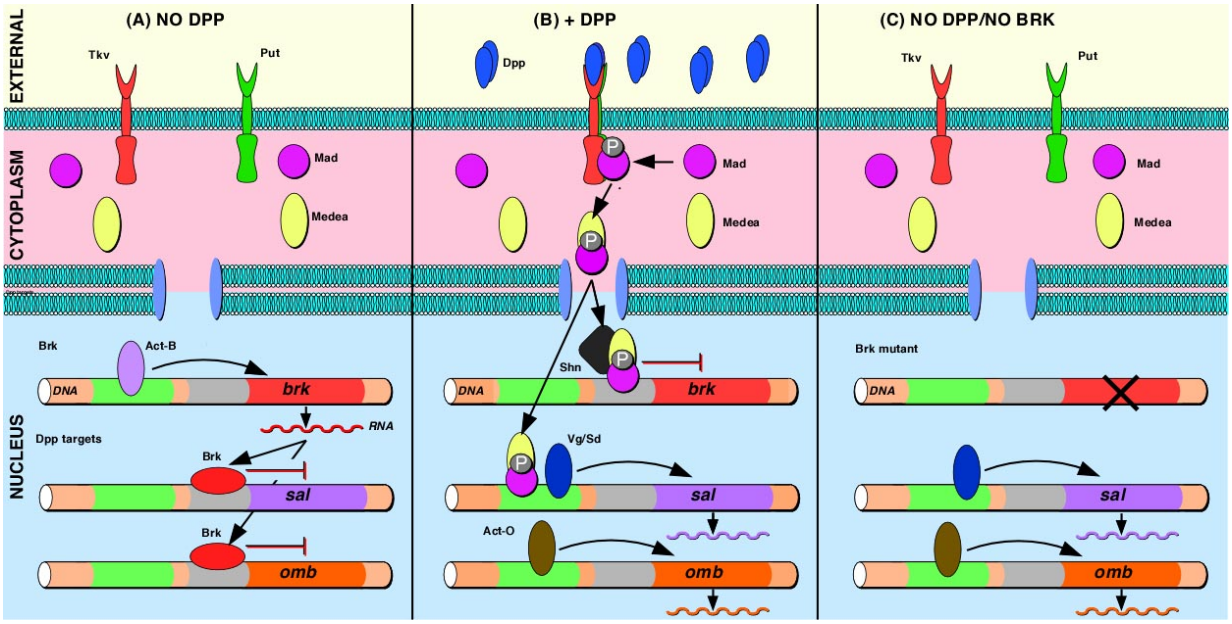

Figure 1-7: Model of regulation of gene expression by Dpp and Brk in the *Drosophila* wing.
(A) In the absence of Dpp signaling, the R-Smad, Mad, and the Co-Smad, Med remain in the cytoplasm allowing high-level expression of *brk* under the control of a yet unidentified activator, Act-B. Brk protein binds at the response elements of Dpp target genes repressing their expression. (B) In the presence of Dpp signaling, Mad is recruited to the activated receptors where it is phosphorylated and thus translocates to the nucleus with Med where it binds response elements in the *brk* gene and, in combination with Shn, represses the expression of *brk*. The absence of Brk allows the transcriptional activation of Dpp targets by tissue specific transcriptional activators, sometimes Mad and Med themselves. (C) In the absence of Dpp and Brk, Dpp targets are expressed due to the sufficiency of their tissue specific activators.

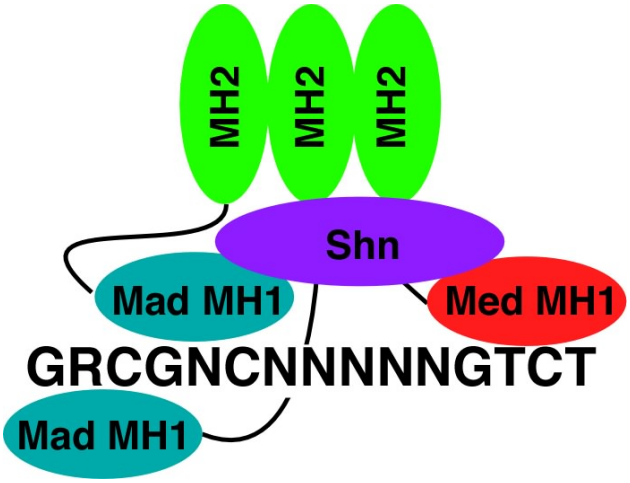

Figure 1-8: Model of the MMS complex bound to the *brk* S.
Figure adapted from Gao et. al (2005). The Mad/Med heterotrimer appears to consist of two Mad MH1 domains contacting the *brk* S DNA at the GRCGNC sequence and one Med MH1 domain contacting DNA at the GTCT sequence. The MH2 domains are thought to interact and contribute to the formation of the heterotrimer. Shn is recruited to the five basepairs of DNA between the Mad and Med binding site and is shown to contact the Mad and Med MH1 domains because it is not recruited in the absence of these proteins.

1.17.1 Key differences exist between activation and repression by pMad

The key factor in the determination of whether the binding sites will activate or repress transcription is based on the binding site position—binding sites with five basepairs of sequence between them will recruit Shn while those spaced closer or further away will not (Muller et al., 2003; Pyrowolakis et al., 2004). Thus, it is important to distinguish the repressive MMS complex-forming Mad and Med binding sites from those where Mad and Med act as transcriptional activators. Activating Mad binding sites are typically G/C rich and are difficult to identify because pMad is not a terribly efficient DNA-binding protein; nevertheless, the binding sites often contain a GCCGnCGC motif (Kim et al., 1997; Massague et al., 2005; Xu et al., 1998). Therefore, the Dpp signaling pathway appears to split into two major branches in the nucleus downstream of the activated nuclear pMad/Med complex: a branch involved in *brk*

repression that is Shn-dependent and a Shn-independent branch involved in the activation of gene expression.

1.18 DPP-RESPONSIVE SILENCER ELEMENTS

When the consensus sequence for the MMS complex is used to scan the *Drosophila* genome, about 350 putative silencers can be identified (Pyrowolakis et al., 2004). Functional MMS complex-dependent silencers can be found not only in *brk*, but also in other *Drosophila* genes repressed by Dpp signaling; thus, the *brk* repression element as well as these other silencers can be categorized as Dpp-responsive silencer elements (Pyrowolakis et al., 2004).

One Dpp-responsive S identified by genome searches is located in the 5'untranslated region of *bag of marbles* (*bam*), a gene that encodes the key regulator determining asymmetric division of the *Drosophila* germline stem cell (Chen and McKearin, 2003a). *bam* transcription is specifically repressed in germline stem cells by Dpp signaling through a transcriptional S element to which pMad and Med have previously been shown to bind (Chen and McKearin, 2003a; Chen and McKearin, 2003b). Sequence similarity between the *bam* S and *brk* S suggested both recruited Shn; this was confirmed by gel shift analysis conducted in the Basler lab (Muller et al., 2003). Additionally, substitution of the *brk* S with the *bam* S along with the *brk* enhancer and *lacZ* resulted in a reporter gene expression pattern identical to that achieved when using the *brk* S (Muller et al., 2003). Therefore, Dpp represses the transcription of genes other than *brk* directly by utilizing Shn and the formation of the MMS complex.

When sequence alignments were conducted, all the conditions described above to be important for MMS complex formation and subsequent repression were shared between the *brk*

S and *bam* S. The *brk* gene was found to contain a total of ten silencers, most likely because *brk* requires an S near each of the different enhancers required to drive expression in various tissues or because the interpretation of the Dpp gradient might require a combinatorial activity of several silencer elements (Pyrowolakis et al., 2004; Yao et al., 2008).

1.19 THESIS AIMS

The primary goal of my project was to understand how a *brk* expression gradient is established from a complementary gradient of BMP signaling and how it is then used to generate spatial patterns of gene expression. The preceding introduction has illustrated the importance of transcription factor gradients due to the ability of these gradients to repress different targets above distinct thresholds, thereby allowing targets to be expressed at different positions along an axis to control differentiation of specific cell types in these positions.

This project began from unpublished, preliminary studies that suggested Brk acts as a transcriptional repressor to negatively autoregulate its expression (see Chapter 2). I set out to understand how this negative autoregulation occurs. Does Brk repress its own expression by binding putative Brk binding sites in its own regulatory regions? Does it work through the silencer or enhancer portion of its regulatory element? Does it interact with other proteins previously described to control Brk (e.g. the MMS complex) to exert transcriptional repression upon itself?

I also sought to determine what response elements contribute to (are necessary for) graded *brk* expression. Initial studies published by the Basler lab, as discussed above, presented a response element that drove a reporter gene in a pattern similar to *brk*; however, its expression

could not exactly recapitulate endogenous *brk* expression (Muller et al., 2003). I determined that this response element failed to rescue a *brk* mutant, thus confirming the imprecise nature of the reconstituted response element expression. Therefore, I sought to determine what other response elements and recruited transcription factors were necessary to establish a proper *brk* expression gradient.

The studies I have performed have added two additional requirements for the establishment of the *brk* expression gradient. While it was previously thought that one positive input (activation by a yet unidentified activator at the enhancer) and one negative input (repression by the MMS complex on the *brk* S) created graded *brk* expression, my studies demonstrated that additional negative and positive influences are necessary. This information greatly contributes to the understanding of how the *brk* expression gradient is established in the correct location so that it can properly generate spatial patterns of gene expression that are required for proper embryo/tissue morphogenesis.

2.0 GENERATION OF THE BRK EXPRESSION GRADIENT REQUIRES BRK TO NEGATIVELY AUTOREGULATE

2.1 INTRODUCTION

2.1.1 Comparison of *brk* expression to that of pMad and downstream targets *sal* and *omb*

Previous studies demonstrated *brk* is expressed in lateral-to-medial gradients in the anterior and posterior halves of the wing pouch (Campbell and Tomlinson, 1999; Jazwinska et al., 1999b; Minami et al., 1999; Muller et al., 2003). Initially we re-examined the *brk* expression gradient in more detail and compared it to that of pMad, which is directly responsible for its establishment (Muller et al., 2003), and to downstream targets *sal* and *omb*, which are directly regulated by Brk (Barrio and de Celis, 2004; Sivasankaran et al., 2000). Brk expression was monitored using either a Brk-specific antibody (anti-brk), or with the enhancer trap, brk^{XA} (with anti-ßgal); both demonstrated graded expression in the wing pouch (Fig. 2-1 A). pMad expression was revealed using an antibody against the phosphorylated form and antibody staining was clearly graded from the medial peaks in the A and P compartments, but the gradient was fairly sharp and levels drop to background quite rapidly so that antibody staining appeared at this background level over most of the region where *brk* expression was graded (Fig. 2-1 Bii). Note that this reflects the limits of our ability to detect physiological levels of pMad with the antibody because genetic

studies demonstrated that pMad is required to repress *brk* even in the regions where pMad levels detected with the antibody are at background levels (Campbell and Tomlinson, 1999). However, this suggested that relatively low levels of pMad are sufficient to repress *brk*.

Closer examination revealed a slight difference in the gradient profile of the anti-brk staining compared to that of the enhancer trap, with the latter gradient being shifted more medial than the former (Fig. 2-1 A). This could be explained by a number of reasons: first, there may have been a real difference in *brk* RNA versus protein expression, second, the enhancer trap may not have faithfully reproduced *brk* expression, third, if the gradient was not static, perdurance of ß-gal protein may have accounted for this difference, or lastly, the most likely explanation, the ßgal antibody was simply more sensitive than the anti-brk antibody. Whether these staining patterns actually reflected where *brk* is expressed can be judged by comparing them to where Brk is known to repress target genes, as described below.

Two of the best-characterized targets of Brk are *sal* and *omb*, with the limits of *sal* expression being more medial than that of *omb* (Fig. 2-1 C) (Lecuit et al., 1996; Nellen et al., 1996). Loss of *brk* results in the expansion of both the *sal* and *omb* domains (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999), so *brk* must be expressed in the cells immediately lateral to the *sal* expression domain. Comparison of the *brk* enhancer trap staining with that of *sal* shows that in the anterior compartment, the edge of the *sal* expression domain coincides with the site at which ßgal expression is first detected (Fig. 2-1 C) and indicates this enhancer trap is probably a good reflection of where *brk* is actually expressed. Overall, these results strongly support the hypothesis that more Brk is required to repress *omb* than is required to repress *sal* (Moser and Campbell, 2005).

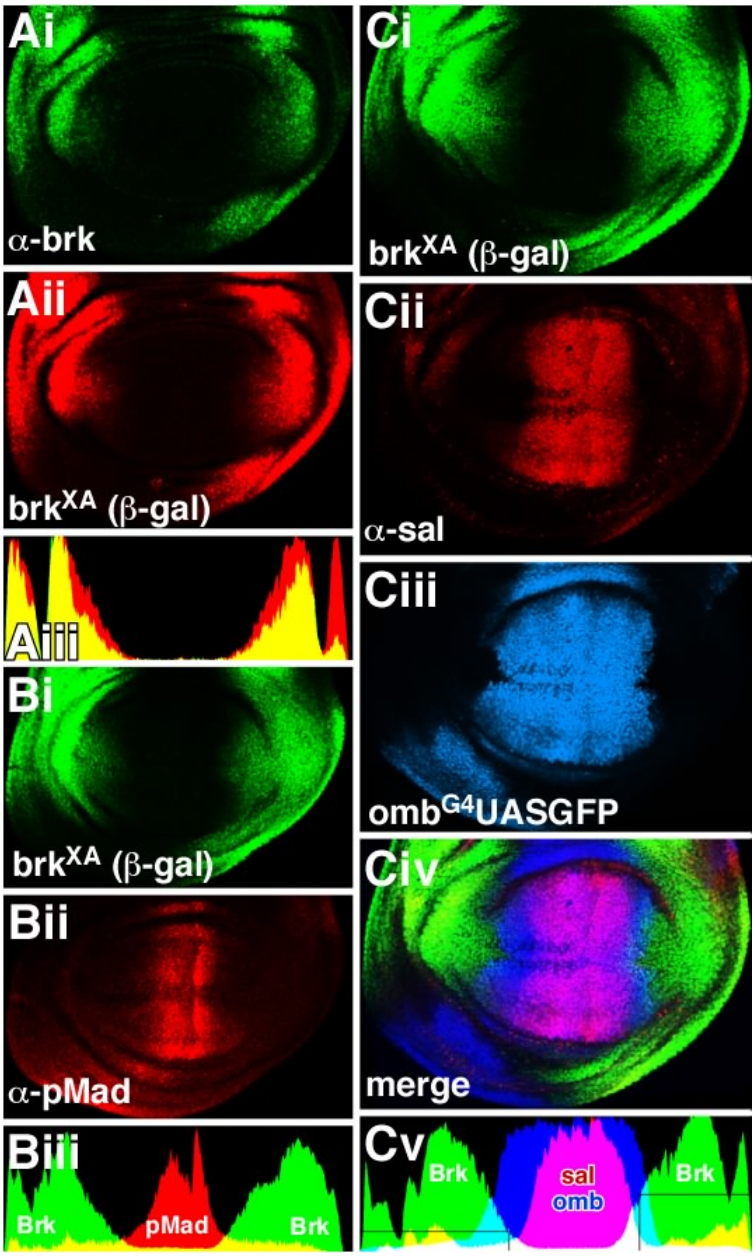

Figure 2-1: Expression of *brk* and Brk target genes in wildtype wing discs.
Figure published in Moser and Campbell (2005). (A) Expression of *brk* revealed with a specific antibody (Ai; green) and a brk-*lacZ* enhancer trap (Aii; brk^{XA}/+; red). (Aiii) An intensity profile across the A/P axis (from left to right, averaging the region between the two horizontal lines in Aii centered on the dorsoventral boundary) shows the lateral-to-medial gradient of expression in the anterior and posterior halves of the wing pouch; there is a slight difference in the profile of the gradients revealed by the antibody and enhancer trap. (B) Comparison of *brk* expression (Bi; brk^{XA}/+; green) to that of pMad (Bii; red). (Biii) An intensity profile shows that although pMad was graded, at the levels of detection, this gradient does not extend significantly into the region where *brk* is graded. (C) Comparison of brk-*lacZ* expression (Ci; brk^{XA}/+; green) to that of Brk targets, *sal* (Cii, red) and *omb* (Ciii; blue). (Civ) Merge panel of (Ci), (Cii), and (Ciii). The intensity profile (Cv) shows that there is little overlap between *sal* and *brk* in the anterior, but this is not the case in the posterior, suggesting *sal* is repressed above a higher threshold of Brk (indicated by the lines) in the posterior compared to the anterior. (ßgal expression in brk^{XA}, pMad, and *sal* expression were detected using specific antibodies; *omb* expression was detected using omb-Gal4; UAS-GFP).

2.1.2 Autoregulation by Brk is also important for *brk* expression

The studies described above clearly demonstrated that a gradient of *brk* expression is essential for both maintaining the normal morphology of the wing disc and establishing nested patterns of gene expression along the A/P axis; they demonstrated that the extracellular Dpp protein gradient establishes the *brk* gradient by generating an intracellular gradient of pMad which, in combination with the co-Smad, Med, binds to silencer elements upstream of *brk* and recruits Shn to repress transcription (Muller et al., 2003; Pyrowolakis et al., 2004). However, *brk* expression has also been suggested to be negatively regulated by Brk itself (Hasson et al., 2001) and the Campbell lab initially investigated the role Brk plays in establishing its own expression pattern by analyzing what happens to the *brk* expression gradient in the absence of Brk protein. This was achieved initially by monitoring ßgal expression in the brk^{XA} enhancer trap.

The *brk* mutant, brk^{XA}, has a *lacZ* P-element inserted just upstream of the transcription start site (Campbell and Tomlinson, 1999) and wing discs from brk^{XA} mutant hemizygotes have a strong *brk* phenotype that is associated with markedly reduced levels of Brk protein (as described in Chapter 1). In brk^{XA}/+ heterozygous discs, ßgal expression is similar to that of Brk protein, showing a clear lateral-to-medial graded expression in the anterior and posterior halves of the wing pouch (Fig. 2-2A). However, in brk^{XA} mutant discs, although ßgal is still expressed, there is a dramatic alteration in both the level and pattern of expression compared to that in heterozygous discs; the general level of expression is increased (Fig. 2-2 D) (this was partly, but probably not entirely, due to dosage compensation as *brk* is located on the X chromosome). Although expression is still absent from the medial region of the wing disc, there is very little graded expression such that there is a fairly sharp boundary between cells expressing ßgal and those not (Fig. 2-2). When compared to wildtype discs, it is apparent that the normal pattern of

no *brk* expression in the medial region, high levels in the lateral region, and graded levels in the mediolateral region is modified in the *brk* mutant discs to no expression in the medial region and high levels in both the mediolateral and lateral regions; in other words, the region that shows graded expression in wildtype discs has high levels of expression in the mutant (Fig. 2-2 C, F). One possible reason for this difference could be that pMad levels are modified in the mutant discs. However, examination of pMad expression in the mutant discs revealed that there is no apparent difference compared to that in wildtype discs (Fig. 2-2 E, F). This indicated that Brk is required to repress itself to generate the gradient of expression (Moser and Campbell, 2005).

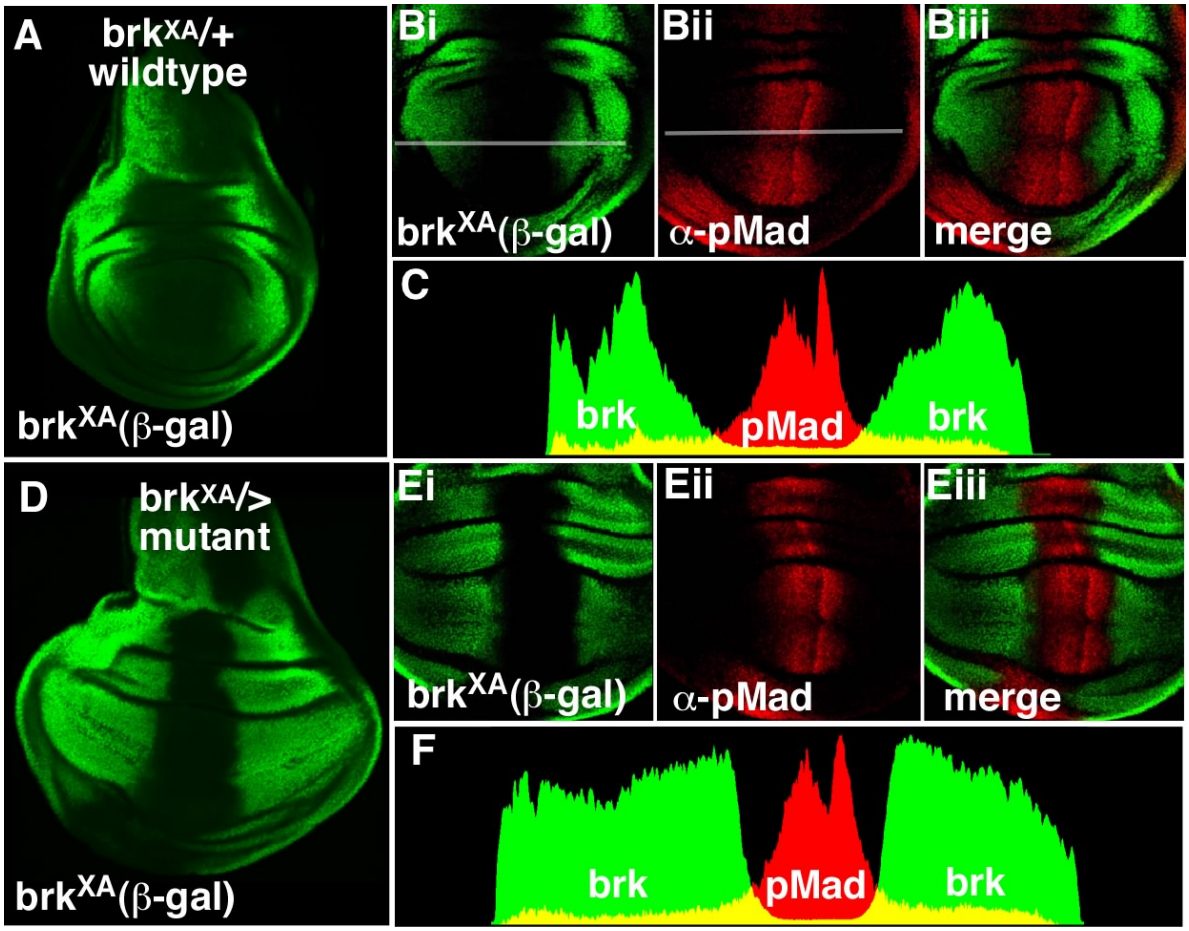

Figure 2-2: The *brk* expression gradient is lost in a *brk* mutant.
Figure adapted from Moser and Campbell (2005). (A) Phenotypically wildtype disc from a brk^{XA}/+ animal stained for ßgal expression. (Bi) The same disc as (A) with the central region enlarged. (Bii) The same disc as (Bi) stained for pMad expression. (Biii) Merge of (Bi) and (Bii). (C) Intensity profile of ßgal and pMad expression across the A/P axis in a brk^{XA}/+ wildtype disc. pMad is expressed in the central region of the disc and *brk* expression levels are high laterally while graded *brk* expression is found in the mediolateral region of the disc with expression absent medially. (D) Hemizygous mutant brk^{XA} disc stained for ßgal expression in parallel with the disc in (A) at the same magnification as the disc in (A). The general levels of expression are clearly higher, expression extends more medially, and expression is not graded. (Ei) The same disc as (D) with the central region enlarged. (Eii) The same disc as (Ei) stained for pMad expression. (Eiii) Merge of (Ei) and (Eii). (F) Intensity profile of ßgal and pMad expression across the A/P axis in brk^{XA} mutant disc. The graded *brk* expression demonstrated in (C) is lost in the mutant where there is a fairly sharp boundary between high levels of expression laterally/mediolaterally and no expression medially.

2.1.3 Aims of these studies

This project began from preliminary studies that suggested Brk acts as a transcriptional repressor to negatively autoregulate its own expression (Hasson et al., 2001; Moser and Campbell, 2005). I set out to determine how this negative autoregulation occurs. Did Brk repress its own expression by binding putative Brk binding sites in its own regulatory regions? Did it work through the silencer or enhancer portion of its regulatory element? Additionally, could it interact with other proteins previously described to control *brk* expression (e.g. the MMS complex) in order to exert transcriptional repression upon itself?

2.2 MAD AND MED REGULATE *BRK* EXPRESSION

Studies described above suggested that *brk* expression is dependent upon Brk autoregulation; a proper *brk* expression gradient is not formed in the absence of Brk repression (Moser and Campbell, 2005). To test if pMad and Med are required to repress *brk* expression, pMad and Med mutant clones were generated and subsequent *brk* expression, as well as *brk* target gene expression, was analyzed.

pMad and Med mutant clones were created in third instar wing discs (Fig. 2-3 Ai, Bi, Ci). When pMad and Med mutant clones were examined in the central and mediolateral portions of the wing disc (where both proteins are typically expressed), an upregulation of *brk* was observed (Fig. 2-3 Aii, Bii, Cii). Therefore, pMad and Med are required to repress *brk* expression in the central and mediolateral regions of the wing disc. pMad and Med mutant clones in lateral regions of the wing discs (where pMad and Med are typically not expressed) did not demonstrate

any effect on *brk* expression indicating that pMad and Med do not function to repress *brk* expression in the lateral most regions of the wing disc. Further examination of *brk* mis-expression in Med mutant clones in the central portion of the wing disc demonstrated that mis-expression of Brk repressed *sal* and *omb* expression in the central portion of the wing disc where both *sal* and *omb* normally exhibit high levels of expression (Fig. 2-3 Biii, Ciii). These results indicated that *brk* expression is dependent upon repression by pMad and Med in the mediolateral and medial regions of the wing disc.

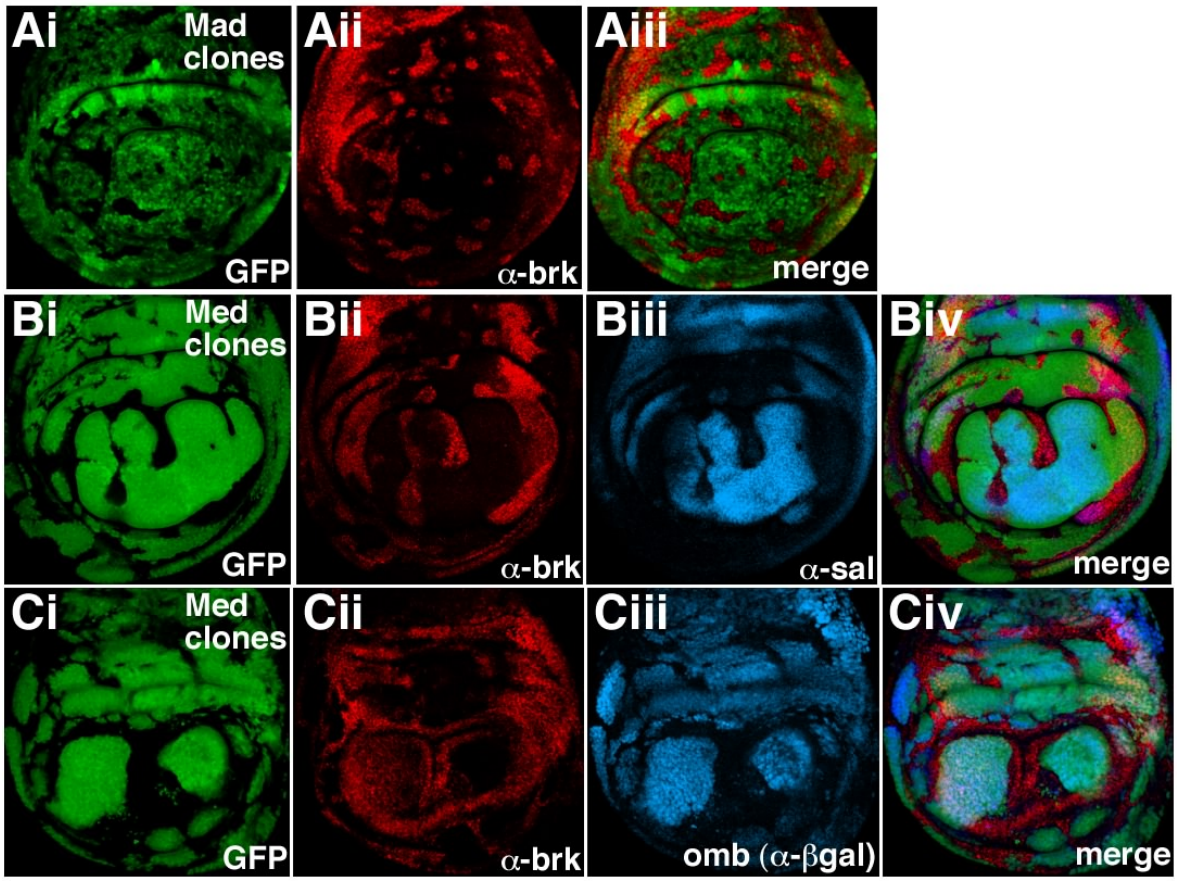

Figure 2-3: Mad and Med are required to repress *brk* expression.
(A) Mad mutant clones. (Ai) Mad mutant clones are marked by a loss of GFP. (Aii) *brk* expression is upregulated in mutant clones (anti-brk). (Aiii) merge of (Ai) and (Aii). (B) and (C) Med mutant clones. (Bi) Med mutant clones are marked by a loss of GFP. (Bii) *brk* expression is upregulated in mutant clones (anti-brk). (Biii) *sal* expression (anti-sal) is absent in the medial region in mutant clones where Brk is mis-expressed. (Biv) merge of (Bi), (Bii), and (Biii). (Ci) Med mutant clones are marked by a loss of GFP. (Cii) *brk* expression is upregulated in mutant clones (anti-brk). (Ciii) *omb* expression (omb-*lacZ*, anti-ßgal) is absent in the medial region in mutant clones where Brk is mis-expressed. (Civ) merge of (Ci), (Cii), and (Ciii).

2.3 BRK AUTOREGULATES THE B14 & B315 RESPONSE ELEMENTS

I previously presented evidence (in Section 2.1) that suggested Brk plays a role in establishing its own expression pattern by comparing ßgal expression of the brk^{XA} enhancer trap in mutant hemizygotes and phenotypically wildtype heterozygotes. Further evidence was obtained below through analyzing the expression of *brk* reporters in wing discs containing *brk* null mutant clones. In this section, two previously published response elements, B14 and B315, were used to support the hypothesis (Muller et al., 2003).

2.3.1 Brk autoregulates B14 in the wing disc

The expression of the *brk* reporter B14X was examined in wing discs containing *brk* null mutant clones. Recall that the B14 genomic fragment possesses the Mad/Med/Shn (MMS) silencer element as well as an enhancer element and was demonstrated to drive *lacZ* expression in a pattern similar to that of the endogenous gene (Muller et al., 2003). B14X is this B14 genomic fragment driving *lacZ* reporter gene expression and was obtained from the Basler lab (construct described in more detail in Chapter 3) (Muller et al., 2003).

B14X expression was dramatically upregulated in *brk* null mutant clones located in the mediolateral and lateral regions of the wing disc, but B14X was not expressed in clones located in the medial regions (Fig. 2-4). This indicated that Brk functions to repress B14X expression in the lateral and mediolateral regions of the wing disc but does not function in the medial region of the disc where pMad functions to repress *brk* expression. Thus, the B14 response element is autoregulated by Brk (Moser and Campbell, 2005).

2.3.2 Brk represses B14 in the embryo

To verify that the same autoregulation occurs in embryos, the B14 response element was utilized to drive a GFP reporter gene (B14B) and tested in embryos (construct described in more detail in Chapter 3). B14B drove reporter gene expression in embryos ubiquitously, apart from the ventrolateral region where *dpp* is expressed; in this region, reporter gene expression was repressed (Fig. 2-4 E). This expression is similar to that described previously for B14X and is expected based on what is known about *dpp* expression (Muller et al., 2003). Our anti-brk antibody does not work on embryos, therefore, a UAS-Brk protein with a C-terminal HA tag (UAS-3PF3-HA) (Winter and Campbell, 2004) was driven with the engrailed^{Gal4} (en^{G4}) driver; this construct drove UAS-3PF3-HA in stripes consistent with en^{G4} expression (Fig. 2-4 F). Upon examination of B14B expression in these embryos, ectopically expressed Brk was found to repress B14B expression (Fig. 2-4 G). This result was also obtained for B14X (data not shown). These results indicated that Brk represses B14 in embryos, just as it does in wing imaginal discs (Section 2.3.1).

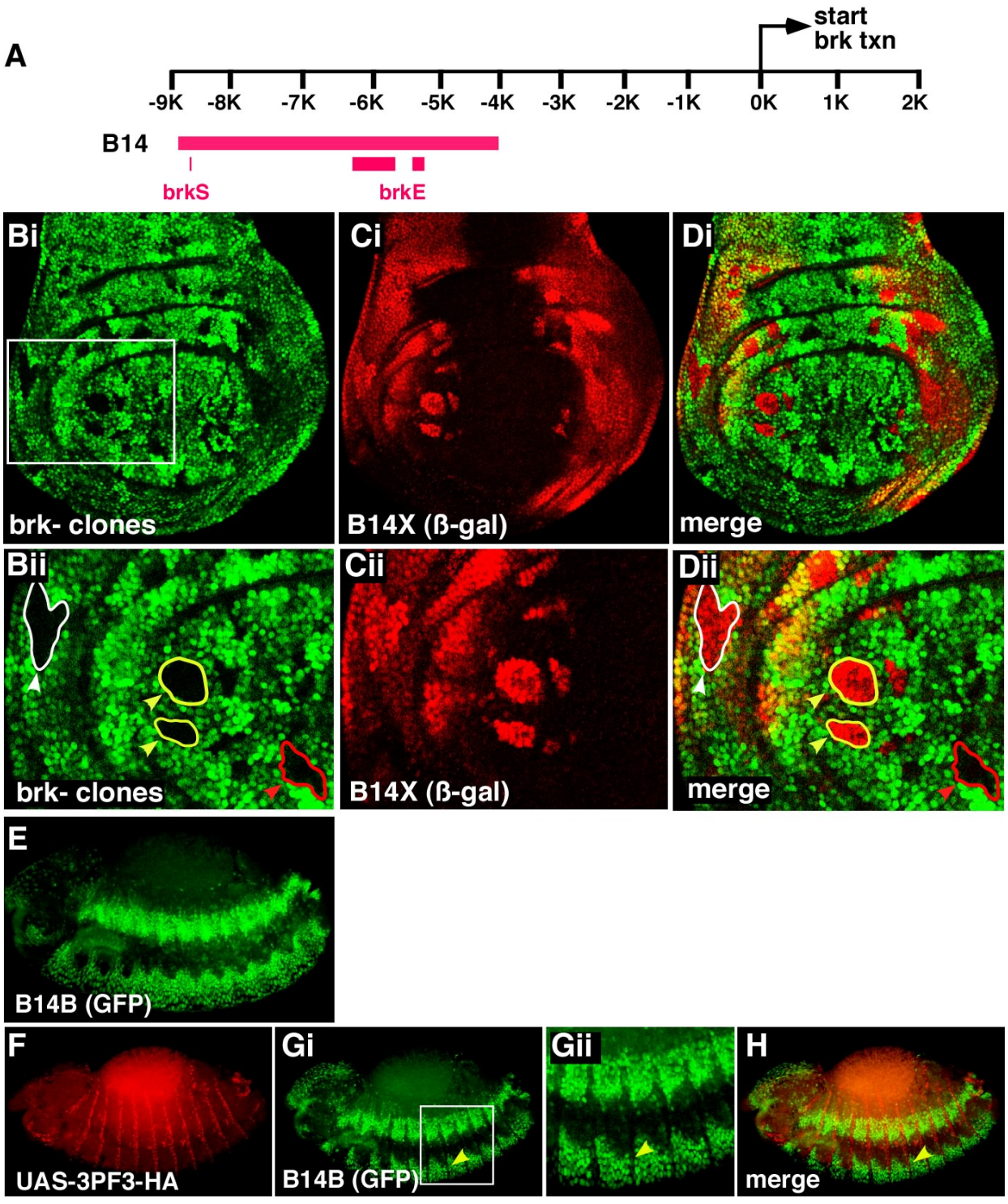
A
start
brk txn
-9K
-8K
-7K
-6K
-5K
-4K
-3K
-2K
-1K
0K
1K
2K
B14
brkS
brkE
Bi
brk- clones
Ci
B14X (ß-gal)
Di
merge
Bii
brk- clones
Cii
B14X (ß-gal)
Dii
merge
E
B14B (GFP)
F
UAS-3PF3-HA
Gi
B14B (GFP)
Gii
H
merge

Figure 2-4: Brk represses/autoregulates the B14 response element.
(Adapted from Moser and Campbell, 2005). (A) Schematic diagram of the B14 response element located upstream of the start of *brk* transcription as well as the enhancer (E) and silencer (S) regions of which B14 is composed. (B-D) Brk loss of function clones demonstrate upregulation of B14X indicating Brk is required to repress B14X. (Bi) *brk* null clones are marked by a loss of GFP. (Bii) magnification of the region outlined in white in (Bi) where a lateral clone is marked in white, several mediolateral clones are marked in yellow, and a medial clone is marked in red. (Ci) B14X expression demonstrates an upregulation of reporter gene expression in lateral *brk* null clones and in mediolateral clones but no upregulation of reporter gene expression in medial regions where pMad is sufficient for repression indicating that Brk autoregulates the B14X construct. (Cii) magnification of (Ci). (Di) merge of panels (Bi) and (Ci). (Dii) Magnification of (Di) where clones are marked as described in (Aii). B14X expression is off in the medial clone but is upregulated in the mediolateral clone to levels similar to those laterally. The lateral clones also demonstrate an upregulation of expression. (E) Expression of the B14B reporter in wildtype embryo. (F-H) Embryo where UAS-3PF3-HA (full-length wildtype Brk) is driven with the en^{G4} driver and B14B expression examined. (F) UAS-3PF3-HA driven with the en^{G4} driver and detected with anti-HA. (G) B14B expression (detected with GFP). (Gi) B14B expression is repressed in the regions where UAS-3PF3-HA is driven (yellow arrowhead) indicating that Brk represses the B14B construct in the embryo, when compared to (E). (Gii) Magnification of the region of (Fi) outlined in the box with repressed region indicated by yellow arrowhead. (H) Merge panel of (F) and (Gi) where region repressed is marked with yellow arrowhead.

2.3.3 Brk autoregulates B315 in the wing disc

To examine if the B315 response element is also regulated by Brk (as is B14), B315X reporter gene expression was examined in *brk* null clones. B315X is comprised of the S and E portions of the B14 genomic fragment driving *lacZ* reporter gene expression and was obtained from the Basler lab (construct described in more detail in Chapter 3) (Muller et al., 2003).

B315X expression was dramatically upregulated in *brk* null mutant clones located in the lateral and portions of mediolateral clones of the wing disc (Fig. 2-5). B315X was not upregulated in clones located in the medial regions (Fig. 2-5). This indicated that Brk is functioning to repress B315X expression in the lateral and mediolateral regions of the wing disc but does not function in the medial region of the disc where pMad functions to repress *brk* expression. B315X is not expressed in embryos most likely because it does not contain an embryonic enhancer element, so it was not tested for repression by Brk as described for B14 in

Section 2.3.2. Altogether, these resulted indicated that the B315 response element is autoregulated by Brk.

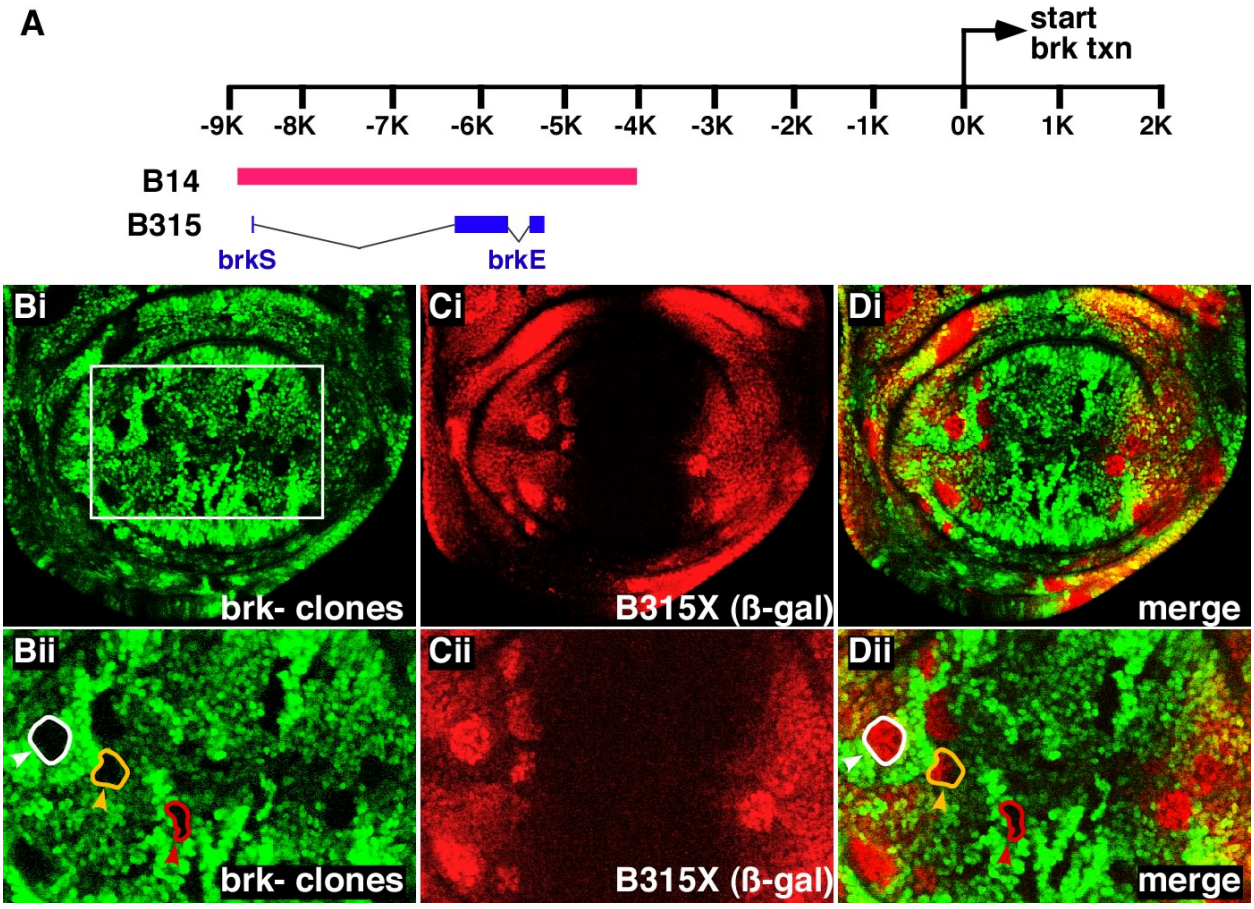

Figure 2-5: Brk represses/autoregulates the B315 response element.
(A) Schematic diagram of the B14 and B315 response elements located upstream of the start of *brk* transcription. B315 consisted of two regions: 1) an enhancer (E) region and 2) the silencer (S) region. (B-D) Brk loss of function clones demonstrate upregulation of B315X indicating Brk is required to repress B315X. (Bi) *brk* null clones are marked by a loss of GFP. (Bii) magnification of the region outlined in white in (Bi) where a lateral clone is marked in white, a mediolateral clone is marked in yellow, and a medial clone is marked in red. (Ci) B315X expression demonstrates an upregulation of reporter gene expression in the lateral *brk* null clone and in portions of the mediolateral clone but no upregulation of reporter gene expression in medial regions where pMad is sufficient for repression indicating that Brk autoregulates the B315X construct. (Cii) magnification of (Ci). (Di) merge of panels (Bi) and (Ci). (Dii) Magnification of (Di) where clones are marked as described in (Bii). B315X expression is off in the medial clone but is upregulated in the lateral portion of the mediolateral clone to levels similar to that laterally. The lateral clones also demonstrate an upregulation of expression.

2.4 BRK REQUIRES ITS DBD TO AUTOREGULATE

As described earlier, the *brk* gene encodes a nuclear, 704 amino acid protein with a sequence-specific N-terminal HTH DNA binding motif (Campbell and Tomlinson, 1999). The DBD is used to interact directly with the *cis*-regulatory elements of a number of Dpp target genes, allowing Brk to repress their expression (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001; Sivasankaran et al., 2000; Zhang et al., 2001). DNA binding site selection and footprinting studies identified that Brk binds to many Dpp target genes via the sequence GGCGYY (Sivasankaran et al., 2000; Zhang et al., 2001). Target gene repression by Brk requires its DBD as well as a repression domain/motif (Winter and Campbell, 2004).

To begin to determine the mechanism utilized by Brk to autoregulate, we tested a *brk* DBD mutant for its ability to autoregulate. brk^{F124} is an EMS point mutant with an amino acid substitution (R82W) in the recognition helix of its DNA binding domain; it was determined to have little or no repressive activity (Winter and Campbell, 2004). brk^{F124} mutant clones were created in wing imaginal discs (Fig. 2-6). The discs were stained with an anti-brk antibody that detected endogenous (wildtype) Brk as well as the mutant protein produced from the brk^{F124} mutant. Upon examination of anti-brk staining in brk^{F124} homozygous mutant clones, an upregulation of Brk was observed compared to the staining observed for nearby heterozygous tissue (Fig. 2-6 Aii). This indicated that brk^{F124} homozygous mutant clones are not able to autoregulate Brk, thus leading to the over-expression detected with anti-brk compared to nearby heterozygous tissue. This indicated that Brk needs its DBD to autoregulate.

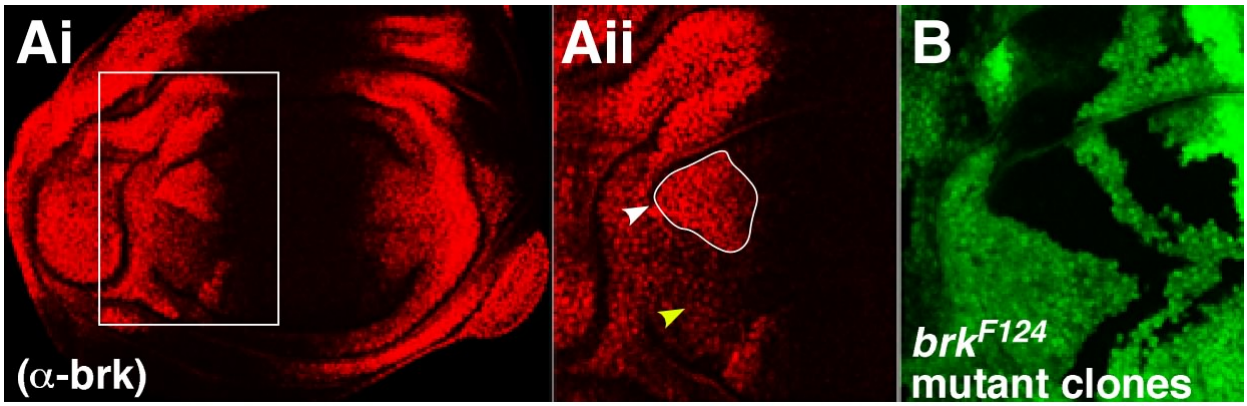

Figure 2-6: *brk*^{F124} homozygous mutant clones demonstrate Brk requires its DBD to autoregulate.
(Ai) Anti-brk staining reveals staining from endogenous (wildtype) Brk as well as that of *brk*^{F124}. White box indicates region enlarged in (Aii) and (B). (Aii) Mutant clone (outlined in white, white arrowhead) demonstrates an upregulation of Brk (detected with anti-brk) compared to the heterozygous tissue below it (yellow arrowhead) indicating that *brk*^{F124} fails to autoregulate in the mutant clones thereby indicating Brk requires its DBD for autoregulation. (B) *brk*^{F124} homozygous mutant clones are marked by a loss of GFP.

2.5 BRK MOST LIKELY DOES NOT INTERACT DIRECTLY WITH DNA TO AUTOREGULATE

Because Brk appears to function as a transcriptional repressor that requires its DBD to autoregulate its own expression, several possibilities exist as to the mechanism(s) Brk may utilize to confer its autoregulatory inhibition. One possibility is that Brk binds to cognate, well-characterized Brk binding sites contained within its regulatory regions to repress its own expression. A second possibility is that Brk utilizes its DBD to bind non-cognate sites on DNA to autoregulate. A third possibility is that Brk interacts indirectly with DNA to autoregulate by associating with other proteins bound to DNA. The ability of Brk to interact directly with DNA will be examined in this section.

2.5.1 Brk does not repress by binding cognate Brk binding sites

Because Brk binds directly to the *cis*-regulatory elements of a number of Dpp target genes to repress their expression, it is possible that it uses its DBD to bind Brk binding sites in the *brk* regulatory regions to autoregulate (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001; Sivasankaran et al., 2000; Zhang et al., 2001). As mentioned previously, DNA binding site selection and footprinting studies identified that Brk binds to many Dpp target genes via the sequence GGCGYY in order to repress (Sivasankaran et al., 2000; Zhang et al., 2001). Therefore, the putative Brk binding sites consisting of the GGCGYY sequence were identified in the *brk* regulatory region and their positions indicated in or near the B14 and B315 response elements (Fig. 2-7). The B14 response element possesses 15 cognate Brk binding sites between the silencer and enhancer (one of these sites is contained within the enhancer). The B315 response element consists of a 53bp silencer and an 860bp enhancer, which contains one Brk site.

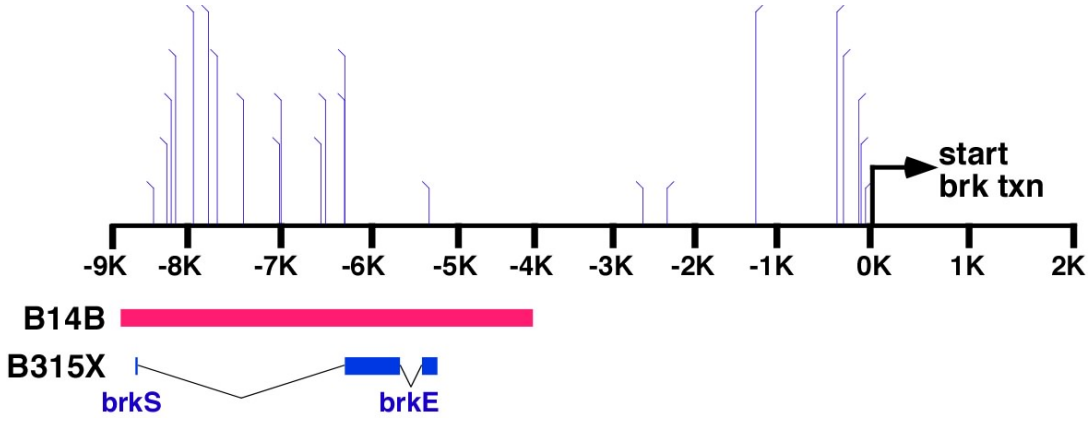

Figure 2-7: Brk binding sites in the B14 and B315 response elements.
Schematic diagram of the B14 (pink) and B315 (blue) response elements (Muller et al., 2003) and the nearby putative cognate Brk binding sites (blue lines above sequence) located upstream of the start of transcription. B315 consisted of two regions: 1) an enhancer (E) region, which alone drives reporter gene expression ubiquitously across the wing disc, and 2) the silencer (S) region, which, when combined with the E, drives reporter gene expression in a pattern very similar to B14. B14B is the B14 response element that drives GFP reporter gene expression and B315X is the B315 response element that drives *lacZ* reporter gene expression.

Both the B14B and B315X constructs are autoregulated by Brk (Figs. 2-4 and 2-5). Both constructs contain putative Brk binding sites. B14B contains 15 sites where 7 are clustered. B315X contains one putative site that it shares with the 15th site in B14B.

To determine if Brk binds to one or more of the putative Brk binding sites contained within the B14B construct to autoregulate its expression, Brk mutant clones were created in the B14Δ2M15B construct in wing discs. The B14Δ2M15B construct contains B14B, except its first 14 Brk binding sites are removed and the 15th site is mutated (Fig. 2-8 A). Therefore, an upregulation of B14Δ2M15B reporter gene expression in *brk* mutant clones would indicate that the construct is autoregulated by Brk even though no cognate Brk binding sites are contained within the construct. Upon examination of B14Δ2M15B reporter gene expression in wing discs containing *brk* null mutant clones, an upregulation of reporter gene expression was observed indicating that Brk can autoregulate the B14Δ2M15B construct in wing discs even though it contains no cognate Brk binding sites (Fig. 2-8 B).

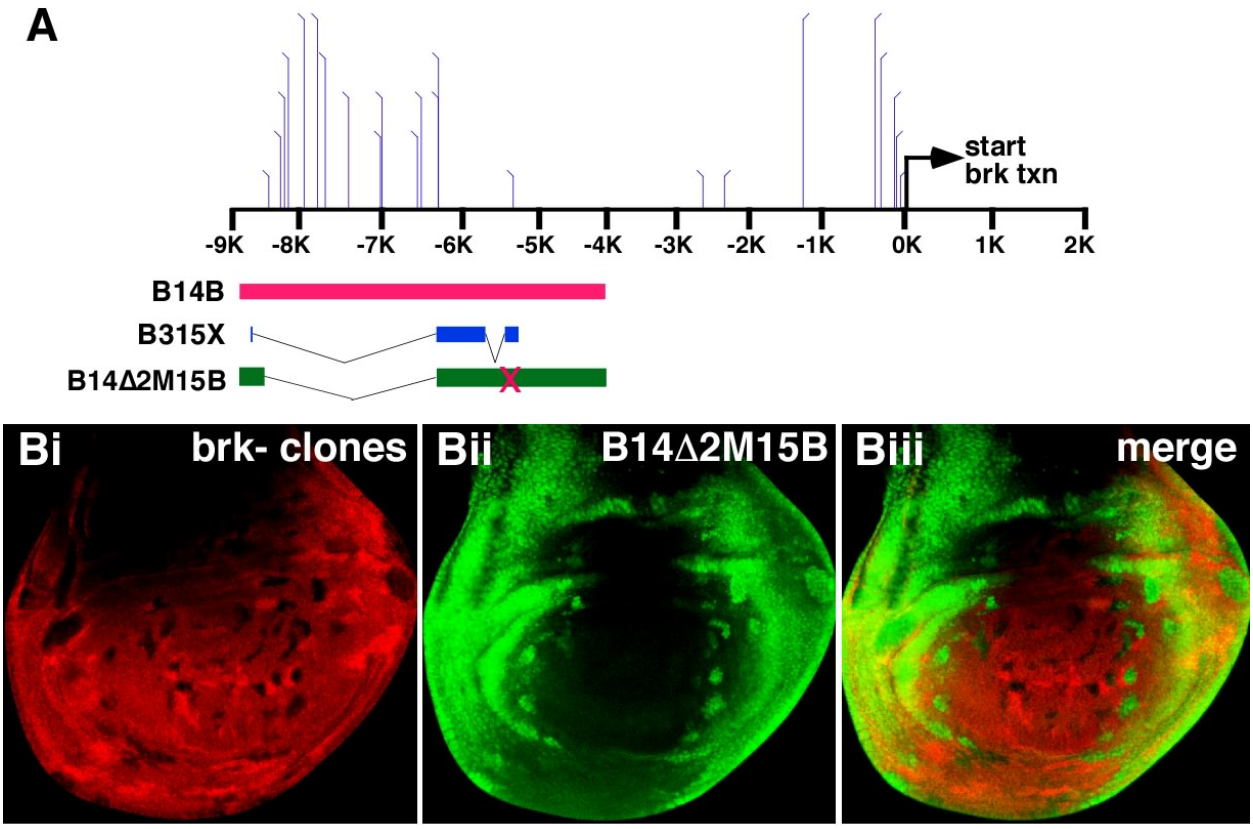

Figure 2-8: B14Δ2M15B contains no cognate Brk binding sites but is autoregulated by Brk.
(A) Schematic of the *brk* genomic region and the B14Δ2M15B construct. Blue lines above the sequence indicate putative Brk binding sites. (B) Brk autoregulates the B14Δ2M15B construct in wing discs. (Bi) Brk null mutant clones are marked by a loss of *lacZ* (anti-ßgal, red). (Bii) B14Δ2M15B (GFP) is upregulated in *brk* mutant clones indicating that the B14Δ2M15B construct is regulated by Brk. (Biii) Merge panel of (Bi) and (Bii).

To determine if Brk binds the one putative Brk binding sites contained within the B315X construct to autoregulate its expression, Brk mutant clones were created in the B315M15X construct in wing discs. The B315M15X construct contains B315X, except that its only putative Brk binding site is mutated (Fig. 2-9 A). Therefore, an upregulation of B315M15X reporter gene expression in *brk* mutant clones would indicate that the construct is autoregulated by Brk. Upon examination of B315M15X reporter gene expression in wing discs containing *brk* null mutant clones, an upregulation of reporter gene expression was observed indicating that Brk autoregulates the B315M15X construct in wing discs even though it contains no cognate Brk

binding sites (Fig. 2-9 B). B315X and B315M15X are not expressed in embryos, so this study was not confirmed in embryonic assays.

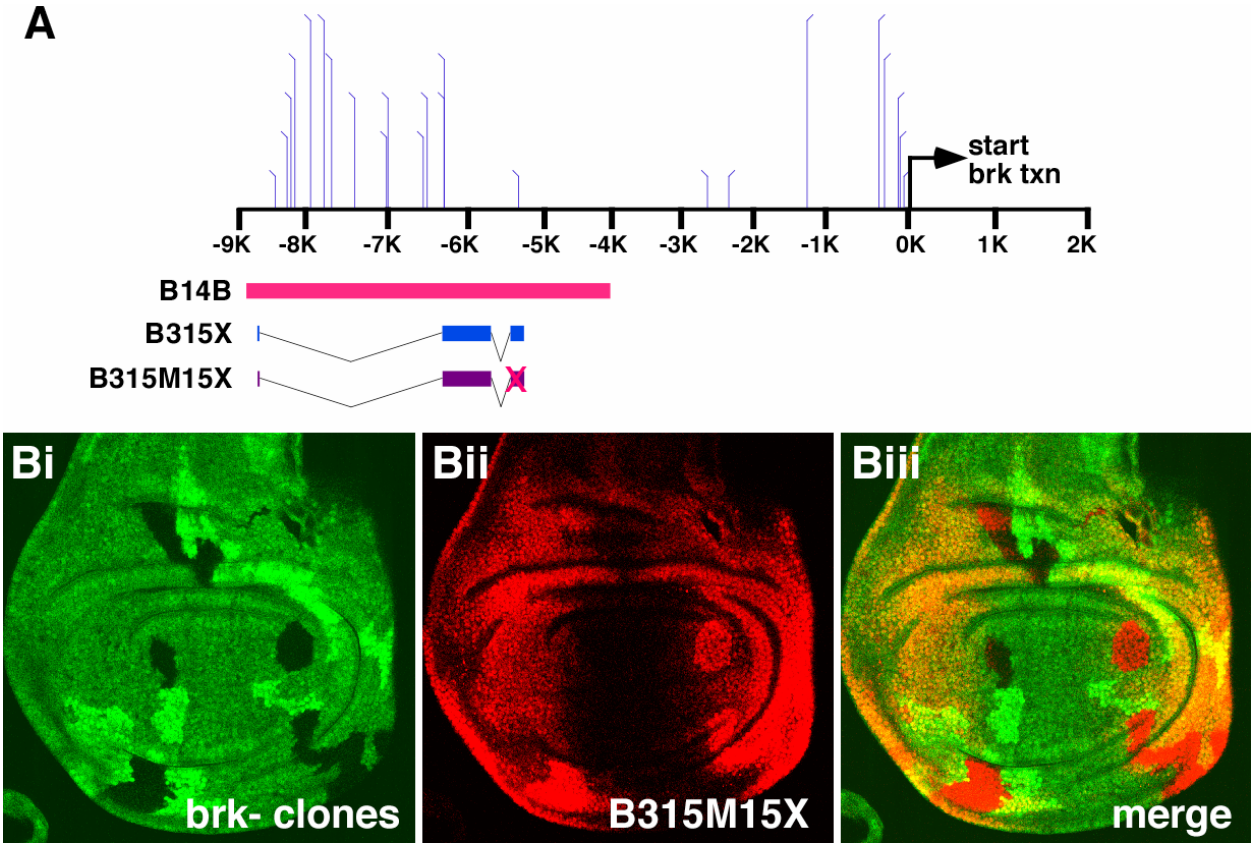

Figure 2-9: B315M15X contains no cognate Brk binding sites but is autoregulated by Brk.
(A) Schematic of the *brk* genomic region and the B315M15X construct. Blue lines above the sequence indicate putative Brk binding sites. (B) Brk autoregulates the B315M15X construct in wing discs. (Bi) Brk null mutant clones are marked by a loss of GFP(green). (Bii) B315M15X (anti-ßgal, red) is upregulated in *brk* mutant clones indicating that the B315M15X construct is regulated by Brk. (Biii) Merge panel of (Bi) and (Bii).

Altogether, the results obtained for the B14Δ2M15B and B315M15X constructs indicated that the while both of these constructs contain no cognate Brk binding sites, they are both autoregulated by Brk. Therefore, Brk can autoregulate independently of binding its own cognate Brk binding sites on DNA.

2.6 BRK APPEARS TO FUNCTION AT THE SILENCER TO NEGATIVELY AUTOREGULATE

For Brk to autoregulate the B14 and B315 response elements (Figs. 2-4 and 2-5), it must act on some portion of the response elements. To determine if Brk functions to autoregulate through the S or E portions of the B14 and B315 response elements, a construct was created and its reporter gene expression analyzed in *brk* mutant clones (Fig. 2-10 A). The expression of *dpp* within the wing disc is controlled by an enhancer, dpp E; therefore, combination of the dpp E with the brk S created the construct dppE-brkS. This construct drove reporter gene expression in the lateral regions of the disc (Fig. 2-10 B) whereas the dppE alone drives ubiquitous expression in the wing pouch, much like the brk E alone (Muller et al., 2003). It should be noted that the dppE, which drives expression throughout much of the wing disc, is not expressed like the endogenous *dpp* gene as the dppE is a minimal enhancer, not the entire collection of response elements necessary to drive *dpp* expression. When *brk* homozygous mutant clones were created and dppE-brkS reporter gene expression analyzed, the dppE-brkS construct was upregulated in mutant clones indicating that it is autoregulated by Brk (Fig. 2-10 C).

Both the brkS-brkE (B315X) construct as well as the dppE-brkS construct were shown to be autoregulated by Brk, even though they contained different enhancers (dppE and brkE). The dppE contains no Brk binding sites and has no sequence similarity to the brkE. Additionally, the brk S contains no Brk binding sites and the brk S in combination with the brk E (which contains one Brk site) is still autoregulated when its only Brk binding site is mutated (B315M15X) (Figure 2-9). It appears that Brk autoregulates through the S; this autoregulation may occur by Brk binding to non-cognate sites on DNA (as the S contains no Brk sites) or by indirectly binding DNA.

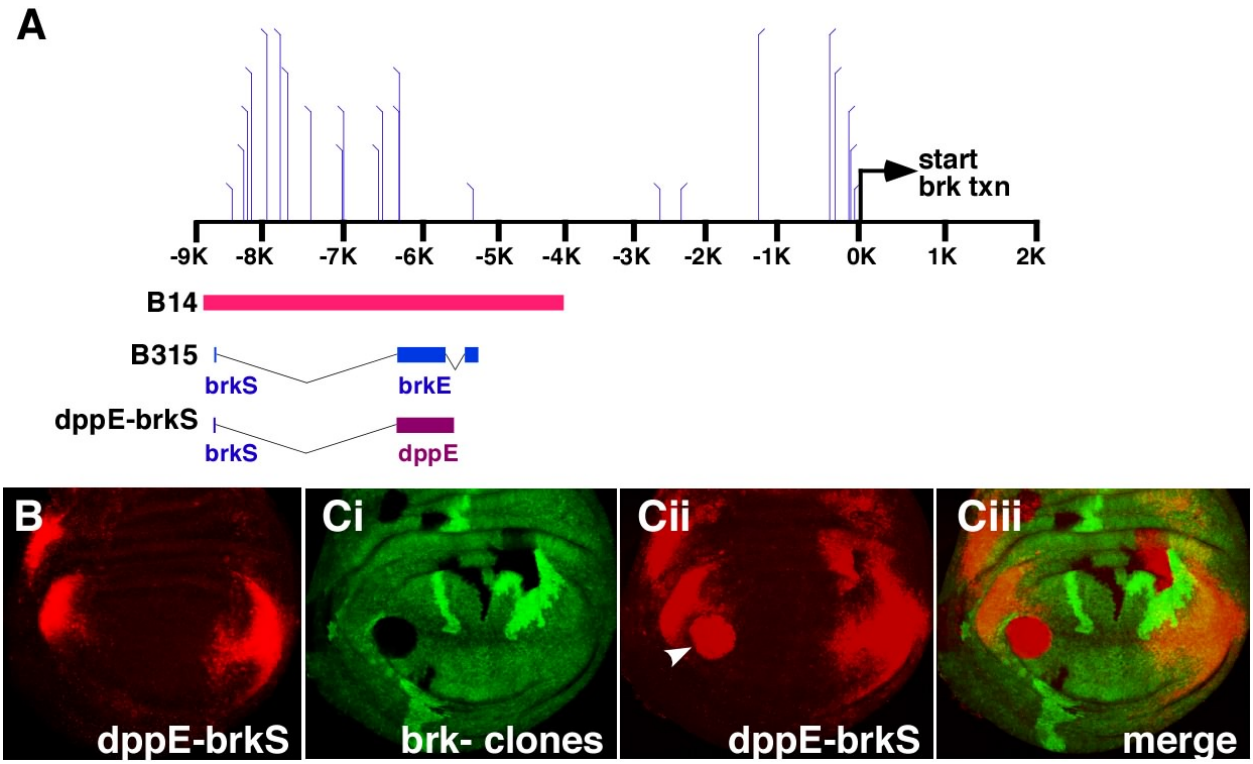

Figure 2-10: Brk appears to autoregulate through the S.
(A) Schematic of the *brk* genomic region, the B14 and B315 response elements, and the constructs created. Blue lines above the sequence indicate cognate Brk binding sites. (B) dppE-brkS *lacZ* expression (anti-ßgal, red). (C) *brk* null mutant clones in dppE-brkE indicate that dppE-brkS is autoregulated by Brk. (Ci) *brk* homozygous mutant clones marked by a loss of GFP (green). (Cii) dppE-brkS *lacZ* reporter gene expression (anti-ßgal, red). White arrowhead indicates an upregulation of expression in a *brk* mutant clone. (Ciii) Merge panel of (Ci) and (Cii). (D) brkE-bamS *lacZ* expression (anti-ßgal, red). (E) *brk* null mutant clones in brkE-bamS indicate that brkE-bamS is autoregulated by Brk. (Ei) *brk* homozygous mutant clones marked by a loss of GFP (green). (Eii) brkE-bamS *lacZ* reporter gene expression (anti-ßgal, red). White arrowhead indicates an upregulation of expression in a *brk* mutant clone. (Eiii) Merge panel of (Ei) and (Eii).

2.7 BRK AUTOREGULATES BY INTERACTING WITH pMAD AS PART OF THE MMS COMPLEX

The evidence I have presented above suggests that Brk autoregulates through the silencer. Several possibilities exist to describe how Brk autoregulates at the S. One possibility is that Brk binds to the S even though this element contains no standard Brk binding sites. Initial EMSA

studies with a bacterially expressed GST-fusion of the Brk N-terminus (first 101 amino acids, including the DNA binding domain) suggested that any binding is very weak, but the interaction is not well characterized and it is unclear if the Brk fusion protein containing the DNA binding domain would bind to any sequence non-specifically (data not shown). Studies of this nature are typically conducted using enriched S2 cell lysates on EMSAs; therefore, this study awaits further evaluation.

A second possibility, which cannot be ruled out, is that Brk represses an activator of another transcriptional repressor (that in turn represses Brk) in order to autoregulate. This activator would have to act at the S (as Brk autoregulation occurs through this element) and interact with the MMS complex thus making it highly coincidental if this scenario is the true mechanism of Brk autoregulation. Chromatin IP experiments (described in section 2.9) will address this possibility. If ChIP indicates Brk is present at the S, then this possibility will be ruled out.

A third more likely possibility is that Brk autoregulates at the S by interacting with other proteins known to function at the S, specifically members of the MMS complex. The MMS complex is formed when pMad and Med bind directly to the S of *brk* and then recruit Shn (Muller et al., 2003; Pyrowolakis et al., 2004). *brk* is not repressed when (1) the *brk* S is missing or mutated, (2) Dpp input is prevented, or (3) when the C-terminal zinc fingers of Shn are deleted/mutated; the formation of the MMS complex is dependent upon these same requirements (Pyrowolakis et al., 2004).

If Brk interacts with the MMS complex in order to autoregulate, several predictions can be made and tested about this interaction. (1) Brk autoregulation should be pMad dependent. If pMad cannot function as a transcriptional repressor with the MMS complex, no Brk should be

present at the S and autoregulation should cease. (2) Brk should interact with one or more of the component proteins of the MMS complex. These predictions are tested below.

2.7.1 Autoregulation by Brk is dependent on pMad activity

If Brk autoregulates by interacting with the MMS complex at the S, the first prediction to be tested is that Brk autoregulation should be pMad dependent. To demonstrate that pMad is necessary to repress *brk* expression in embryos as it is in discs (Fig. 2-3), studies were performed with an inhibitory Smad, UAS-Dad, driven at high levels with the en^{G4} system. Recall that Dad is an I-Smad that acts antagonistically to pMad and Med (Schmierer and Hill, 2007; ten Dijke et al., 2000).

Upon examination, the B14X reporter gene expression was expressed in almost all of the ectoderm of stage 11 embryos apart from its exclusion from a ventrolateral stripe where *dpp* is expressed at this stage (Fig. 2-11 A). When UAS-Dad was ectopically expressed in stripes (Fig. 2-11 B) (thus removing or significantly reducing pMad from those regions) across the embryo, B14X reporter gene expression was no longer repressed; in the ventrolateral region, it now extended through the stripes of UAS-Dad expression. This indicated that pMad activity is necessary to repress the B14 response element, and thus Brk, as was previously demonstrated in discs (Fig. 2-11 B-D).

As shown above, ectopically driving UAS-3PF3-HA with en^{G4} in embryos repressed B14 expression, indicating that ectopically driven Brk autoregulates the B14 response element (Fig. 2-4 D-G). To determine if pMad plays a role in this autoregulation of Brk, studies were carried out where UAS-Dad and UAS-3PF3-HA were driven with the en^{G4} system in combination. If

Brk was no longer able to repress B14 when an I-Smad was over-expressed, it would indicate that Brk autoregulation is dependent upon pMad.

Indeed when UAS-Dad and UAS-3PF3-HA were driven in the same embryo and B14X reporter gene expression assayed, it was determined that ectopically expressed Brk fails to repress B14 in the absence of pMad (Fig. 2-11 E-H). This result is consistent with the notion that Brk on its own is not sufficient for autoregulation to occur and supports the hypothesis that Brk autoregulates by interacting with the MMS complex.

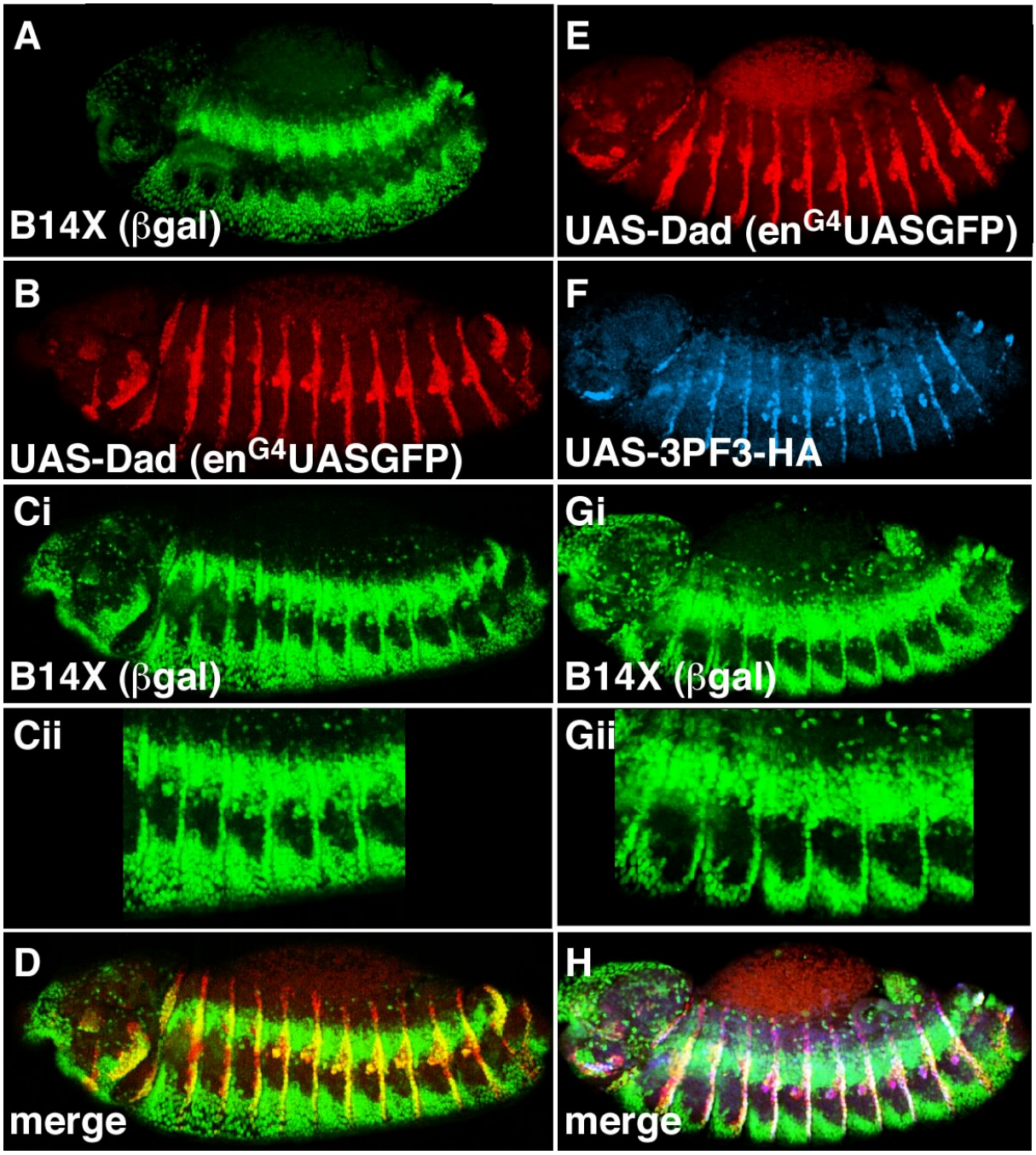

Figure 2-11: Autoregulation by Brk is dependent upon pMad activity.
(A) Expression of B14X (anti-ßgal) in an embryo. (B-D) The same embryo was utilized to show that pMad is necessary for *brk* repression. (B) UAS-Dad is driven with en^{G4}UASGFP. (Ci) B14X (anti-ßgal) expression extends into the stripes of expression where UAS-Dad is driven. (Cii) Magnification of (Ci). (D) Merge panel of (B) and (Ci). (E-H) The same embryo was utilized to show that pMad is necessary for *brk* to autoregulate. (E) UAS-Dad is driven with en^{G4}UASGFP. (F) UAS-3PF3-HA is driven with en^{G4}UASGFP and detected with anti-HA (blue). (Gi) B14X (anti-ßgal) expression extends into the stripes of expression where UAS-Dad is driven indicating ectopically expressed Brk cannot repress (as it did in the absence of the UAS-Dad transgene) without pMad. (Gii) Magnification of (Gi). (H) Merge panel of (E), (F), and (Gi).

2.7.2 Brk and pMad interact *in vivo*

If Brk autoregulates by interacting with the MMS complex at the S, the second prediction to be tested is that Brk interacts with one or more of the components of the MMS complex. To test this hypothesis, I sought to: (1) confirm the expression of an *in vitro* translated Brk protein and (2) determine if pMad and Brk co-immunoprecipitate (co-IP) from enriched embryonic lysates. The embryonic lysates used in these studies were of the following genotype: $\frac{\text{UAS-3PF3-HA}}{+\text{ or }>}$; $\frac{\text{engrailed}^{\text{Gal4}}}{+}$; $\frac{\text{UAS-tkv}^{\text{QD}}}{\text{Ubiq-Myc-Mad}}$. The UAS-3PF3-HA construct (Winter and Campbell, 2004) and the UAS-tkv^{QD} construct were driven by en^{G4} and Myc-tagged Mad was ubiquitously expressed. A constitutively active form of the tkv receptor (tkv^{QD}) was also co-expressed because nuclear translocation of pMad and Med requires activation of the Dpp signal transduction cascade (Raftery and Sutherland, 1999).

2.7.2.1 *in vitro* translated Brk protein immunoprecipitates with multiple antibodies

To evaluate if the Brk protein could be immunoprecipitated, radiolabeled full-length Brk protein with a C-terminal HA tag (3PFT) was transcribed and translated *in vitro* using a rabbit reticulocyte system. To confirm the later usage of multiple antibodies, this protein was then subjected to immunoprecipitation experiments using two anti-brk antibodies, two-anti-HA antibodies, one non-specific antibody control, and a no antibody control (Fig. 2-12). 3PFT was efficiently immunoprecipitated by both anti-brk antibodies as well as both anti-HA antibodies (Fig. 2-12 A). As a control, radiolabeled Luciferase protein lysate was similarly treated. Luciferase was not immunoprecipitated by any of the antibodies (Fig. 2-12 B) indicating that the immunoprecipitations observed for 3PFT were specific to Brk.

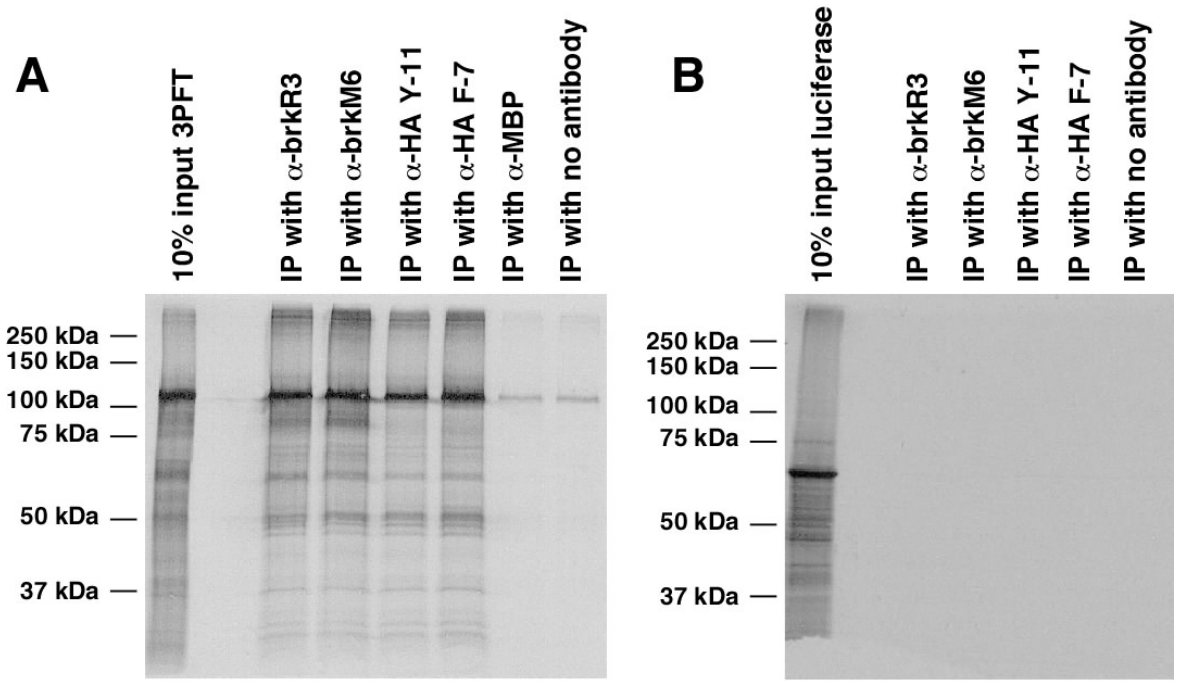

Figure 2-12: *in vitro* translated Brk immunoprecipitates with multiple antibodies.
9% SDS-PAGE with radiolabeled full-length *in vitro* translated Brk with a C-terminal HA tag (3PFT). (A) 3PFT immunoprecipitated with two anti-brk antibodies as well as with two anti-HA antibodies. 3PFT immunoprecipitated only very weakly with no antibody or with a non-specific (anti-MBP) antibody control. (B) Luciferase control protein did not immunoprecipitate with either anti-brk antibody or with either anti-HA antibody.

2.7.2.2 Western blot analysis of Brk with anti-HA and anti-brkR3 antibodies

Experiments were conducted to test the ability of the anti-HA and anti-brk antibodies to detect Brk protein from embryonic lysates enriched for UAS-3PF3-HA protein. Initially, the two anti-HA antibodies were utilized as they are commercially available and quantities are not limiting. The anti-HA F-7 antibody was unable to detect Brk from 0.13 mg of Brk-enriched embryonic lysate while it was able to detect Slpr, a control HA-tagged protein, from only 30μg of Slpr-enriched embryonic lysate (Fig. 2-13 A). Similar results were obtained for the anti-HA Y-11 antibody (data not shown). An anti-HA antibody detected Brk from an embryonic lysate

enriched for UAS-3PF3-HA when the sample was first enriched for UAS-3PF3-HA by immunoprecipitation, but even then, the band detected was very weak (Fig. 2-13 C).

Because the anti-HA antibodies did not efficiently recognize the Brk protein in embryonic lysates, the anti-brk antibodies were examined by Western blot. The anti-brkR3 antibody recognized Brk protein at both wildtype levels as well as that enriched from the UAS-3PF3-HA construct in embryos (Fig. 2-13 B). The anti-brkM6 antibody failed to recognize the Brk protein on Western blot analysis (data not shown). The anti-brkR3 antibody also recognized a large band above 250kDa that was thought to include a complex of the Brk protein with other proteins in the embryonic lysate that was not denatured (Dr. Jeffrey Brodsky, personal communication).

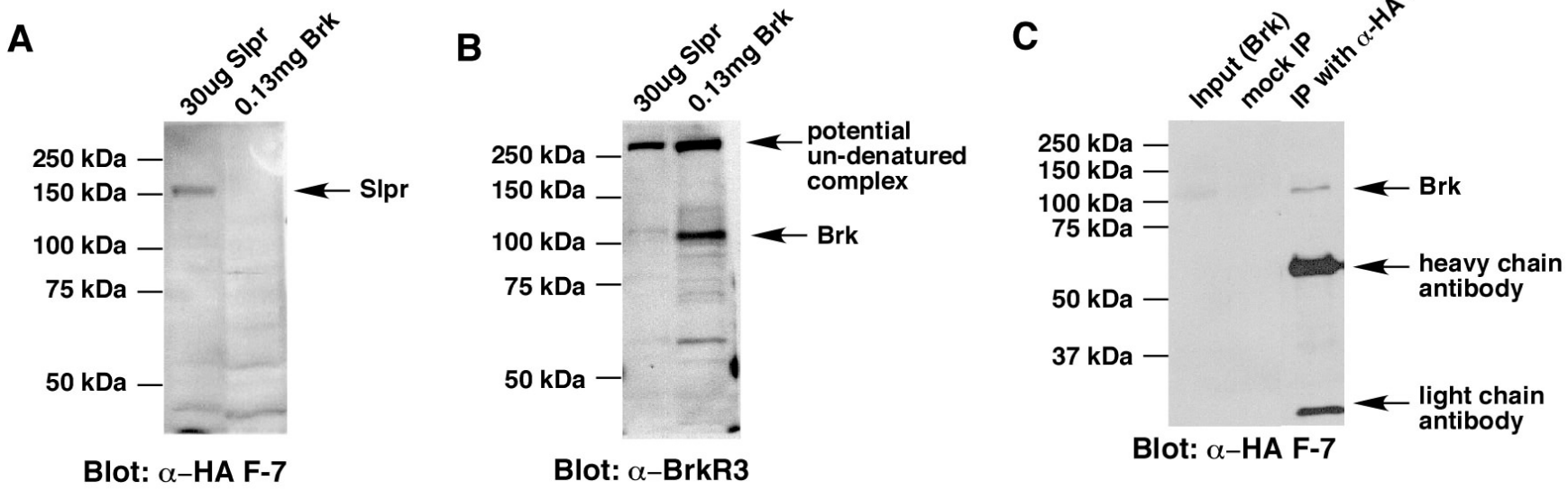
A
30ug Slpr
0.13mg Brk
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
Slpr
Blot: α–HA F-7
B
30ug Slpr
0.13mg Brk
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
potential
un-denatured
complex
Brk
Blot: α–BrkR3
C
Input (Brk)
mock IP
IP with α-HA F-7
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
Brk
heavy chain
antibody
light chain
antibody
Blot: α–HA F-7

Figure 2-13: Western blot of Brk with anti-brkR3 and anti-HA.
Embryonic lysate enriched for Brk is of the genotype: UAS-3PF3-HA/+; en^{G4}/+; UAS-tkv^{QD}/Ubiq-myc-Mad. (A-B) Same blot stripped and re-probed. (A) Western blot analysis of 30µg embryonic lysate enriched for a control HA-tagged protein, Slpr (gift from Stronach lab) and 0.13mg embryonic lysate enriched for Brk with the anti-HA F-7 antibody. Slpr control protein is detected with this antibody but Brk protein is not. (B) Same blot as (A). Western blot analysis with anti-brkR3. Endogenous Brk is detected very weakly in the lane enriched (via the UAS-Gal4 system) for Slpr control protein while an enrichment of UAS-3PF3-HA is detected in the lane enriched for Brk protein. Upper band detected by the anti-brkR3 antibody is a complex of the Brk protein with other proteins in the lysate. (C) Western blot with anti-HA F-7 of 0.13mg embryonic lysate enriched for Brk immunoprecipitated with the anti-HA F-7 antibody. Input sample is very weakly recognized by the anti-HA F-7 antibody, but a faint band representing UAS-3PF3-HA is detected in the IP lane. No band is detected for the mock IP (no antibody added).

2.7.2.3 Western blot and IP analysis of Brk and Mad

Molecular mass predictions for UAS-3PF3-HA predicted that the tagged protein should have a molecular mass of approximately 85kDa. The endogenous protein and UAS-3FP3-HA protein from embryonic lysates as well as the 3PFT protein generated by *in vitro* transcription/translation all ran on SDS-PAGE at a molecular mass slightly above 100kDa. This is consistent with other published results (Sivasankaran et al., 2000).

To evaluate the ability of the anti-brkR3 and anti-myc•HRP antibodies to immunoprecipitate Brk and myc-tagged Mad, embryonic lysates enriched for UAS-3PF3-HA and Ubiq-myc-Mad were prepared and boiled for 5 minutes in 1% SDS. The concentration of SDS was then reduced and the lysate immunoprecipitated and subsequently blotted with the anti-brkR3 antibody or the anti-myc antibody. Upon analysis, the UAS-3PF3-HA protein detected upon Western blot analysis of the immunoprecipitation reaction from embryonic lysates boiled in 1% SDS indicated that the tagged Brk protein had a molecular mass slightly over 100kDa (Fig. 2-14 A). This result indicated that the anti-brkR3 antibody successfully immunoprecipitated and detected the Brk protein.

Molecular mass predictions for myc-tagged Mad predicted a protein of approximately 60kDa and this was the mass of the protein detected upon Western blot analysis of immunoprecipitated myc-mad (Fig. 2-14 B). This result indicated that the anti-myc•HRP antibody successfully immunoprecipitated and detected the Brk protein.

While only one band is recognized by the anti-brk antibody in input samples, three bands are recognized when the lysate is immunoprecipitated and blotted with the anti-brk antibody; one band is the same size as the input, while one band is slightly smaller and one band is slightly larger (Fig. 2-14 A). One possible explanation could be that the protein in the IP that is slightly smaller than (un-tagged endogenous) Brk in the embryonic input lysate is a degradation product while the protein that is slightly larger in the IP than the protein observed in the embryonic input lysate is the HA-tagged version of Brk. This could indicate that the protein most often found in the enriched embryonic lysate and detected with the anti-brk antibody is modified and does not contain its HA tag (the middle band). This would explain the observation that the HA-tagged Brk protein is very difficult to blot with an anti-HA antibody. If this were the case, it would be expected that any time HA-tagged Brk protein is recognized, it would be slightly larger on a blot than the endogenous protein recognized by the anti-brk antibody. It appears that the small amount of HA-tagged Brk protein recognized upon IP and Western blotting with anti-HA is slightly larger than that observed when blotting with an anti-brk antibody (compare Fig. 2-13 B and C). An alternative possible explanation for the three Brk protein bands could be that the Brk protein is post-translationally modified.

Further experiments to confirm the identity of the Brk and myc-tagged Mad bands observed on Western blots would include performing IPs and Western blots on tissue from *brk*

mutants (and blotting with anti-brk) and tissue not carrying the myc-tagged Mad construct (and blotting with anti-myc).

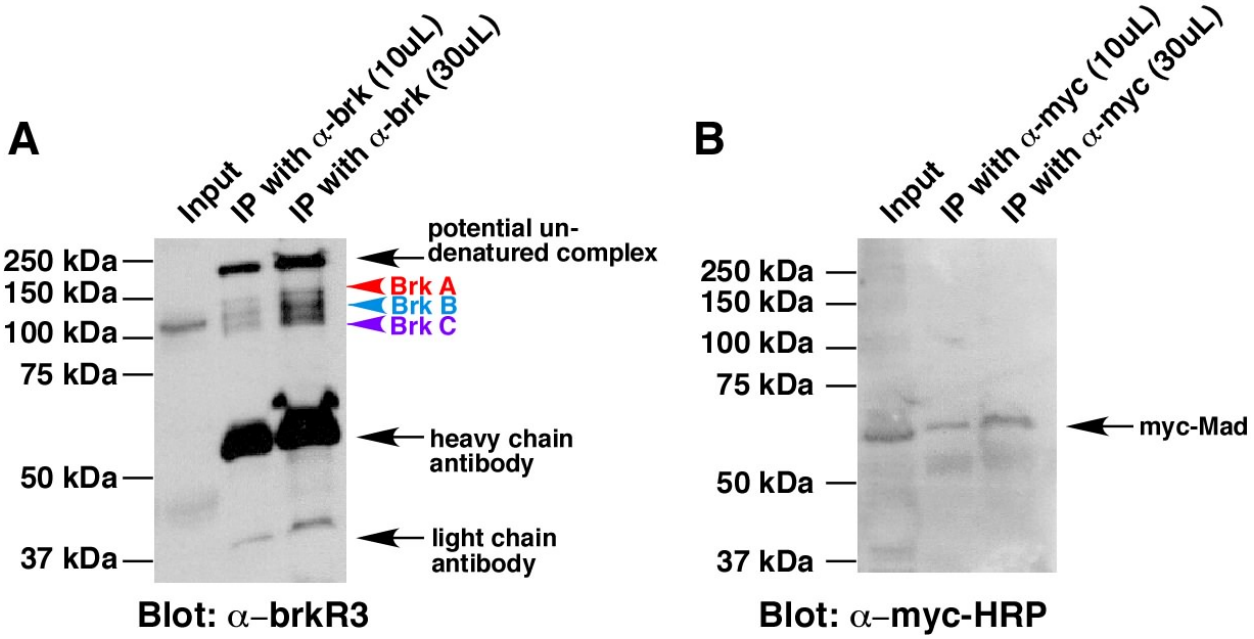

Figure 2-14: Immunoprecipitation analysis of Brk and Mad from embryonic lysates treated with 1% SDS. 9% SDS-PAGE. Embryonic lysate was of the genotype: UAS-3PF3-HA/+; en^{G4}/+; UAS-tkv^{QD}/Ubiq-myc-Mad and was treated with 1% SDS before use (input and IP reactions). (A) Blot with anti-brkR3 of IP with anti-brkR3 identifies Brk with a molecular mass just over 100kDa and that Brk immunoprecipitates with anti-brkR3. Two volumes of IP reaction (10µL and 30µL) were run on the gel. The three Brk proteins immunoprecipitated are labeled A, B, and C (see description Section 2.7.2.3). Potentially un-denatured protein complex is as described in Fig. 2-13. (B) Blot with anti-myc-HRP of IP with anti-myc identifies myc-tagged Mad with a molecular mass of approximately 60kDa and that Myc immunoprecipitates with anti-myc. Two volumes of IP reaction (10µL and 30µL) were run on the gel. Heavy and light chain antibodies are not recognized because the primary antibody is conjugate to HRP indicating no secondary antibody was utilized.

2.7.2.4 Brk and Mad co-immunoprecipitate *in vivo*

Because the studies described above support the hypothesis that Brk on its own is not sufficient for autoregulation and indicate that pMad activity is necessary for this repression, I sought to determine if Mad and Brk interact *in vivo* in embryonic lysates. If so, immunoprecipitation with

an antibody directed against one of the proteins (or its tag) and subsequent Western blot analysis with an antibody directed against the other protein (or its tag) would result in a band on the blot.

Upon immunoprecipitation of the embryonic lysate with anti-myc, which recognized the myc-tagged Mad protein, and subsequent Western blot with anti-brkR3, a band was identified for Brk (Fig. 2-15). This result indicated that Brk co-immunoprecipitates with myc-tagged Mad, thereby indicating that Mad and Brk interact *in vivo*. The complimentary experiment was then performed. When the immunoprecipitations were performed to IP UAS-3PF3-HA with anti-brkR3, anti-HA F-7, or anti- HA Y-11 and Western blot was then performed with anti-myc, no interaction was seen (data not shown). This result does not necessarily indicate that the proteins do not interact, as it is most likely due to the poor/in-efficient ability of the anti-myc-HRP antibody to detect proteins on Western blot (as seen in Fig. 2-14 B). In all, the ability of Brk and Mad to co-IP together indicates that the two proteins interact *in vivo*.

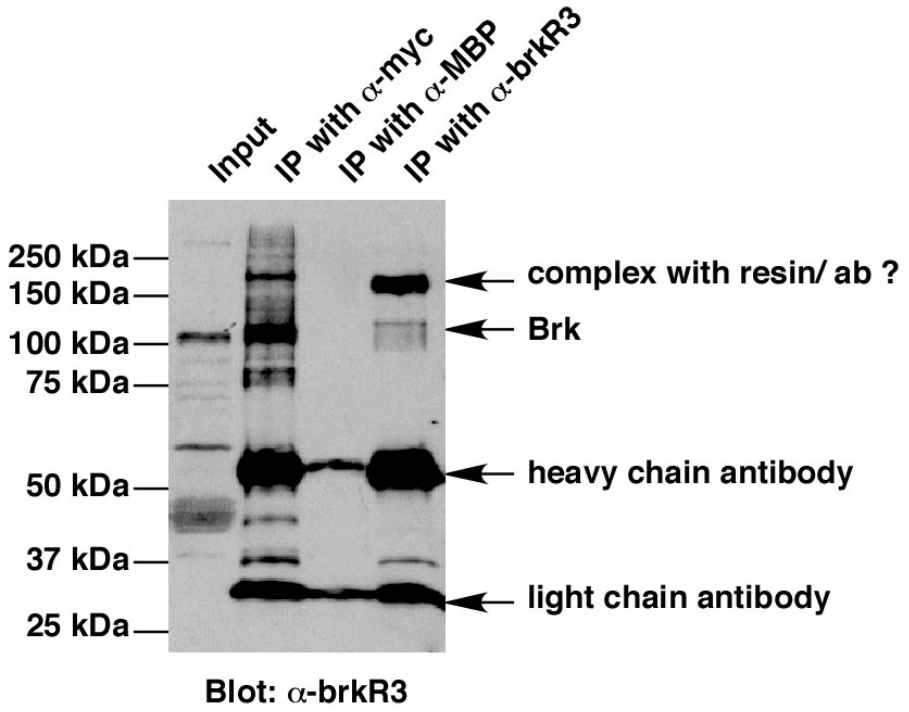

Figure 2-15: Brk and Mad co-IP *in vivo*.
9% SDS-PAGE and subsequent Western blot analysis with anti-brkR3 antibody. Embryonic lysate is of the genotype: UAS-3PF3-HA/+; en^{G4}/+; UAS-tkv^{QD}/Ubiq-myc-Mad. Anti-brkR3 detects the approximately 100kDa Brk protein (lane 1). Immunoprecipitation with anti-myc and blot with anti-brkR3 detects the Brk protein indicating that Brk and myc-tagged Mad interact *in vivo* (lane 2). Brk is not immunoprecipitated with a non-specific antibody (anti-MBP) (lane 3). Brk protein is immunoprecipitated with anti-brkR3 (lane 4).

2.7.3 MBP-pull down experiments indicated that Brk interacts *in vitro* directly with Mad and Med

While the above described co-IP interactions confirm that Brk and Mad interacted *in vivo*, they do not indicate whether this interaction occurs directly or whether or not the interaction is dependent upon the presence of DNA. To determine if Brk interacts directly with Mad and Med *in vitro*, Maltose binding protein pull-down (MBP-PD) experiments were performed. Radiolabeled, *in vitro* transcribed/translated, control protein (Luciferase) or full-length Brk

protein with a C-terminal HA tag (3PFT) were created and subjected to pull downs with MBP control protein, MBP-MadNL, or MBP-MedNL. The MBP-Smad fusions consisted of the N-terminal MH1 domain, which is believed to contact DNA, and the linker region, which is believed to interact with other proteins (Gao and Laughon, 2006).

The results of the PD assay indicated that Brk interacts specifically with Mad and Med but not MBP alone (Fig. 2-16). To determine which region of the Brk protein is responsible for its interactions with Mad and Med, a series of modified and C-terminally truncated Brk proteins were created (Fig. 2-16 A). These proteins were radiolabeled, *in vitro* transcribed/translated, and subjected to MBP-PD analysis with the MBP, MBP-MadNL, and MBP-MedNL proteins.

The first modified protein tested was RH2DT. This modified Brk protein has the second recognition helix of its DNA binding domain deleted. This construct was tested because evidence presented above suggests that Brk may require its DBD to autoregulate (Fig. 2-6). It is possible that Brk requires its DBD to autoregulate because it utilizes this domain to interact with Mad. Upon examination of the RH2DT protein in MBP-PD experiments, it was found to still interact with MBP-MadNL and MBP-MedNL indicating that Brk does not require its DBD for its interactions with Mad and Med. This domain is most likely necessary for a different aspect of Brk autoregulation.

To determine if the C-terminal region of Brk is responsible for its interactions with the Mad and Med proteins, C-terminal truncations of 3PFT were created and subjected to MBP-PD experiments. Stop1T, Stop2T, and Stop3T were tested and seem to interact with MBP-MadNL and MBP-MedNL at levels comparable to those seen with Luciferase control protein. These studies did not conclusively indicate whether or not the C-terminal region of Brk is required for its interactions with pMad and Med.

Because full-length Brk interacts with Mad and Med in the MBP-PD experiments, the interaction it possessed with each of these proteins appears to be direct. It also appears that the interaction occurs independently of DNA *in vitro* (i.e. pMad does not need to bind DNA before it can bind to Brk). Further modified Brk constructs would need to be examined to identify the domain/motif of Brk with which Mad and Med interact.

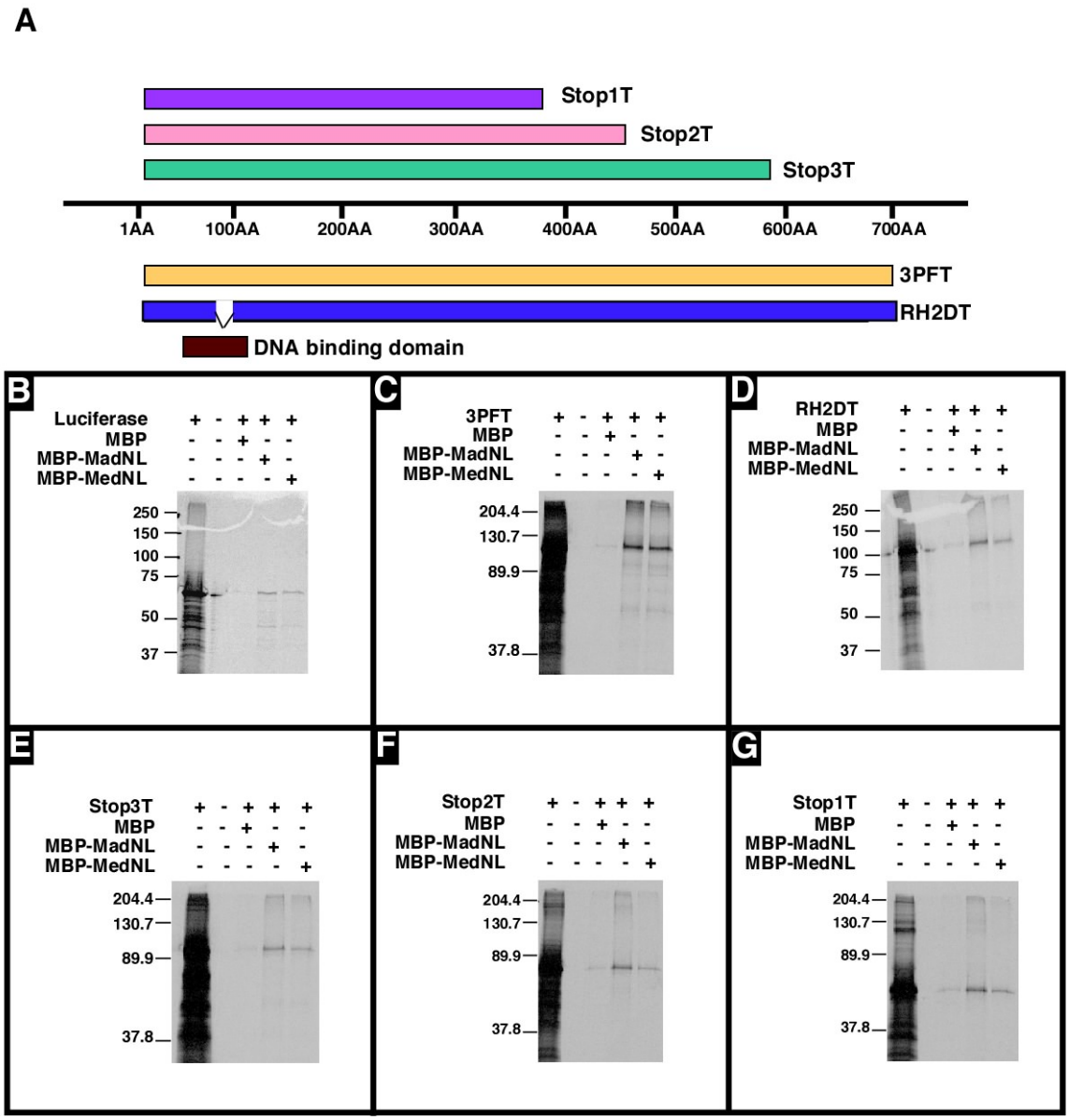

Figure 2-16: Brk interacts directly with Mad and Med independently of DNA.
Input run in each lane is 10% of the protein amount added to PD assay lanes. (A) Schematic of the modified Brk proteins that were radiolabeled, *in vitro* transcribed/translated, and subjected to MBP-PD. (B) Luciferase control protein does not interact with MBP and interacts only very weakly with MBP-MadNL and MBP-MedNL. (C) 3PFT does not interact with MBP and interacts with both MBP-MadNL and MBP-MedNL. (D) RH2DT does not interact with MBP and interacts with both MBP-MadNL and MBP-MedNL. (E) Stop3T does not interact with MBP and interacts at low levels with both MBP-MadNL and MBP-MedNL. (F) Stop2T does not interact with MBP and interacts at low levels with both MBP-MadNL and MBP-MedNL. (G) Stop1T does not interact with MBP and interacts at low levels with both MBP-MadNL and MBP-MedNL.

2.8 CHIP STUDIES TO CONFIRM WHERE BRK ACTS ON B14 TO AUTOREGULATE

To confirm that Brk autoregulates by acting at the S or to determine on what other response element region(s) of B14 Brk acts in order to autoregulate, chromatin immunoprecipitation (ChIP) experiments were carried out. Because previous studies demonstrated that Brk autoregulates B14 in embryos (Fig. 2-4), ChIP studies were conducted utilizing chromatin from embryonic lysates of the following genotype: $\frac{\text{UAS-3PF3-HA}}{+\text{ or }>}$; $\frac{\text{engrailed}^{\text{Gal4}}}{+}$; $\frac{\text{UAS-tkv}^{\text{QD}}}{\text{Ubiq-Myc-Mad}}$ Controls for the IP portion of the experiment included mock IPs (no antibody added) as well as IPs with embryonic lysates not enriched for the HA-tagged Brk protein (when IPs were conducted with anti-HA antibodies). Controls for the PCR portion of the experiment included negative control primers directed against DNA polymerase α-180 and positive control primers directed against a portion of the *UbxB* regulatory region previously demonstrated to bind Brk *in vitro* (Saller and Bienz, 2001; Yamasaki and Nishida, 2006).

2.8.1 Multiple HA and brk antibodies failed in ChIP studies

Initially, antibodies directed against the C-terminal HA tag of the UAS-Brk construct were utilized in ChIP. Both a mouse monoclonal and a rabbit polyclonal HA antibody were used in ChIP reactions with varied amounts of antibody and chromatin. These experiments failed to indicate any enrichment of the tagged Brk protein at *UbxB* or *brk* (data not shown).

The experiment was then repeated with a third anti-HA antibody; however, this antibody was an IgG conjugate to a resin purchased from Santa Cruz Biotechnology. The protocol followed for the ChIP was obtained from Marcie Warner in the Arndt lab and followed, but

again, these experiments failed to indicate any enrichment of the tagged Brk protein at *UbxB* or *brk* (data not shown).

Because the three anti-HA antibodies tested failed in ChIP experiments, I next attempted the experiments utilizing our anti-brk antibody. The polyclonal rat anti-brkR3 antibody was tested in ChIP and failed to indicate an enrichment of tagged Brk; upon further analysis, the antibody was found to be contaminated with DNA; this result was confirmed by PCR using the antibody as a template. Due to the lack of enough anti-brkR3 antibody for affinity purification, the anti-brkR3 antibody was then DNaseI treated in hopes of removing contaminating DNA. ChIP experiments with the DNaseI treated anti-brkR3 antibody failed to demonstrate any enrichment upon PCR analysis indicating that the dialysis conducted in attempts to remove the DNaseI enzyme from the antibody most likely failed (data not shown).

2.8.2 Strategies for further ChIP experiments

Based on the observations that the three anti-HA antibodies as well as the anti-brkR3 antibody failed to ChIP Brk, several strategies were attempted to create more reagents to use in the ChIP experiments in hopes of conducting a functional assay.

(1) Two new UAS-Brk constructs were created: HA3PF3HAU contains both N- and C-terminal HA tags while Flag3PF3HAU contains an N-terminal Flag tag and a C-terminal HA tag. Transformants were obtained for these constructs and enriched embryonic lysates will be created and tested.

(2) The same antigen used to create the anti-brkR3 antibody was injected into six animals in order to generate new anti-brk antibodies. The purpose of these new antibodies was two-fold. First, they may function better for ChIP analysis than the previous anti-brk antibody. Secondly,

enough antibody would be generated that I would be able to affinity purify the antibody in hopes of generating a cleaner antibody for ChIP experiments.

The six new anti-brk antibodies were tested via Western blot and anti-brkGP10 was determined to be the only antibody that could recognize the Brk protein in the enriched embryonic lysate (data not shown). Therefore, this antibody was affinity purified (creating anti-brkGP10AF) and then tested in ChIP experiments using varied amounts of chromatin as well as varied amounts of both anti-brkGP10 and anti-brkGP10AF. The results of a representative experiment failed to indicate any enrichment of the Brk protein at *UbxB* or *brk* compared to a negative control region and to the mock (no antibody added) control (Fig. 2-17).

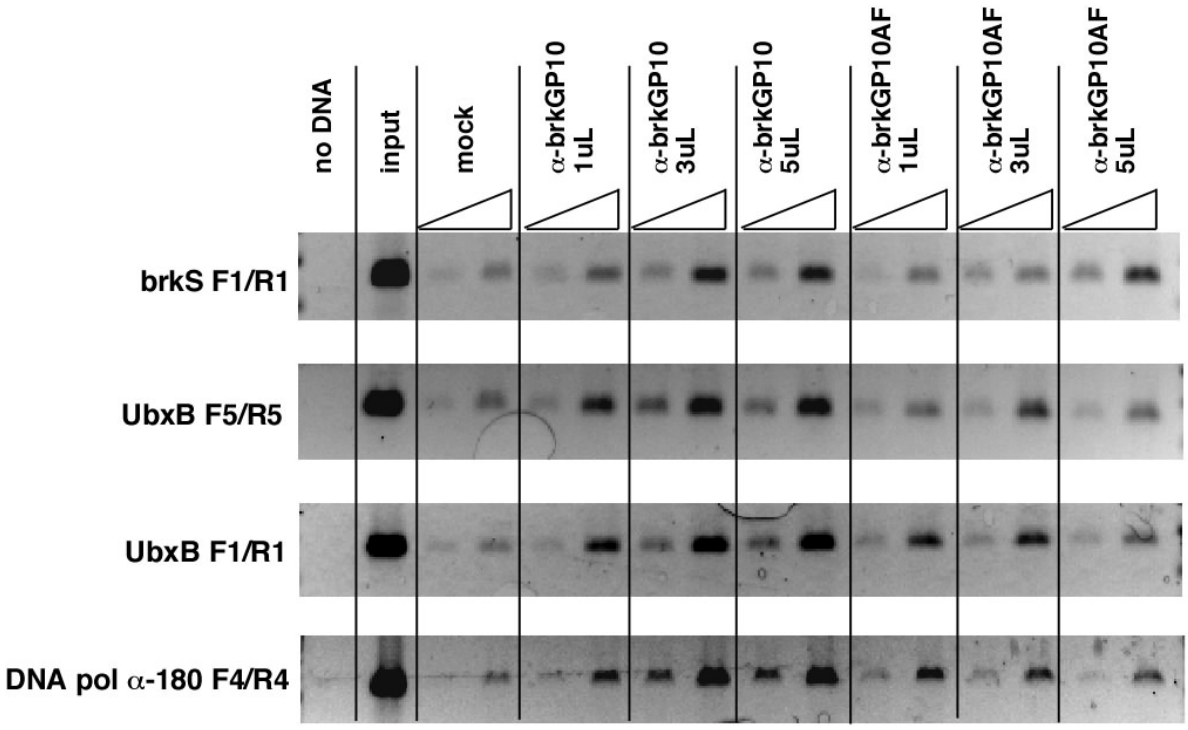

Figure 2-17: Representative ChIP with anti-brk antibodies.
Primer pairs utilized for PCR are indicated on the left. Representative gel of PCR products obtained from ChIP with 100μL chromatin from enriched embryonic lysate. Lane 1 is no DNA added PCR negative control. 1μL purified chromatin was used in input PCR reaction. For lanes 3-16, odd lanes had 1μL purified chromatin added to PCR reaction whereas even lanes had 4μL purified chromatin added to PCR reaction. Indicated above the lanes is the antibody utilized as well as the volume of that antibody (1μL, 3μL, or 5μL) utilized in the ChIP reaction.

2.9 DISCUSSION

When I joined the lab, preliminary studies suggested Brk acts as a transcriptional repressor to negatively autoregulate its own expression (Hasson et al., 2001; Moser and Campbell, 2005). The most convincing evidence in support of this hypothesis are the studies of the brk^{XA} mutant. Wing discs from hemizoygotes have a strong *brk* phenotype that is associated with reduced levels of Brk protein. Heterozygous brk^{XA} discs have reporter gene expression in a pattern similar to that of Brk protein with a lateral-to-medial gradient of expression in the anterior and posterior halves of the wing pouch; however, brk^{XA} mutant discs have a higher level of reporter gene expression that is still absent from the medial region of the disc but have very little graded expression such that there is as a sharp boundary between cells expressing the reporter versus non-expressing cells. I was interested in determining how Brk negative autoregulation functioned in the generation of the *brk* expression gradient.

2.9.1 Brk autoregulates through the S

The ability of Brk to autoregulate the B14 and B315 response elements was evaluated through *brk* mutant clonal analysis. It was determined that Brk autoregulates B14 and B315 in the wing disc and B14 in the embryo. These studies confirm that Brk represses its own response elements and therefore its own expression. Brk does not bind its cognate Brk binding sites contained within the B14 and B315 response elements as the constructs B14Δ2M15B and B315M15X contain no cognate Brk binding sites but are still autoregulated by Brk indicating that Brk most likely autoregulates by interacting indirectly with DNA.

I next sought to determine which portion of the B14 or B315 response elements were utilized by Brk for autoregulation. To this end, I obtained several constructs from the Basler lab that combine different E and S elements and compared their reporter gene expression in *brk* mutant clones to B315, which consists of the brkE and brkS and is autoregulated by Brk (Pyrowolakis et al., 2004). The dppE alone drives ubiquitous reporter gene expression in the wing pouch; upon combination with the brkS to create the construct dppE-brkS, expression is limited to the lateral regions of the disc (Pyrowolakis et al., 2004). When *brk* mutant clones were created in the dppE-brkS construct and reporter gene expression analyzed, the construct was autoregulated by Brk. The dppE and brkE share no common sequence similarity. Therefore, the common sequence element shared between dppE-brkS and B315 is the brkS indicating that Brk may autoregulate through the S. Further experiments would be necessary to confirm this hypothesis—*brk* mutant clones would be created in the dppE and brkE alone to ensure that these constructs are not autoregulated by Brk. While they share no common sequence similarity, it remains possible that they are repressed by Brk, perhaps in a situation where a yet-unknown activator requires Brk to repress at the E, though this possibility remains unlikely.

2.9.2 Brk interacts *in vivo* with pMad to autoregulate

Because the evidence described above suggested that Brk autoregulates through actions at the S, I sought to determine if Brk interacts with other proteins known to function as the S, specifically pMad, a member of the MMS complex recruited to the S and important for its function as a Dpp-responsive silencer element. If Brk autoregulates through members of the MMS complex, it

would be expected that Brk autoregulation would be dependent upon pMad and that Brk should be able to interact with one or more components of the MMS complex.

I demonstrated that autoregulation in embryos by Brk is dependent upon pMad activity. This result is consistent with the notion that Brk on its own is not sufficient for autoregulation and supports the hypothesis that it interacts with pMad in order to autoregulate. In order to further test the hypothesis that Brk interacts with proteins found at the S, I first confirmed Western blot analysis of embryonic lysates enriched for UAS-3PF3-HA and Ubiq-myc-mad, then I sought to determine if the two proteins interact *in vivo*. When the enriched embryonic lysate was subjected to immunoprecipitation with anti-myc and then blotted for anti-brk, it was determined that Brk and pMad interact *in vivo* in this embryonic lysate.

2.9.3 Brk interacts *in vitro* with Mad and Med independently of DNA

MBP-PD experiments were conducted to confirm that Brk and Mad interacted *in vivo* independently of DNA as well as in hopes of identifying the region of the Brk protein required for these interactions. While Luciferase control protein interacts only very weakly with Mad and Med, full-length Brk interacts strongly with both Mad and Med. We suspected the DBD was the region required for Brk to interact with pMad and Med as the brk^{F124} DBD mutant was unable to autoregulate in wing imaginal discs. This was not the case, however, because the RH2DT construct interacts with Mad and Med even though the second recognition helix of the DBD was missing in this protein. The DBD is most likely involved in an aspect of autoregulation outside of interactions with pMad and Med. Additional N- and C-terminally truncated Brk constructs could be evaluated to determine the domain/motif of Brk responsible for its interactions with pMad and Med.

2.9.4 ChIP studies proved difficult in the identification of the region of B14 acted upon by Brk

In order to determine what other portions of the *brk* B14 regulatory region Brk represses, ChIP experiments were commenced. While these studies could not tell us if Brk acted directly on DNA to autoregulate or worked through binding partners, they would confirm where on B14 (or the entire *brk* upstream region) Brk acted. A failure of these studies to identify a region could also indicate that Brk autoregulation occurs by Brk repressing an activator of another repressor that then acts on Brk. This possibility, however, is unlikely as the unknown repressor would be predicted to function through the S. Initial ChIP experiments were performed utilizing antibodies directed against the C-terminal HA tag carried by the Brk protein over-expressed in the embryonic lysates utilized in these studies. Three different anti-HA antibodies failed to indicate Brk was bound more prevalently either at regions contained within B14 or the *UbxB* response element when compared to control PCR primer pairs. This may be due to the fact that the anti-HA antibodies poorly recognize the tagged Brk protein via Western blot or may simply be due to the fact that the antibodies do not work well on ChIP. An anti-brk antibody was attempted, but this also failed to indicate Brk was bound at regions contained within B14 or the *Ubx* B response element when compared to control PCR primer pairs.

2.9.4.1 New constructs and antibodies generated for ChIP

In attempts to successfully perform Brk ChIP analysis, (1) I created an N-terminally and C-terminally HA tagged Brk protein and an N-terminally Flag and C-terminally HA tagged Brk protein to over-express in embryos utilized in ChIP studies, and (2) I created six new antibodies and affinity purified one new anti-brk antibody generated in a guinea pig using the same antigen

as the anti-brk antibody (anti-brkR3) described above. Because the C-terminal HA tag was not recognized on Western blot analysis and seemed to not be detected by the three anti-HA antibodies I tried on ChIP, I hoped that either the N-terminal HA or Flag tags would be more accessible and prove more useful for ChIP.

The one new anti-brk antibody tested as well as the affinity-purified version of the same antibody failed to demonstrate an enrichment of Brk at B14. While we generated six new antibodies, I only tested one. The other five could also be tested in future ChIP experiments, though they were unable to detect Brk in embryonic lysates via Western blotting.

2.9.4.2 A lack of controls contributed to ChIP failure

Ultimately, I believe that many of the difficulties faced with the ChIP studies stemmed from the lack of a suitable positive control for use in the studies. The failure of these experiments may indicate that the alternative mechanism of Brk autoregulation is functioning (Brk repressing an activator of an unknown transcriptional repressor of Brk); however, the ChIP experiments also failed to indicate an enrichment of Brk at *UbxB*, indicating that the controls for the experiment failed for reasons described below.

We lacked a suitable region to utilize as a positive control for PCR. *zen* and *UbxB* elements have been confirmed *in vitro* via EMSAs to be bound by Brk but never by ChIP. While I utilized the *UbxB* response element as a positive control in my ChIP studies, *UbxB* is only expressed in a small proportion of cells in the embryo (Saller and Bienz, 2001). Therefore, it is likely that it is difficult to detect Brk interactions at this element. The only other control region Brk has been identified to repress was the *zen* promoter; Brk represses *zen* but *zen* is only expressed during hours 2-4 of embryonic development and attempts to obtain enough chromatin

from embryos in this early time point in development failed (Rushlow et al., 2001). *zen* primers were not tested as control PCR primers for these reasons.

2.9.4.3 Further ChIPs would utilize wing disc tissue in hopes of detecting interactions of Brk on B14

The region of the embryo where UAS-3PF3-HA is driven with UAS-tkv^{QD} and functions to repress B14 is only a very small proportion of the size of the entire embryo. We confirmed that Brk autoregulates in embryos (as in wing discs) in order to validate using enriched embryo extracts for ChIP and IP; indeed, IP experiments demonstrated that pMad and Brk interacted *in vivo* in embryos. While attempts at ChIP were made with a large amount of chromatin to attempt to compensate for the fact that proportion of en^{G4} expressing cells was low and expression of UAS-Brk was driven at high levels, background signal remained too high. These experiments could be repeated utilizing tissue from third-instar wing imaginal discs as the en^{G4} driver promotes high levels of expression in half of the wing disc. Controls would include primers directed against regions of *sal* and *omb* previously characterized by our lab to be responsive to Brk repression (GC, unpublished results). While large amounts of tissue would need to be dissected, it is possible that enough chromatin with the proper interactions may be isolated. Additionally, antibodies could be generated against a different antigen/region of the Brk protein in hopes that if the particular antigen we used is masked when Brk is bound to other proteins, other regions of Brk may be more accessible.

2.9.5 Brk negative autoregulation is key to establishing a gradient of *brk* expression

I began these studies because I was interested in learning how the *brk* expression gradient is established. Ultimately, the studies described above have demonstrated that the generation of the Brk expression gradient requires Brk to negatively autoregulate. pMad and Med are also required to repress Brk, which does not seem to function by binding cognate Brk binding sites on DNA. It appears likely that Brk functions to autoregulate by working at the S portion of the B14 response element and may require its DBD for this autoregulation to occur. Brk appears to autoregulate by functioning as a member of a protein complex with pMad.

There are at least three reasons why *brk* negatively autoregulates, which will be discussed in more detail in Chapter 4. First, Brk negative autoregulation may act as a feedback mechanism to ensure that the *brk* expression gradient is stable. Second, pMad levels appear to be the same in the presence or absence of Brk, but mediolateral cells that are lacking Brk are unable to repress *brk*. Brk may allow cells to repress *brk* and thereby respond to low levels of Dpp signaling. Third, and most importantly, Brk negative autoregulation appears to be necessary in order to establish graded expression of *brk* from the pMad gradient, modulating the simple threshold response of *brk* to pMad (as demonstrated by examining the *lacZ* reporter gene expression in the brk^{XA} mutant) to a continuous response creating a situation where the *brk* expression pattern is an equilibrium response to the amount of each input present at each position in the gradient. Without Brk negative autoregulation, graded *brk* expression would not be achieved.

3.0 PROPER BRK GRADIENT PLACEMENT REQUIRES ACTIVATION BY pMAD AT A NEWLY-DEFINED SEQUENCE ELEMENT

3.1 INTRODUCTION

As described in detail above, the Basler lab set out to understand the role played by Dpp in the generation of the *brk* expression gradient by identifying the regulatory elements of the *brk* gene important for the regulation of its expression. Recall that they identified B14 as a 4.7 kb response element fragment located about 5 kb upstream of the start of *brk* transcription (Muller et al., 2003). Also as described in detail above, recall that B315 is a response element consisting only of the enhancer as well as a silencer from B14 (Muller et al., 2003).

Also recall that pMad can function not only to repress the transcription of genes (as discussed above for its role at the *brk* S), but also to activate transcription of target genes based on its binding site context. pMad can directly activate Dpp-dependent transcription of genes; the decision of activation or repression by pMad is determined by the Mad protein binding sites contained within the regulatory regions of Dpp-responsive genes where binding sites that are not oriented exactly five basepairs away from Med binding sites (and thus do not allow the recruitment of Shn and subsequent formation of the MMS complex) allow for activation of transcription (Certel et al., 2000; Kim et al., 1997).

3.1.1 Aims of these studies

In addition to my goals of determining the mechanism by which Brk autoregulated (Chapter 2), I sought to determine what response elements contributed to defining the exact graded profile (both the position and slope) of *brk* expression. This is important because Dpp target genes such as *sal* and *omb* are repressed by Brk and are differentially sensitive to the amount of Brk present in the gradient; without proper *brk* expression, target genes are not expressed properly. Initial studies published by the Basler lab, as detailed in Chapter 1, presented a response element that drove a reporter gene in a pattern similar to *brk*; however, its expression could not recapitulate exactly *brk* expression (Muller et al., 2003). Here I show that this response element fails to rescue a *brk* mutant therefore, I sought to and was successful in identifying other response elements and recruited transcription factors that are necessary to establish a proper *brk* expression gradient.

3.2 B14 & B315 ARE NOT THE MINIMAL RESPONSE ELEMENTS REQUIRED FOR BRK EXPRESSION

Initial studies published by the Basler lab utilized a series of enhancer-testing constructs composed of various upstream regions of *brk* to screen for response elements that drove a reporter gene in a pattern similar to *brk* (Muller et al., 2003). However, our interpretation of the figures led us to believe that the reporters failed to recapitulate *brk* expression in two ways: 1) the reporter gene expression did not appear to extend as far medially across the A/P axis in the wing disc as our staining with an anti-brk antibody or enhancer trap and 2) the reporter gene

expression driven by the response elements was not graded like expression revealed with our anti-brk antibody or our enhancer trap, brk^{XA}. To test these observations, I obtained the previously published fly lines that consisted of B14, the minimal upstream response element published to drive reporter gene expression in a *brk* pattern, and B315, the fragments of B14 containing only a short silencer and enhancer (Fig 3.1).

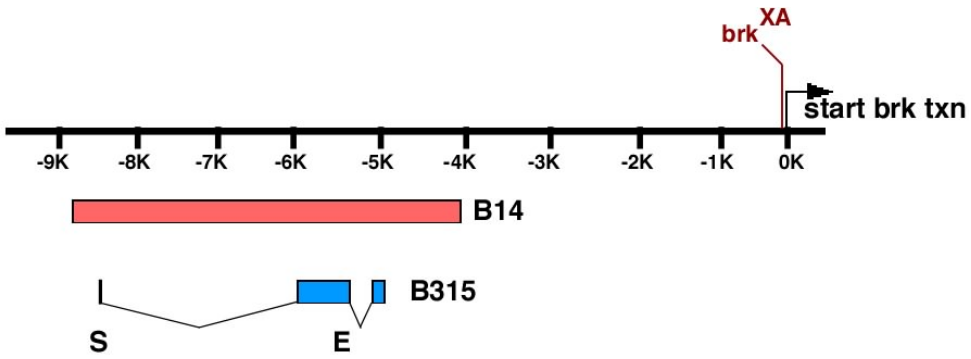

Figure 3-1 : B14 and B315 response elements.
Schematic representation of the B14 (pink) and B315 (blue) response elements (Muller et al., 2003), the *brk* transcription start site, and the insertion location of the brk^{XA} mutant (Campbell and Tomlinson, 1999). B315 consists of two regions: 1) an enhancer (E) region, which alone drives reporter gene expression ubiquitously across the wing disc, and 2) the silencer (S) region, which, when combined with the E, drives reporter gene expression in a pattern very similar to B14 (Muller et al., 2003).

Both the B14 and B315 response elements are in the vector pX27, which allows testing of the elements by examining the expression pattern of *lacZ* reporter utilized by this vector. The B14 and B315 response elements driving the *lacZ* expression in the pX27 vector are identified as B14X and B315X, respectively. I initially began my studies by repeating the X-gal staining published on B14 and B315, along with that of the P-element enhancer trap mutant brk^{XA} which when heterozygous, as described in Chapter 2, drives *lacZ* in an identical expression gradient and pattern compared to endogenous *brk*. These studies were undertaken to confirm our doubts that

the published spatial reporter gene expression actually differs from *brk*; however, the X-gal staining reaction in the published pictures may not have been allowed to proceed long enough to detect this type of expression.

3.2.1 X-gal staining of B14X, B315X, and brk^{XA} demonstrates that B14X & B315X fail to recapitulate endogenous *brk* expression

While identifying graded X-gal staining is quite difficult when staining is observed at one time point, it is more obvious if staining is monitored both early when staining first appears as well as later. If the staining exhibited is graded, then there is a medial shift in the expression domain of the staining in the later stained discs as compared to the discs stained for less time as staining becomes apparent in regions where expression is lower. If there is little or no graded expression present, the two time points will appear to have similar staining patterns, with X-gal staining appearing to extend to the same medial region of the disc.

X-gal staining B14X (two lines called B14X-A and B14X-B), B315X, and brk^{XA}/+ wing imaginal discs for both 90 minutes as well as overnight at 37°C confirmed that the reporter gene staining in B14X-A, B14X-B, and B315X never extends as far into the mediolateral region of the A/P axis of the wing disc and is not graded like that of the brk^{XA}-*lacZ* construct (Fig. 3-2). brk^{XA} exhibits graded X-gal staining as judged by the medial shift in expression from early to later staining time points (staining shifts from the white dashed lines in Fig. 3-2 to the red dashed lines). B14X and B315X do not exhibit graded X-gal staining as they do not have a medial shift in expression from early to later staining time points (width of staining at 90 minutes and overnight are the same as judged by the white dashed lines in Fig. 3-2).

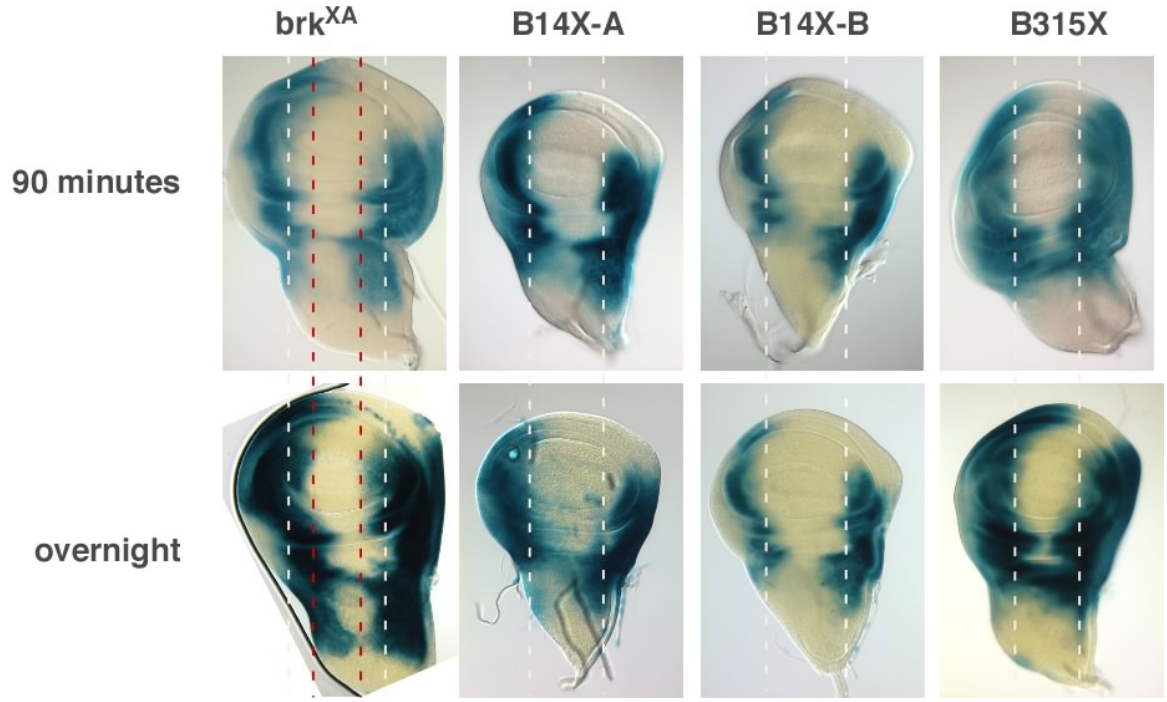

Figure 3-2: X-gal staining of B14X, B315X, and *brk*^{XA} demonstrates that B14X and B315X fail to recapitulate endogenous *brk* staining.
X-gal staining at 90 minutes (top row) and overnight (bottom row) performed at 37ºC. *brk*^{XA} (first column) staining reveals staining extends more medially across the A/P axis of the wing imaginal disc following overnight staining (compare white dashed line and red dashed lines indicating the boundaries of staining). Staining demonstrated by both lines of B14X (columns 2 and 3) and that of B315X (column 4) does not expand medially after overnight staining as indicated by the white dashed line identifying the boundaries of staining.

3.2.2 Immunofluorescence confirms that B14X & B315X fail to recapitulate endogenous *brk* staining

Because it is difficult to detect graded X-gal staining, studies were undertaken with anti-ßgal antibodies as antibody staining can clearly reveal graded expression that X-gal staining cannot. ßgal staining in *brk*^{XA} heterozygotes previously demonstrated a graded expression pattern identical to that of staining with anti-brk antibodies (Chapter 2); therefore, staining with anti-ßgal (of *brk*^{XA}) and anti-brk will be used interchangeably in the remainder of this document. To confirm that the expression patterns from B14X and B315X are not graded like that of *brk*^{XA}, the

ßgal produced from the reporter genes was visualized using an anti-ßgal antibody. B14X, B315X, and brk^{XA} heterozygotes were dissected, fixed, and third-instar wing imaginal discs were stained with anti-ßgal antibodies. An intensity profile was constructed along the A/P axis close to the margin using NIH Object Image to compare the expression profiles of B14X, B315X, and brk^{XA}/+ expression from comparably sized third-instar wing imaginal discs (Fig. 3-3). B14X was found to drive reporter gene expression in a pattern that is not identical to *brk* expression; its expression does not extend as far medially toward the center of the disc as that of brk^{XA}/+. This appears to be largely due to the fact that the gradient in B14X is much more precipitous than the extended graded expression from brk^{XA}. B315X was also found to drive reporter gene expression a pattern that is not identical to *brk* expression; its expression appears to extend more medially toward the center of the disc than that of brk^{XA}/+. Curiously, in the anterior this appears to be because its expression is higher in the region where expression is graded, but the opposite is true in the posterior. These observations confirmed our initial suspicions that neither the B14 nor B315 response elements appears to drive a reporter gene in the correct place/gradient as *brk*.

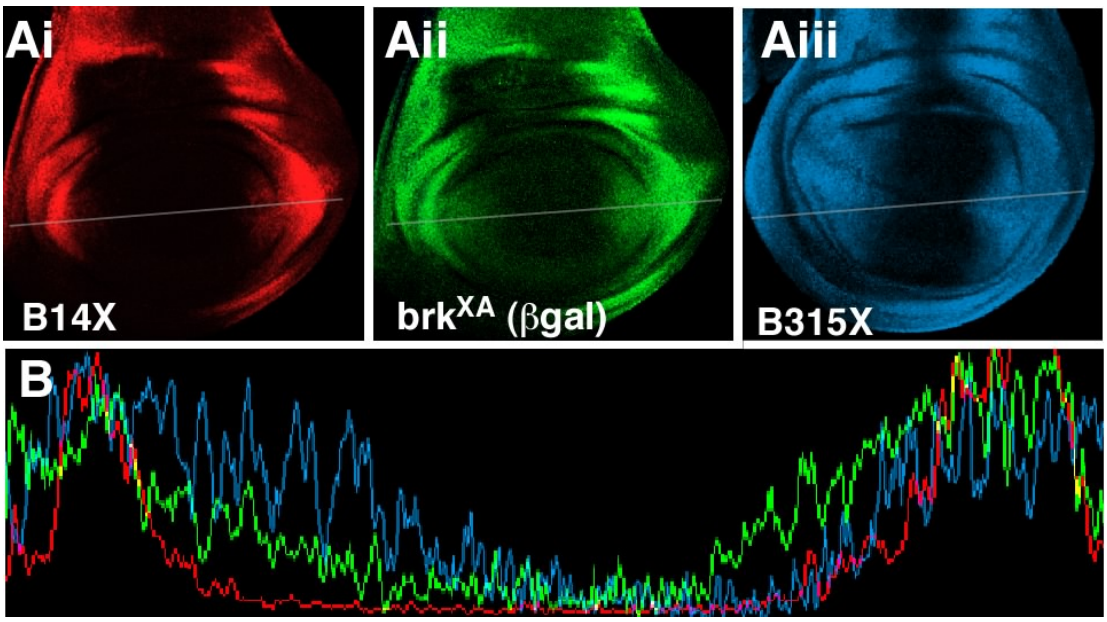

Figure 3-3: Immunofluorescence confirms that B14X and B315X fail to recapitulate *brk* expression.
Antibody staining B14X, B315X, and brk^{XA}/heterozygotes with an anti-ßgal antibody. While all three are different discs, everything possible was done to ensure that similar discs were utilized. (A) *lacZ* reporter gene expression. brk^{XA}/+ expression has previously been demonstrated to be identical to staining with an anti-brk antibody (Chapter 2). Grey lines indicate the region utilized to generate the intensity profile in (B). (B) Intensity profiles of B14X (red), brk^{XA} (green), and B315X (blue) across the center of the wing imaginal discs, from left to right. B14X appears to drive reporter gene expression in a pattern that extends less far medially than that of *brk* while B315X appears to drive reporter gene expression more medially than *brk*.

3.3 VECTOR AND REPORTER GENE DIFFERENCES DO NOT EXPLAIN WHY B14 AND B315 DO NOT DRIVE REPORTER GENE EXPRESSION IDENTICALLY TO BRK

Several possibilities could explain why B14 and B315 fail to drive reporter gene expression in a pattern identical to that of *brk*. First, the pX27 vector may contain cryptic sequences that modify expression driven by B14. Second, the nature of the promoter used to drive reporter gene

expression may be important; the pX27 vector used to test the B14X and B315X response element fragments uses a heterologous *hsp70* minimal promoter. There may be something specific to this promoter that interferes with its ability to drive a normal *brk* pattern, or there may be something unique about the endogenous *brk* promoter that is essential to drive the endogenous expression pattern. Third, B14 may be missing additional essential cis-regulatory elements required to drive the normal pattern of *brk* expression. These possibilities will be examined in the subsequent section.

To test the first and second possibilities, the B14 and/or B315 response element(s) were tested in different enhancer-testing vectors of which one utilized a different promoter (Table 3-1). Also, upon examination of the *hsp70* minimal promoter utilized by many vectors (including pX27), we found that putative Brk binding sites are present within the promoter. These sites could recruit Brk to alter the reporter gene expression driven by the response elements. To determine whether the vector and/or promoter was/were responsible for the inappropriate expression of the reporter from B14X and B315X, the B14 and B315 response elements were tested in vectors that (a) used a different promoter (C4PLZ), (b) did use a standard *hsp70* promoter but were constructed differently (pH-Pelican), or (c) which used an *hsp70* promoter in which the Brk sites were mutated (pHSB); additionally, some of these vectors used (d) GFP or Gal4 (with UAS-GFP) as reporters to rule out possible artifact with using ßgal. All of the combinations of promoters, putative Brk binding sites present in the *hsp70* promoter, and reporter genes are illustrated for the vectors utilized in this study in Table 3-1. The construct designation letter added to the end of B14 or B315 corresponds to the vector utilized. For example, B14Z is constructed in the C4PLZ vector whereas B14B only differs in that it is constructed in the pHSB vector.

Table 3-1: Vectors utilized to study the affects of B14 and B315 in different vectors.

Vector	Construct Designation	Promoter	Brk Sites in Promoter		Reporter
			Present	Mutated	
C4PLZ	Z	transposase	no	no	lacZ
pX27	X	hsp70	yes	no	lacZ
pHPelican	N	hsp70	yes	no	lacZ
pHStinger	R	hsp70	yes	no	GFP
pHSB	B	hsp70	yes	yes	GFP
pHSGal4	G	hsp70	yes	yes	Gal4 (GFP)

3.3.1 The reporter gene can not explain the inability of B14 & B315 to drive reporter gene expression exactly recapitulating *brk* expression

Initially, B315X was re-injected in order to generate more than one transgenic line in the same manner as described by Muller et al. (2003); expression was similar in both my transgenic lines as well as the Basler lab's version of B315X (i.e. none were identical to *brk*) (compare Fig 3-4 A and E). This experiment confirmed that the differences in expression were not due to the transgene insertion site, confirming that multiple inserts of B315X all have similar reporter gene expression, though none are identical to *brk*.

The pH-Pelican utilizes a standard *hsp70* promoter but the vector is constructed differently than pX27. To rule out that B315X does not drive reporter gene expression in a *brk* pattern due to artifacts contained within its pX27 vector (testing possibility b), B315 was constructed in the pH-Pelican vector (B315N) driving *lacZ* expression (Fig. 3-4). Expression of B315N was not identical to *brk* expression indicating that vector artifacts do not explain why B315 does not drive reporter gene expression in a *brk* gradient.

To rule out that the expression driven by B14X and B315X are not artifacts of using ßgal as a reporter gene (testing possibility d), both B14 and B315 were constructed in vectors utilizing GFP as a reporter. These were compared to the *lacZ* expression of B14 and B315 as demonstrated with B315N, B315X, B14X, and B14Z, which all utilize *lacZ* reporters but are not expressed in patterns identical to *brk* (Fig. 3-4 and Fig. 3-5). Upon comparison of reporters utilizing *lacZ* to those utilizing GFP, like B315R (Fig. 3-4 D), we determined that the *lacZ* reporter is not responsible for the failure of the B14X and B315X reporters to recapitulate *brk* expression. Additionally, upon comparison of reporters utilizing *lacZ* to those utilizing Gal4 (and the UAS-Gal4 system to observe expression by crossing to UAS-GFP and visualizing GFP expression in the pattern in which the response element drove Gal4), like B14G (Fig. 3-5 D), it was observed that the type of reporter gene does not cause B14 to be expressed in a pattern identical to *brk*. Therefore, the type of reporter gene utilized (GFP, *lacZ*, or Gal4) is not relevant to the reporter gene expression driven by B14 and B315.

3.3.2 The use of the *hsp70* promoter cannot explain the inability of B14 to recapitulate *brk* expression

To determine if the *hsp70* promoter used by the vector is the reason why B14X does not drive reporter gene expression in a *brk* pattern (testing possibility a), B14 was constructed in the C4PLZ vector (B14Z) which utilizes a transposase promoter and this reporter gene expression was compared to that of the B14 lines obtained from the Basler lab which utilize the *hsp70* promoter (Fig. 3-5). The transposase promoter utilizes a TATA box type promoter as does *hsp70* (Bier et al., 1989; Kaufman and Rio, 1991; O'Kane and Gehring, 1987). The reporter gene

expression driven from B14Z mirrored that of B14 indicating that B14's spatial differences in expression are not due simply to the use of the *hsp70* promoter (compare Fig. 3-5 A and C).

3.3.3 The putative *brk* binding sites are not responsible for the inability of B14 & B315 to recapitulate *brk* expression

To examine if the presence of putative Brk binding sites contained within the *hsp70* promoter of the enhancer-bashing vectors utilized is the reason why B14 and B315 do not drive reporter gene expression in a *brk* pattern (testing possibility c), B14 and B315 were constructed in the pHSB vector where the putative Brk binding sites in the promoter are mutated (B14B and B315B). B14B expression was compared to that driven by B14X. The B315R expression was compared to B315 in the same parent vector, pHStinger, whose putative Brk binding sites in the *hsp70* promoter are intact (B315R). Upon examination of reporter gene expression, it was determined that removing putative Brk binding sites from the promoter does not alter reporter gene expression for either B14 or B315 (compare Fig. 3-5B and Fig. 3-4 B and D). These constructs also do not have expression identical to that of *brk*. Therefore, the presence of putative Brk binding sites in the *hsp70* promoters of enhancer bashing vectors does not explain why the reporter gene expression driven by B14 and B315 is not identical to *brk*.

Overall, by testing both B14 and B315 in a variety of enhancer-bashing vectors, it was determined that the type of promoter utilized by the vector, the presence of putative Brk binding sites in the promoter, and the type of reporter gene whose expression is driven by the response element are not reasons why reporter gene expression driven by B14 and B315 does not match that of *brk*. Therefore, further studies were undertaken to determine if the endogenous promoter

is important or if additional regulatory sequence information is necessary to recapitulate a *brk* expression pattern.

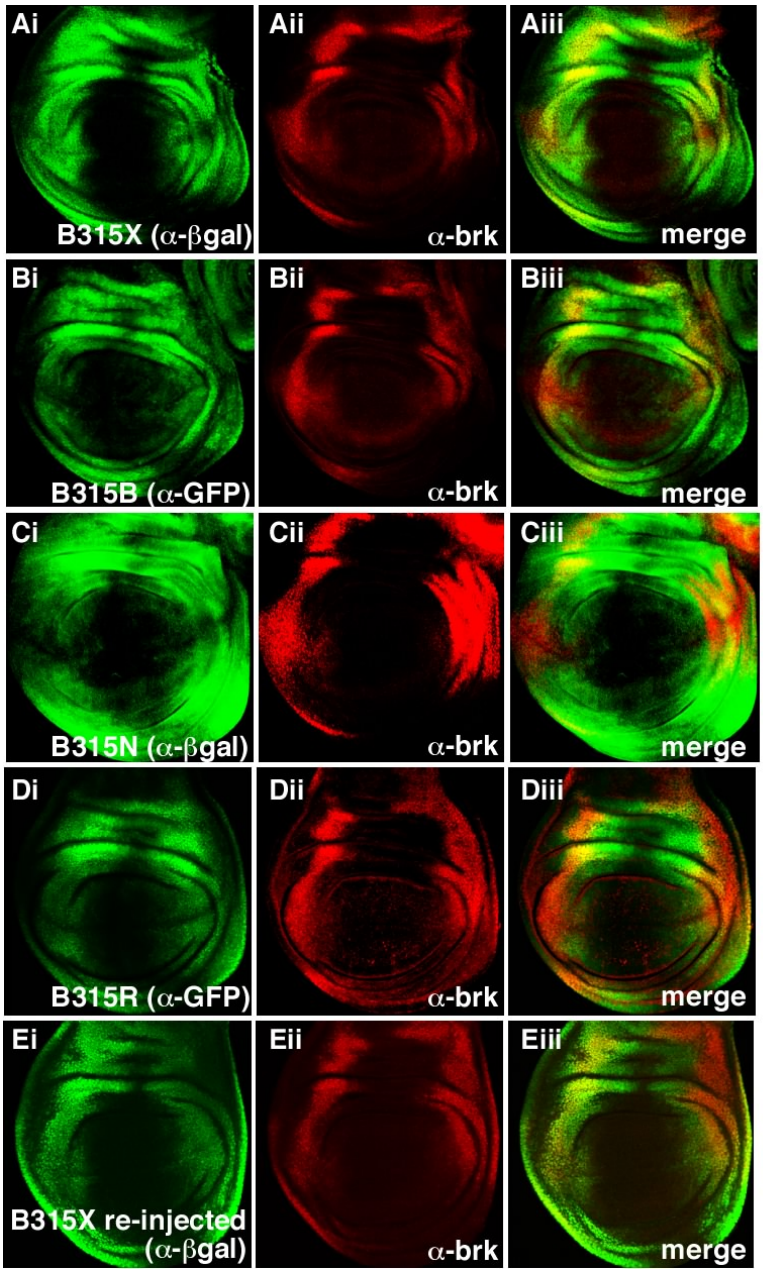

Figure 3-4: Vector differences do not explain why B315 fails to recapitulate *brk* expression.
B315, in various vector backbones, fails to recapitulate endogenous *brk* expression. (Ai) Expression of B315X (anti-ßgal) differs from that endogenous *brk* as demonstrated by staining with an anti-brk antibody (Aii). (Aiii) merge of (Ai-Aii). (Bi) Expression of B315B (anti-GFP) differs from that endogenous *brk* as demonstrated by staining with an anti-brk antibody (Bii). (Biii) merge of (Bi-Bii). (Ci) Expression of B315N (anti-ßgal) differs from that endogenous *brk* as demonstrated by staining with an anti-brk antibody (Cii). (Ciii) merge of (Ci-Cii). (Di) Expression of B315R (anti-GFP) differs from that endogenous *brk* as demonstrated by staining with an anti-brk antibody (Dii). (Diii) merge of (Di-Dii). (Ei) Expression of B315X when re-injected (anti-ßgal) differs from that endogenous *brk* as demonstrated by staining with an anti-brk antibody (Eii). (Eiii) merge of (Ei-Eii).

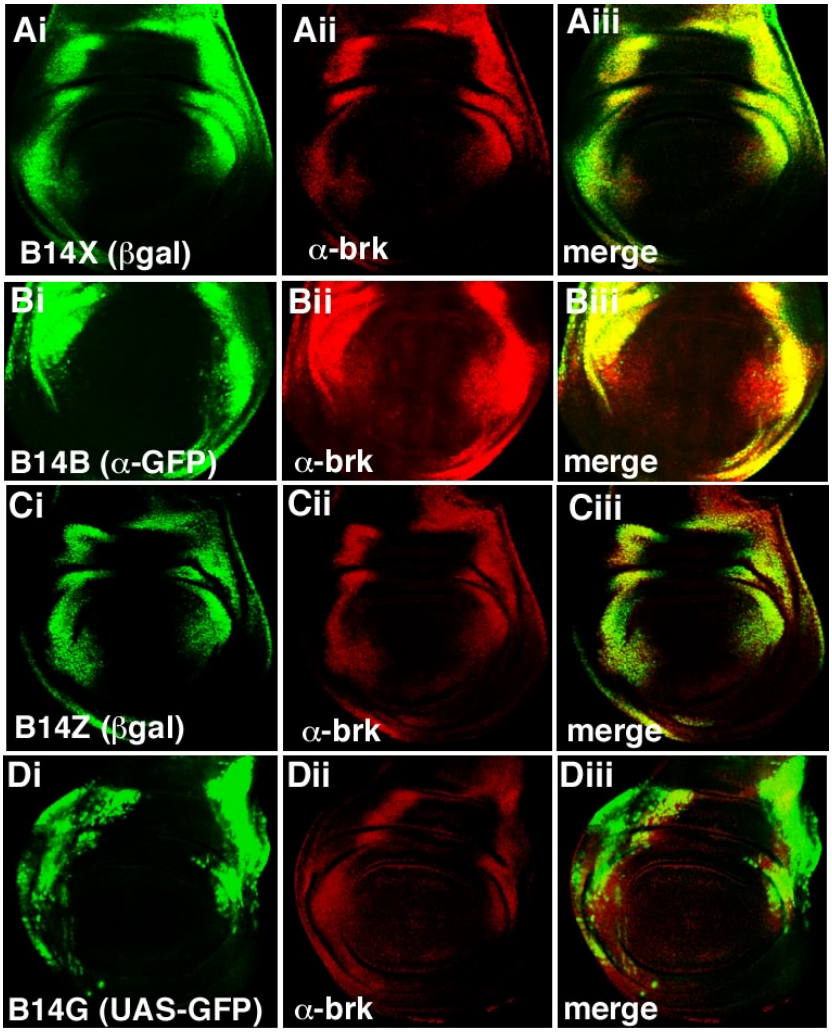

Figure 3-5: Vector differences do not explain why B14 fails to recapitulate *brk* expression.
B14, in various vector backbones, fails to recapitulate endogenous *brk* expression. (Ai) Expression of B14X (anti-ßgal) differs from that endogenous *brk* as demonstrated by staining with an anti-brk antibody (Aii). (Aiii) merge of (Ai-Aii). (Bi) Expression of B14B (anti-GFP) differs from that endogenous *brk* as demonstrated by staining with an anti-brk antibody (Bii). (Biii) merge of (Bi-Bii). (Ci) Expression of B14Z (anti-ßgal) differs from that endogenous *brk* as demonstrated by staining with an anti-brk antibody (Cii). (Ciii) merge of (Ci-Cii). (Di) Expression of B14G (when crossed to UAS-GFP) differs from that endogenous *brk* as demonstrated by staining with an anti-brk antibody (Dii). (Diii) merge of (Di-Dii).

3.4 B14 IS NOT THE MINIMAL RESPONSE ELEMENT NECESSARY TO CREATE THE BRK EXPRESSION PATTERN

Because vector context was determined to not be relevant for the reporter gene expression driven by B14 and B315, I sought to determine if the minimal sequence necessary to create the graded *brk* expression gradient had been identified. I decided to work for the duration of my thesis work with the B14 response element as it is larger than B315. Because initial experiments suggested that B14 and B315 were not the only response element regions necessary to recapitulate *brk* expression, the larger B14 response element was used to determine what additional genomic sequences were necessary to recapitulate *brk* expression and rescue mutants, if necessary. Also, results described in Chapter 2 indicated that B315 is not expressed in embryos, presumably because it does not contain all response elements necessary for embryonic expression—this construct would not be expected to rescue *brk* mutants as it is not expressed embryonically. If additional sequences need to be added to recapitulate the *brk* expression pattern, it would be best to start with the larger response element fragment. All of the reporter gene and rescue constructs created to test this are illustrated in Fig. 3-6. Constructs utilized to test for *brk* mutant rescue contained a "W" at the end of the construct's name indicating that it did not drive a reporter gene (without reporter), but rather drove the *brk* cDNA. Vectors containing putative Brk sites within *hsp70* promoters utilized mutated sites, where appropriate.

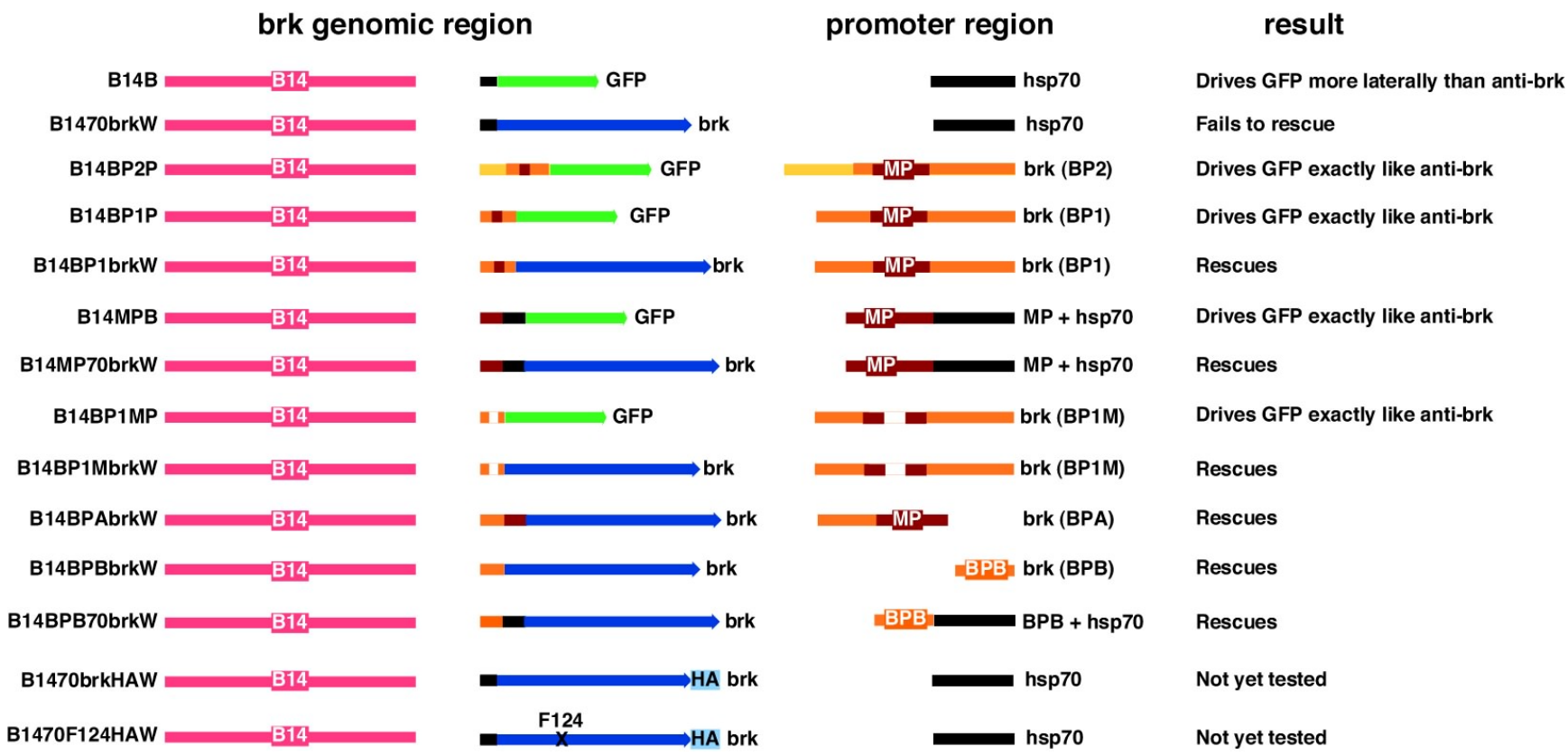
brk genomic region
promoter region
result
B14B
B14
GFP
hsp70
Drives GFP more laterally than anti-brk
B1470brkW
B14
brk
hsp70
Fails to rescue
B14BP2P
B14
GFP
MP
brk (BP2)
Drives GFP exactly like anti-brk
B14BP1P
B14
GFP
MP
brk (BP1)
Drives GFP exactly like anti-brk
B14BP1brkW
B14
brk
MP
brk (BP1)
Rescues
B14MPB
B14
GFP
MP
MP + hsp70
Drives GFP exactly like anti-brk
B14MP70brkW
B14
brk
MP
MP + hsp70
Rescues
B14BP1MP
B14
GFP
brk (BP1M)
Drives GFP exactly like anti-brk
B14BP1MbrkW
B14
brk
brk (BP1M)
Rescues
B14BPAbrkW
B14
brk
MP
brk (BPA)
Rescues
B14BPBbrkW
B14
brk
BPB
brk (BPB)
Rescues
B14BPB70brkW
B14
brk
BPB
BPB + hsp70
Rescues
B1470brkHAW
B14
HA
brk
hsp70
Not yet tested
B1470F124HAW
B14
F124
X
HA
brk
hsp70
Not yet tested

Figure 3-6: Reporter and rescue constructs created.
B14 is represented in pink in the first column. The second column depicts the promoter and either the reporter or the rescue (brk cDNA) used. The third column is a detailed magnification of the promoter region. The fourth column is a description of the result achieved. See text for detailed discussion of the constructs. A "W" at the end of a construct's name indicates it is utilized to test for *brk* mutant rescue.

3.4.1 Creation of a recombinant *Drosophila* line to standardize gene expression/rescue studies

I created a recombinant fly line to utilize in the gene expression and rescue studies. The brk^{XA} hypomorphic mutation and omb^{Gal4} UASGFP were recombined onto the same chromosome. Wildtype third instar wing discs were then dissected and stained to reveal *brk* (brk^{XA}-*lacZ*), *sal* (anti-sal), and *omb* expression (omb^{Gal4} UASGFP) (Fig. 3-7 A) as well as Brk protein staining with an anti-brk antibody (Fig. 3-7 B). All expression patterns were determined to recapitulate previous *brk*, *omb*, and *sal* expression patterns. brk^{XA} mutant wing discs from this line were then examined (Fig. 3-7 C). These animals do not survive past the third instar stage, have no evidence of Brk protein (as determined by a lack of detectable staining with an anti-brk antibody), have no gradient of expression from brk^{XA}-*lacZ* enhancer trap (as described in detail in Chapter 2), and have greatly expanded expression of *omb* due to the absence of Brk which normally represses the expression of *omb* (as described in detail in Chapter 2).

If B14 is truly the minimal response element fragment necessary to drive a reporter gene in a *brk* expression gradient, then the combination of B14 with a promoter to drive the *brk* cDNA should rescue a *brk* mutant. As the utilization of two different heterologous promoters was determined above to not influence reporter gene expression, the rescue experiments were performed utilizing the *hsp70* minimal promoter with the putative *brk* binding sites mutated

(from pHSB); this construct drives the *brk* cDNA as described above and is termed B1470brkW. As the brk^{XA} chromosome carries the forked marker and my transgene carries the white marker, I could determine which males were rescued.

No brk^{XA} rescued male adult flies were recovered in *trans* by the B1470brkW construct. Upon examination of the mutant non-rescue larvae carrying the B1470brkW transgene, the larvae looked exactly like brk^{XA} mutant animals (i.e. they exhibit large discs with the characteristic brk^{XA}-*lacZ* staining pattern of no gradient of expression as well as expanded *omb* expression and no detectable Brk protein) (Fig. 3-7 D). Due to the lack of rescue of the brk^{XA} mutant with B1470brkW, it can be concluded that B14 is not the minimal response element fragment necessary to create a *brk* expression gradient; this result is consistent with the reporter gene assays described in the last section.

Additional confirmation that B14 is not the minimal response element necessary for *brk* expression came from studies utilizing the B14G construct. This construct drives Gal4 in the B14 expression pattern (Table 3-1 and Fig. 3-4). The B14G construct was crossed to UAS-brk and tested for its ability to rescue the brk^{XA} mutant (data not shown). B14G driving UAS-brk fails to rescue a brk^{XA} mutant (data not shown) further confirming the results obtained by B1470brkW indicating that B14 is not the minimal response element necessary for *brk* expression.

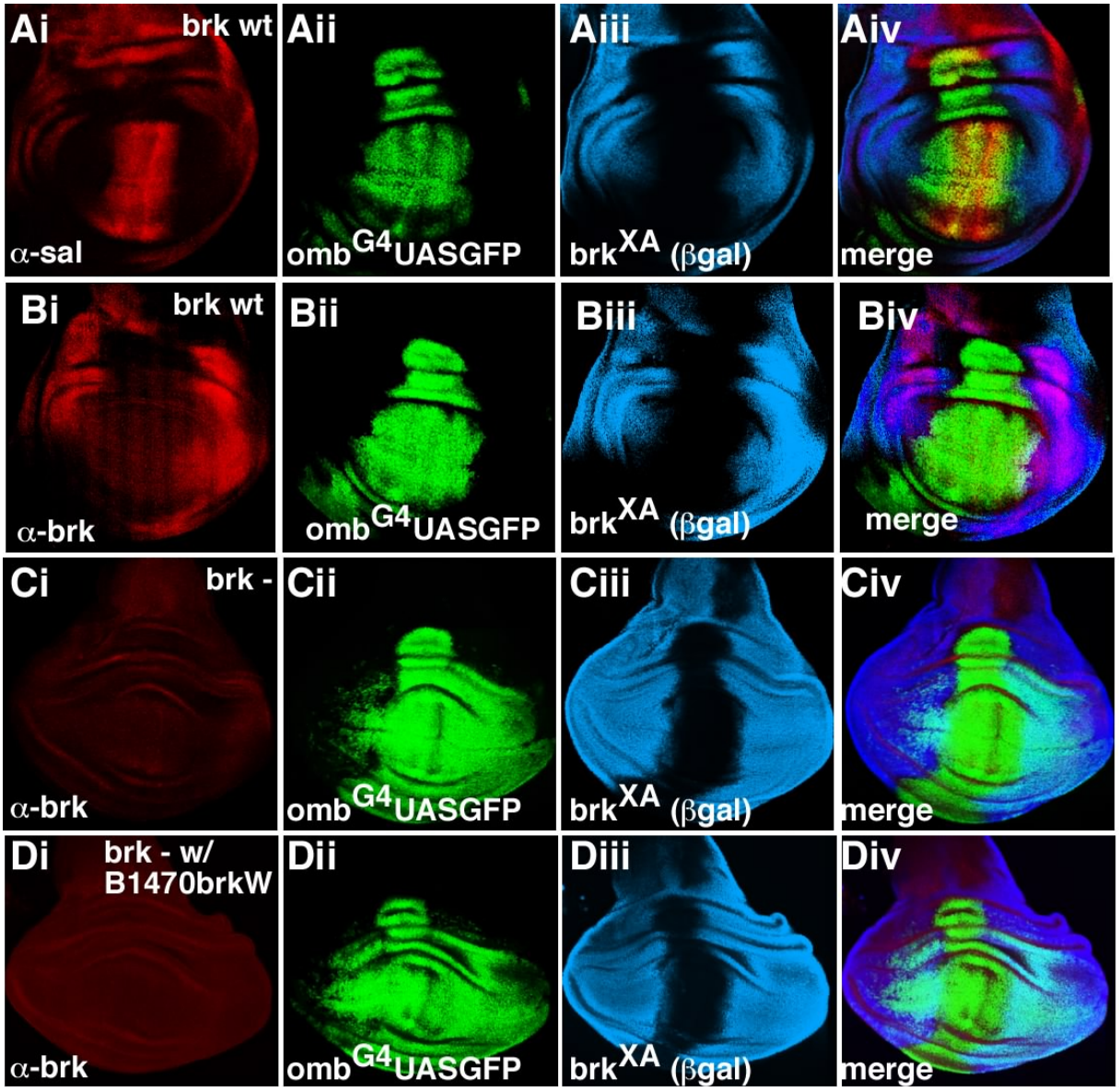

Figure 3-7: B1470brkW fails to rescue a brk^{XA} mutation.
brk^{XA} wildtype and mutant with failed B1470brkW rescue. (A-B) *brk^{XA}* heterozygous wing disc shows proper sal (anti-sal), omb (omb^{Gal4}UASGFP), and Brk (anti-brk, *brk^{XA}-lacZ*) expression. (C) *brk^{XA}* mutant wing disc shows no Brk (evidenced by no staining detected with anti-brk) as well as expanded *omb* (omb^{Gal4}UASGFP) expression and characteristic mutant *brk^{XA}-lacZ* expression. (D) *brk^{XA}* mutant is not rescued by B1470brkW, has no Brk (evidenced by no staining detected with anti-brk) as well as expanded *omb* (omb^{Gal4}UASGFP) expression and characteristic *brk^{XA}-lacZ* expression.

3.5 B14 IN COMBINATION WITH A REGION INCLUDING THE ENDOGENOUS PROMOTER DRIVES REPORTER GENE EXPRESSION IN A BRK PATTERN

The heterologous *hsp70* and transposase promoters utilized in reporter gene assays contains a TATA box style core promoter whereas *brk* appears to utilize a DPE style promoter (reviewed by Smale and Kadonaga, 2003). It is possible that correct *brk* expression and mutant rescue can only be achieved when an endogenous DPE promoter type is utilized.

To examine if utilizing a region including the endogenous promoter instead of a heterologous promoter is necessary for B14 to drive a reporter gene in a pattern exactly like *brk*, B14BP1P and B14BP2P were created by combining B14 with either the BP1 or BP2 fragment (without a heterologous promoter) and driving GFP reporter gene expression (Fig. 3-8).

Both B14BP1P and B14BP2P drove GFP reporter gene expression in a pattern identical to that of *brk*. Therefore, adding a region near the start of *brk* transcription (including the endogenous promoter) to B14 restores reporter gene expression to a pattern identical to *brk*.

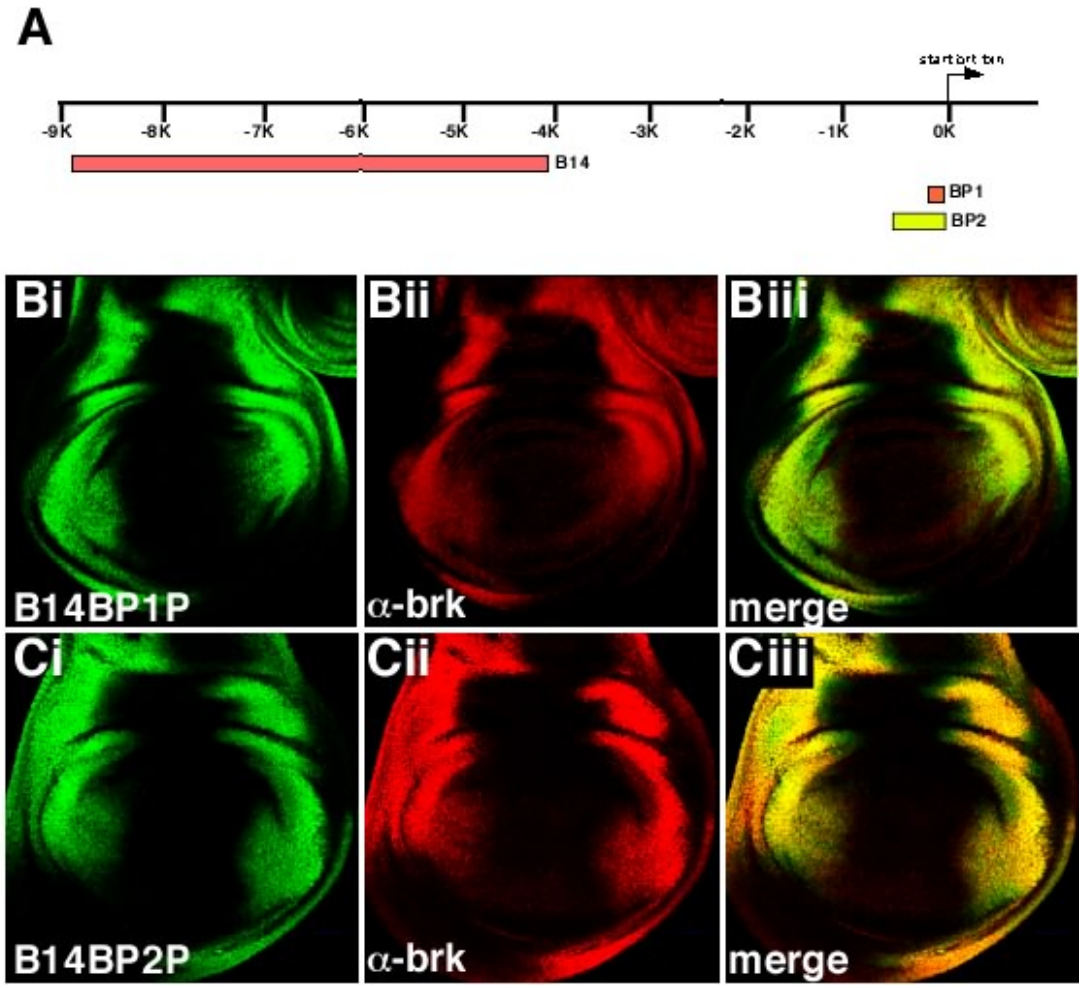

Figure 3-8: B14BP1P and B14BP2P drive GFP in a pattern identical to *brk*.
(A) Schematic of the genomic regions utilized to build constructs. (B) B14BP1P expression is identical to staining with an anti-brk antibody. (Bi) B14BP1P expression (anti-GFP). (Bii) Brk expression (anti-brk). (Biii) Merge panel of (Bi) and (Bii). (C) B14BP2P expression is identical to staining with an anti-brk antibody. (Ci) B14BP2P expression (anti-GFP). (Cii) Brk expression (anti-brk). (Ciii) Merge panel of (Ci) and (Cii).

3.6 A REGION INCLUDING THE ENDOGENOUS PROMOTER CAN COMBINE WITH B14 TO RESCUE A BRK^{XA} MUTANT

To examine if the combination of B14 with BP1 or BP2 are the minimal response element fragments necessary to drive *brk* expression, I sought to determine if they rescued a *brk*^{XA}

mutant. B14BP1brkW and B14BP2brkW were created using B14 and either BP1 or BP2 driving the *brk* cDNA fragment and the constructs tested for their ability to rescue the brk^{XA}omb^{Gal4}UASGFP mutation (see Fig. 3-6).

The B14BP1brkW transgene successfully rescued the brk^{XA} mutant to adulthood where it produced a perfectly formed fly with wings properly sized and patterned upon comparison to wildtype (Fig. 3-9). Upon dissection and examination of third-instar wing discs from these rescued mutants, *omb* expression was normal, brk^{XA} expression was graded like that of *brk*, and Brk obtained from the transgene alone was expressed in the correct pattern and gradient. This result indicated that sequences contained within the BP1 response element are necessary to rescue a *brk* mutant.

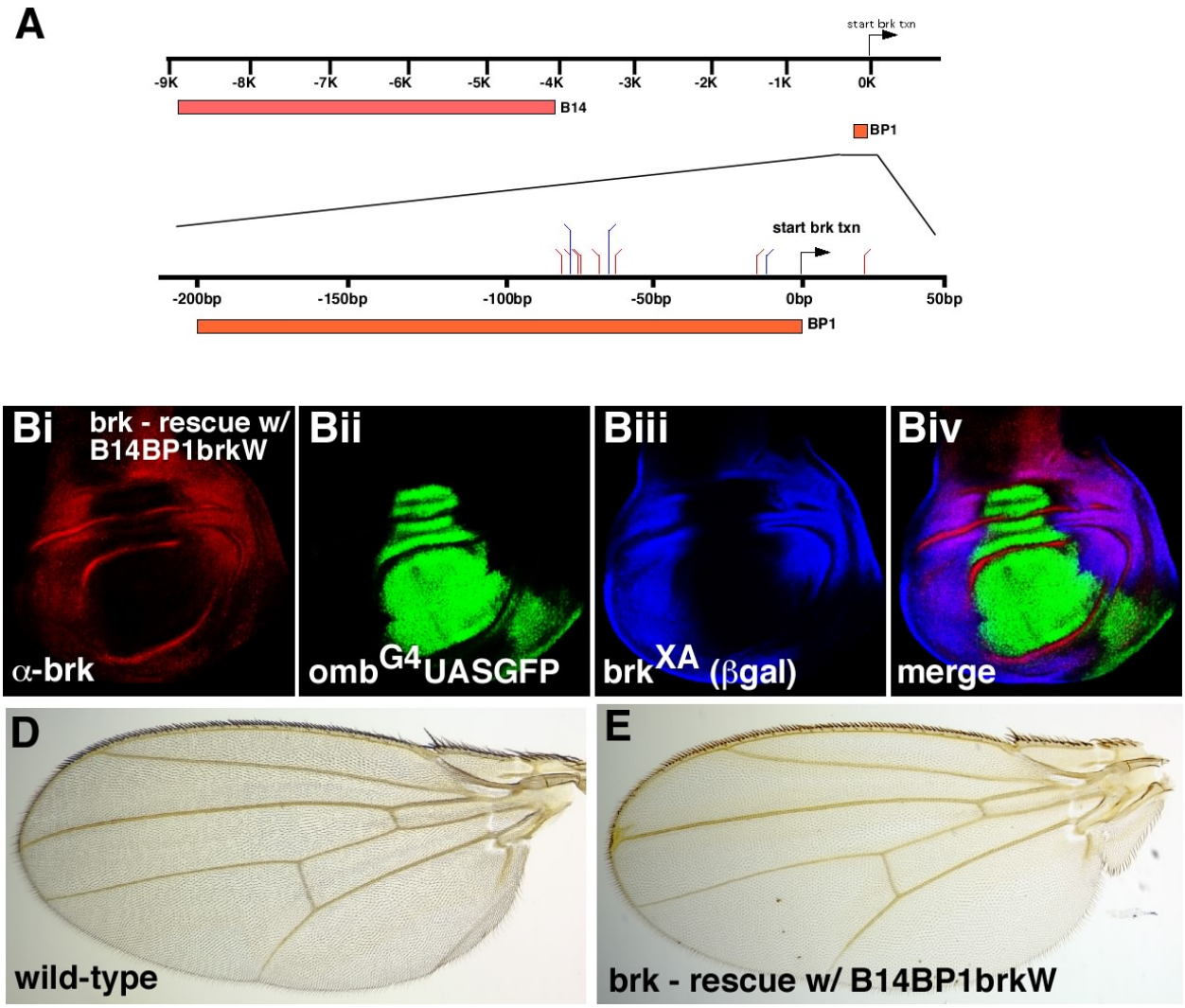

Figure 3-9: B14BP1brkW rescues *brk*^{XA} mutants.
B14 combined with BP1 contains the response elements necessary to properly drive *brk* expression. (A) Schematic of the genomic regions utilized in the study. The red lines represent putative (activating) Mad binding sites while the blue lines represent putative Brk binding sites. (B) *brk*^{XA} mutant discs rescued by the B14BP1brkW transgene. Anti-brk staining (Brk contributed totally from the transgene) demonstrated proper graded *brk* expression; *omb* expression was properly placed, and *brk*^{XA}-*lacZ* expression demonstrates a non-mutant pattern. (D-E) Wildtype and *brk*^{XA} mutant rescue wings photographed at the same size. (D) Wildtype wing. (E) *brk*^{XA} mutant rescued with B14BP1brkW transgene was correctly sized and patterned.

3.7 MAD ACTIVATION NEAR THE BRK ENDOGENOUS PROMOTER IS NECESSARY FOR GRADED BRK EXPRESSSION

Because B14BP1brkW rescues the *brk*^{XA} mutation, two possibilities exist as to why this construct could rescue while B1470brkW failed to rescue. The first possibility is that the type of promoter utilized by the two constructs is important. B1470brkW uses an *hsp70* (TATA box) minimal promoter while *brk* contains no TATA box and uses DPE promoter elements. *brk* rescue may not be possible if a promoter that is too strong or non-native is utilized. The second possibility why B14BP1brkW rescues *brk*^{XA} while B1470brkW fails to rescue is that sequences necessary for correct gradient placement and expression (and therefore necessary for rescue) are located within BP1 but outside of the promoter itself.

3.7.1 *brk*^{XA} drives graded expression without utilizing the *brk* endogenous promoter

To examine if B14BP1brkW rescues the *brk*^{XA} mutant due to the presence of the *brk* endogenous promoter, I examined reporter expression from two P-element enhancer trap insertions in *brk* that drove *lacZ* reporter gene expression (Campbell et al., 1999). These P-elements were compared to Brk expression with an anti-brk antibody (Fig. 3-10). While *brk*^{XA} has graded expression that extends as far medially across the A/P axis of the wing disc as anti-brk, *brk*^{X47} does not. *brk*^{X47} expression is confined to more lateral regions of the wing disc. It should be noted that the *brk*^{XA} and *brk*^{X47} enhancer traps drive *lacZ* expression utilizing *hsp70* promoters in PZ elements. Because *brk*^{XA} expression is identical to *brk*, it is difficult to support the proposal that the nature of the promoter utilized (*hsp70* or endogenous *brk*) is important to generate graded expression.

Sequences distal to the promoter that are still positioned upstream of the promoter in brk^{XA} but not in brk^{X47} are most likely the factors responsible for graded expression in brk^{XA} and in *brk*.

The sequences included in the region upstream of the insertion site of brk^{XA} were examined. Most of BP1 is included in this region, and the most striking sequence element includes a cluster of overlapping putative Mad and Brk binding sites termed MP (Fig. 3-10). The Mad binding sites contained within this region are characteristic of the sites known to be utilized when pMad acted as a transcriptional activator with the typical GC consensus sequences (described in detail in Chapter 1), not the repressive sites that recruit the MMS repression complex. In addition to repressing as a member of the MMS complex, pMad can also directly activate Dpp-dependent transcription of genes; the decision of activation or repression by pMad is determined by the Mad protein binding sites contained within the regulatory regions of Dpp-responsive genes where GC-rich binding sites that are not situated exactly five basepairs away from Med binding sites (thus not allowing for the subsequent recruitment of Shn) allow for activation of transcription (Certel et al., 2000; Kim et al., 1997). It was hypothesized that sequences included in BP1 contribute to its *brk* expression pattern and rescue when combined with B14 to drive Brk. This is consistent with the observation that if B14B would be transcriptionally activated by pMad, its expression would be predicted to shift medially (and thus be expressed more like *brk*). brk^{X47} most likely drives reporter gene expression in a *brk*-like pattern because the B14 element can presumably act upstream or downstream, thereby regulating brk^{X47}, but it may also be that the activating Mad sites need to be close to the promoter to be effective in generating graded expression.

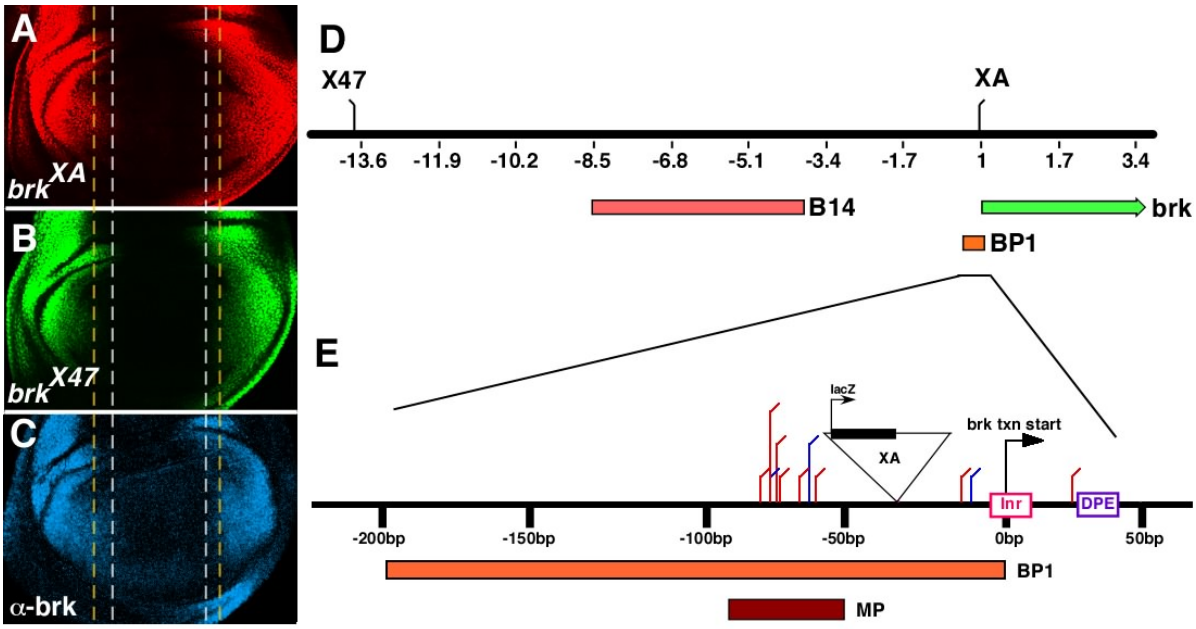

Figure 3-10: Expression and insertion sites of different *brk* enhancer traps.
Insertion sites and expression of brk^{X47} and brk^{XA} compared to anti-brk. (A-B) Wing imaginal discs from flies carrying *brk* enhancer traps. (A) brk^{XA} (lacZ enhancer trap; ß-gal expression). (B) brk^{X47} (lacZ enhancer trap; ß-gal expression). Comparison of expression reveals that the limit of reporter gene expression in brk^{XA} along the A/P axis (marked by the white dashed lines) is more medial than in brk^{X47} (marked by the yellow dashed lines). (C) Staining of a wildtype animal with anti-brk is identical to that of brk^{XA} (expressed as far medially as white dashed lines). (D) Diagram of the *brk* genomic region showing the *brk* transcriptional unit, the B14 and BP1 response elements, and the enhancer trap insertion sites. (E) Magnification of the region in D including BP1, the brk^{XA} insertion, the endogenous *brk* promoter elements (DPE, Inr), and the *brk* transcription start site. Mad binding sites are shown with red lines and Brk binding sites with blue lines. The Inr (pink) and DPE (purple) promoter elements are depicted with boxes on the sequence. The P-element in brk^{XA} is a PZ element with an *hsp70* promoter oriented so that the lacZ gene is immediately downstream of the promoter and this Mad/Brk element.

3.7.2 Mad binds MP *in vitro* in EMSA studies

To test the hypothesis that activating Mad binds the MP response element to generate graded expression when combined with B14, the ability of pMad to bind the MP region was tested *in vitro* using electromobility shift assays (EMSAs). A 40 basepair double-stranded probe was generated against the MP region as well as a probe with the putative Mad binding sites mutated to use in competition experiments. Plasmids that expressed the MBP fusion protein alone or

conjugated to Mad were obtained, expressed in bacteria, and proteins purified for use (Gao et al., 2005). The Mad protein is known to consist of two Mad Homology domains (MH1 and MH2) joined by a linker region. Other labs have never successfully purified the entire Mad protein as the MH2 domain has been shown to antagonize DNA binding (Xu et al., 2005). Therefore, the fusion protein created, MBP-MadNL, contained the MH1 plus linker region and was previously described to be successful in EMSAs (Kim et al., 1997).

To begin these experiments, it was confirmed that the negative control, MBP protein, does not bind the MP probe on EMSA; therefore, any binding observed with MBP-MadNL would presumably be due to the MadNL portion of the protein (Fig. 3-11). As a positive control to ensure that the purified fusion protein generated functions for EMSA experiments, the ability of MBP-MadNL to bind a *UbxB* probe was tested, as previously reported (Kirkpatrick et al., 2001; Saller and Bienz, 2001). This binding was competed away with excess cold wildtype *UbxB* probe; however, binding was not competed away with excess cold probe in which the Mad binding sites were mutated. This experiment served to validate that MBP-MadNL functionally binds probes containing its binding sites on EMSAs as well as validating the nature of the point mutations in probes created for *UbxB* to eliminate the ability of Mad to recognize and bind its binding sites; these same point mutations were created in my MP probe.

MBP-MadNL was next tested with the MP probe and was found to bind and generate a shifted complex. This binding was competed away by adding excess cold MP probe; however, this binding was not competed away by adding excess cold mutant probe where the putative Mad binding sites were mutated, thus indicating that the MBP-MadNL protein binds the Mad binding sites in the MP response element *in vitro*.

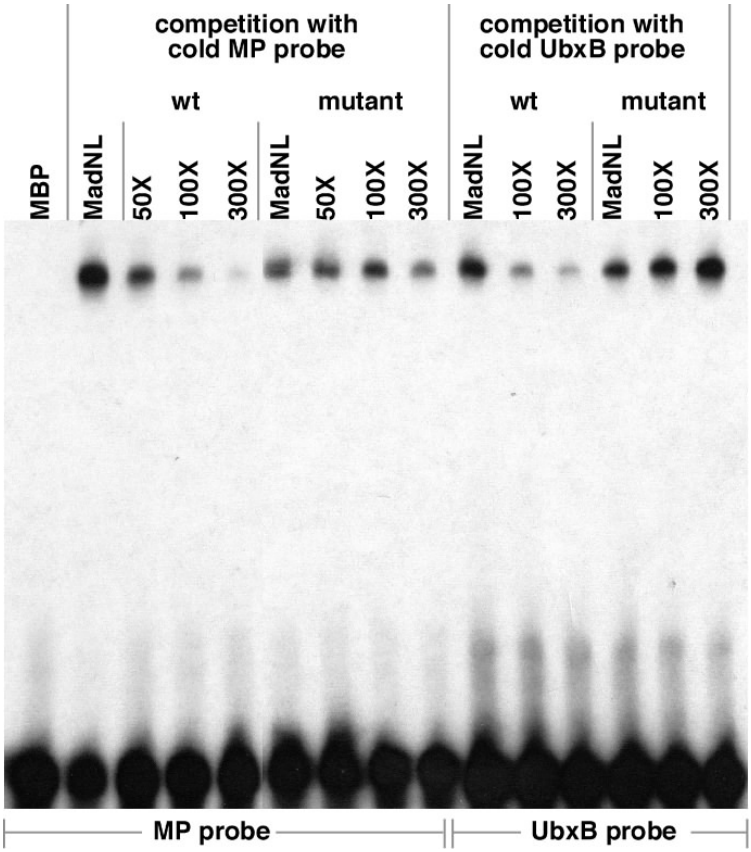

Figure 3-11: Mad binds MP on EMSAs.
EMSA experiment where MBP alone fails to bind the MP probe, but MBP-MadNL protein binds the probe. This binding is competed away with cold wildtype probe but is not competed away with cold mutant probe where the putative Mad binding sites are mutated. MBP-MadNL also binds to the UbxB control probe while MBP alone does not (not shown). This binding is competed away with cold wildtype control probe but is not competed away with cold mutant control probe where the putative Mad binding sites are mutated.

3.7.3 B14MPB drives reporter gene expression in a perfect *brk* pattern

If sequences contained within BP1 and also lying upstream of the insertion site of the brk^{XA} enhancer trap are necessary in combination with B14 for perfect *brk* expression and *brk* mutant rescue, an obvious potential region to test is the MP cluster of activating Mad binding sites contained within BP1 (Fig. 3-10 E). As discussed in detail earlier, pMad can both activate and repress transcription of target genes based on its binding site context. Based on this information,

a possible hypothesis describes a situation where activation by pMad at the MP element is necessary in the mediolateral region of the wing disc to pull the expression of B14 more medial. This could explain why B14 is not expressed as far medially across the A/P axis as *brk*—B14 does not contain this potential element that may be activated by pMad. Activation by pMad could be required in the BP1 element to extend expression, generate the graded *brk* pattern, and allow subsequent rescue.

To test this possibility, B14 was combined with the MP region (35 basepairs in length) and placed into the pHSB vector (creating B14MPB); this combination of response elements plus the *hsp70* promoter (with *brk* binding sites mutated) drove GFP reporter gene expression (Fig. 3-12). B14MPB was able to drive reporter gene expression in a pattern identical to *brk* (Fig. 3-12). This result supported the notion that the MP response element contains information necessary for proper *brk* expression; in addition, this supported the hypothesis that the expression and rescue provided by B14BP1P and B14BP1brkW, respectively, is not due to their usage of the endogenous *brk* promoter as B14MPB does not use the endogenous promoter yet retains the ability to drive perfect *brk*-like expression. It should also be noted that the levels of reporter gene expression driven by B14MPB were much higher than those driven by B14BP1P. Therefore, lower levels of reporter gene expression are not necessary to allow B14BP1P to drive *brk*-like expression as B14MPB drives high levels of expression like endogenous *brk*.

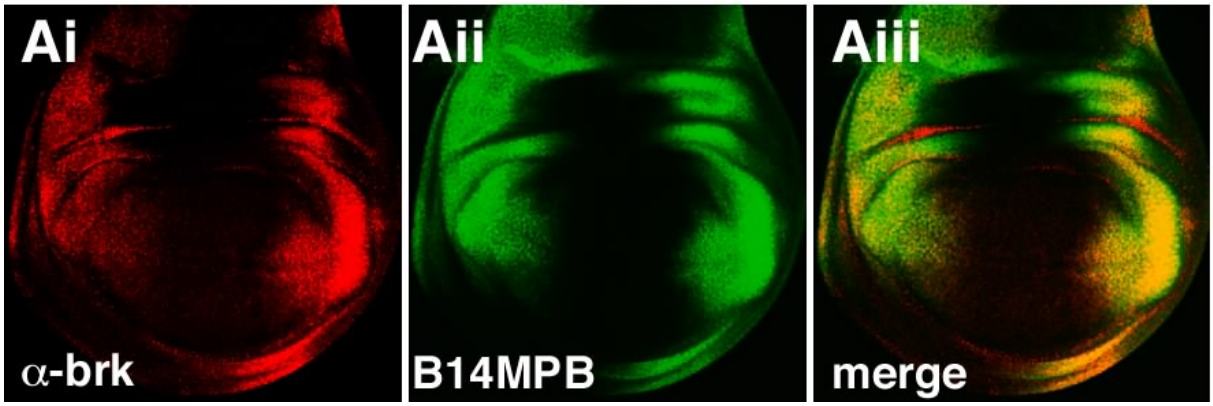

Figure 3-12: Reporter gene expression driven by B14MPB is identical to *brk*.
(Ai) *brk* expression in B14MPB animals (anti-brk). (Aii) GFP reporter gene expression from the B14MPB transgene (anti-GFP). (Aiii) merge of (Ai) and (Aii).

3.7.4 B14MP70brkW rescues a brk^{XA} mutant

Because B14MPB expression looks exactly like endogenous *brk* expression, we tested whether the MP response element is the only fragment necessary in combination with B14 to rescue the brk^{XA} mutant; I created B14MP70brkW that utilizes the two response elements and an *hsp70* promoter to drive the *brk* cDNA and tested for its ability to rescue the brk^{XA} mutation (Fig. 3-13).

The B14MP70brkW construct, upon comparison to B1470brkW (which fails to rescue brk^{XA}), only differs in that it contains the 35 basepair MP response element. This small region allowed rescue of the brk^{XA} mutant by B14MP70brkW (Fig. 3-13). The rescue also confirmed that the type of promoter utilized (*hsp70* or endogenous/DPE style) is not the determinant that prompts rescue of mutants—it is the sequence of the response elements that creates the *brk* expression gradient and subsequent rescue.

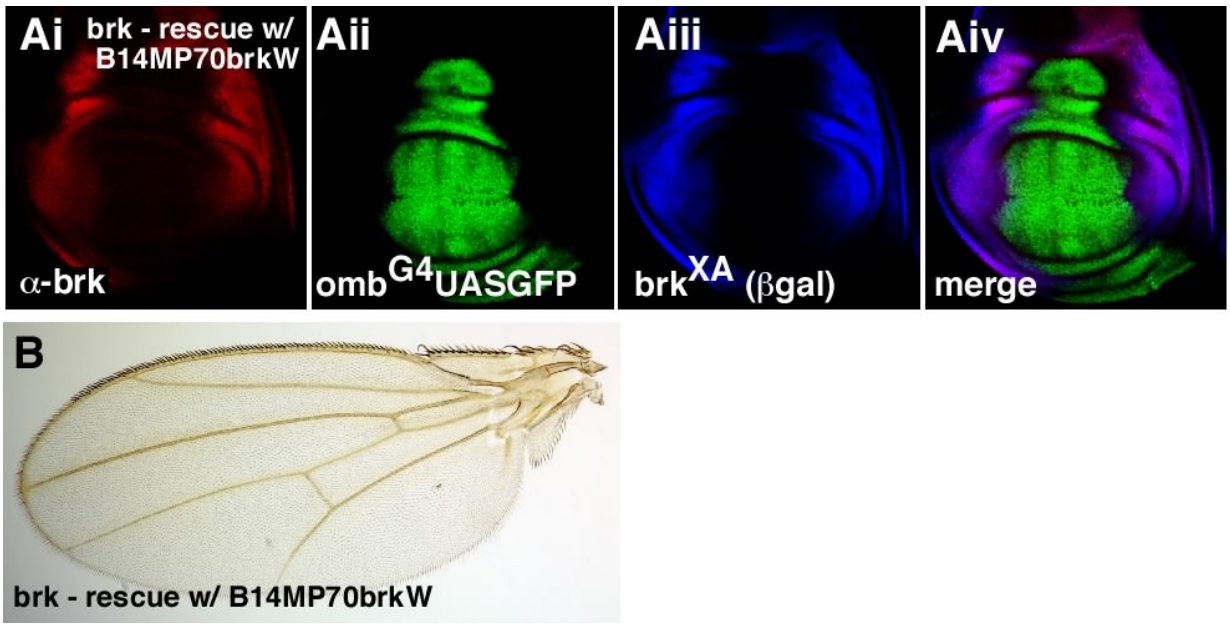

Figure 3-13: B14MP70brkW rescues *brk^{XA}*.
(A) Third instar imaginal discs from *brk^{XA}* mutant rescued with the B14MP70brkW transgene. Anti-brk staining demonstrates Brk in the correct expression gradient and *omb* expression is correctly placed while the *brk^{XA}-lacZ* reporter demonstrates a wildtype expression pattern. (B) Adult *brk^{XA}* mutant wing rescued with the B14MP70brkW transgene is of the proper size and patterning as wildtype wings.

3.7.5 The Mad binding sites in MP are activating sites

Because the B14BP1brkW and B14MP70brKW constructs contain additional basepairs of sequence between their B14 response element and promoter, two possible explanations exist for their *brk* mutant rescue while B1470brkW fails to rescue. (1) The addition of the MP element between B14 and the promoter adds additional spacing necessary between the two elements for rescue, or (2) the MP element contains additional sequence information necessary for rescue. The first possibility is not true: B1470brkW contains 64 basepairs of DNA between its B14 and *hsp70* promoter while B14MP70brkW contains 68 basepairs, including its MP element, between its B14 and *hsp70* promoter elements. This indicates that the addition of spacing between the

B14 and promoter elements does not cause B14BP1brkW and B14MP70brkW to rescue a *brk* mutant; the rescue is due to the sequences contained within the MP element.

The addition of the 35 basepair MP response element is the factor that permits B14MP70brkW to rescue a *brk* mutant, presumably due to activation of the construct by pMad at MP. To confirm that these sites are really activating Mad sites, I tested the sites in a reporter that was already known to contain activating Mad sites, the Vestigal quadrant enhancer (VgQ) (Fig. 3-14) (Certel et al., 2000; Kim et al., 1997). This enhancer was previously shown to contain two activating Mad sites that are essential for it to drive reporter gene expression in the wing pouch. The VgQ enhancer in our modified pHSB vector similarly drives GFP expression in the wing pouch; this expression is dependent upon the presence of the Mad sites because when they are removed in VgQnomadB, reporter gene expression is lost, indicating that expression of GFP is dependent upon pMad both binding and activating the construct (Fig. 3-14).

To test the Mad binding sites contained within MP for their ability to recruit pMad and activate expression, the binding sites were cloned into the VgQ enhancer in place of its own activating Mad binding sites. The resulting construct, VgQMPB, drove reporter gene expression in the central region of the wing disc like VgQB (Fig. 3-14). This expression was determined to be dependent on the presence of the activating Mad sites, as demonstrated by the lack of reporter gene expression from the construct VgQnomadB. This result indicates that the Mad binding sites in MP recruit activating pMad.

To further confirm that the reporter gene expression seen in VgQMPB is dependent on pMad, pMad null (Mad^{1-2}) clones were created and reporter gene expression from VgQMPB analyzed (Fig. 3-14). In clones lacking pMad, reporter gene expression from VgQMPB was lost, indicating that its expression is dependent upon pMad binding to the Mad binding sites contained

in MP to activate expression of the construct and, thus, its reporter. This result confirms the notion that activating pMad binds to the MP element and also supports the hypothesis that pMad binds to the MP element to activate *brk* expression (while pMad also represses *brk* expression through its actions at the silencer as a member of the MMS complex, Chapter 2).

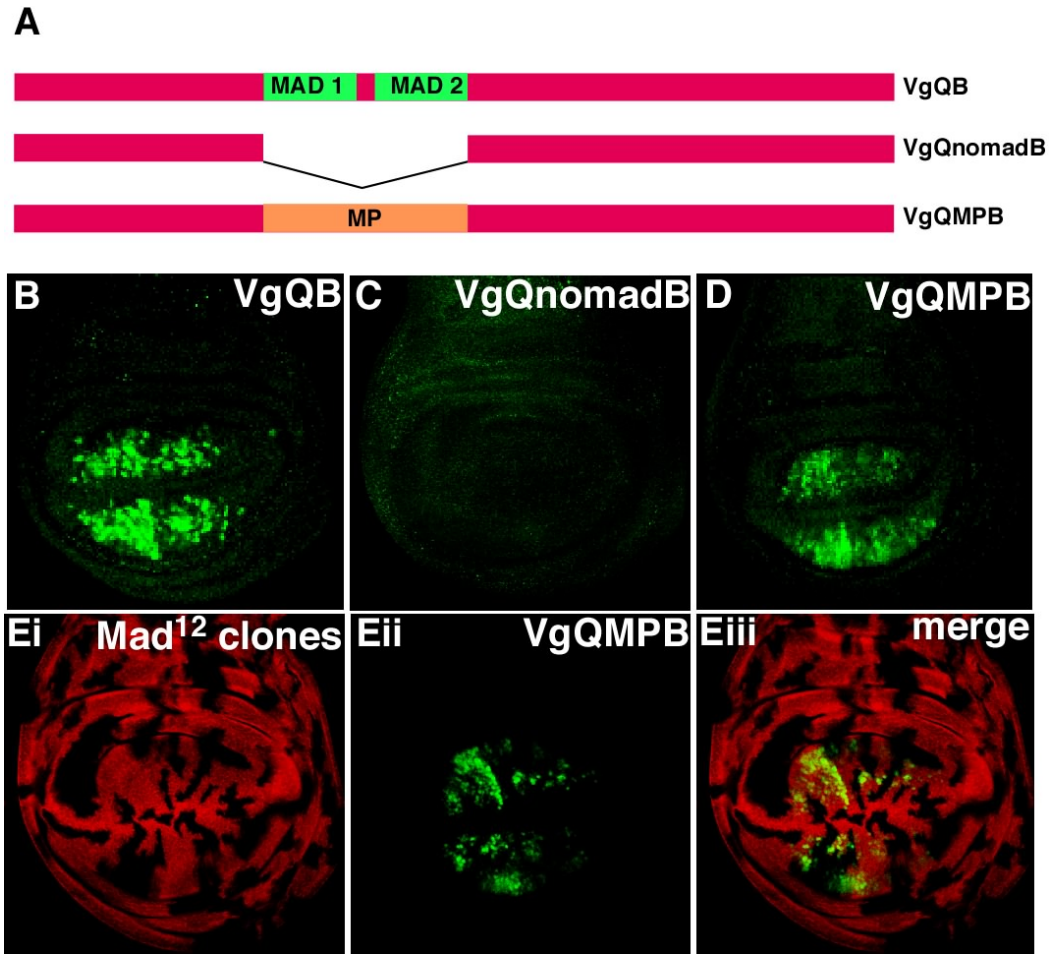

Figure 3-14: The Mad binding sites in MP function as activating Mad binding sites.
(A) Schematic of the constructs created. (B) VgQB expression is found in the central portion of the wing disc. (C) Removal of the activating Mad binding sites from VgQB creates VgQnomadB and results in a loss or reporter gene expression. (D) Addition of the MP cluster of Mad binding sites to VgQnomadB restores reporter gene expression. (E) Reporter gene expression in VgQMPB is lost in Mad^{12} null mutant clones (marked by a loss of ß-gal (red) expression). (Ei) Mad clones are marked by a loss of ßgal staining (red). (Eii) VgQMPB expression (anti-GFP, green) is absent from Mad mutant clones. (Eiii) merge of (Ei) and (Eii).

3.8 THE MP RESPONSE ELEMENT HAS A REDUNDANT ELEMENT NEAR THE BRK ENDOGENOUS PROMOTER

3.8.1 Other sequences in BP1 rescue a *brk*^{XA} mutant

If the activating Mad sites contained within the MP element are the portion of BP1 that is necessary upon combination with B14 to allow rescue, then removal of these sites should return GFP reporter gene expression to that demonstrated by B14B and should cause a loss of rescue. Alternatively, redundant elements (to MP) may exist within BP1 that would still allow for proper reporter gene expression and subsequent rescue. To demonstrate the necessity of activation by pMad at the MP response element (in combination with B14 for rescue), a GFP reporter construct, B14BP1MP, as well as a rescue construct, B14BP1MbrkW, were designed (Fig. 3-15). These constructs contain B14 and the BP1 endogenous promoter/response element with the cluster of activating Mad sites contained within the MP response element removed.

When reporter gene expression from B14BP1MP was examined, it was determined to look identical to anti-brk staining (Fig. 3-15). Additionally, B14BP1MbrkW was able to rescue a *brk*^{XA} mutant to adulthood where its wings were perfectly sized and formed. These results suggest that other redundant elements are contained in BP1 that would also allow for proper reporter gene expression and rescue if tested individually.

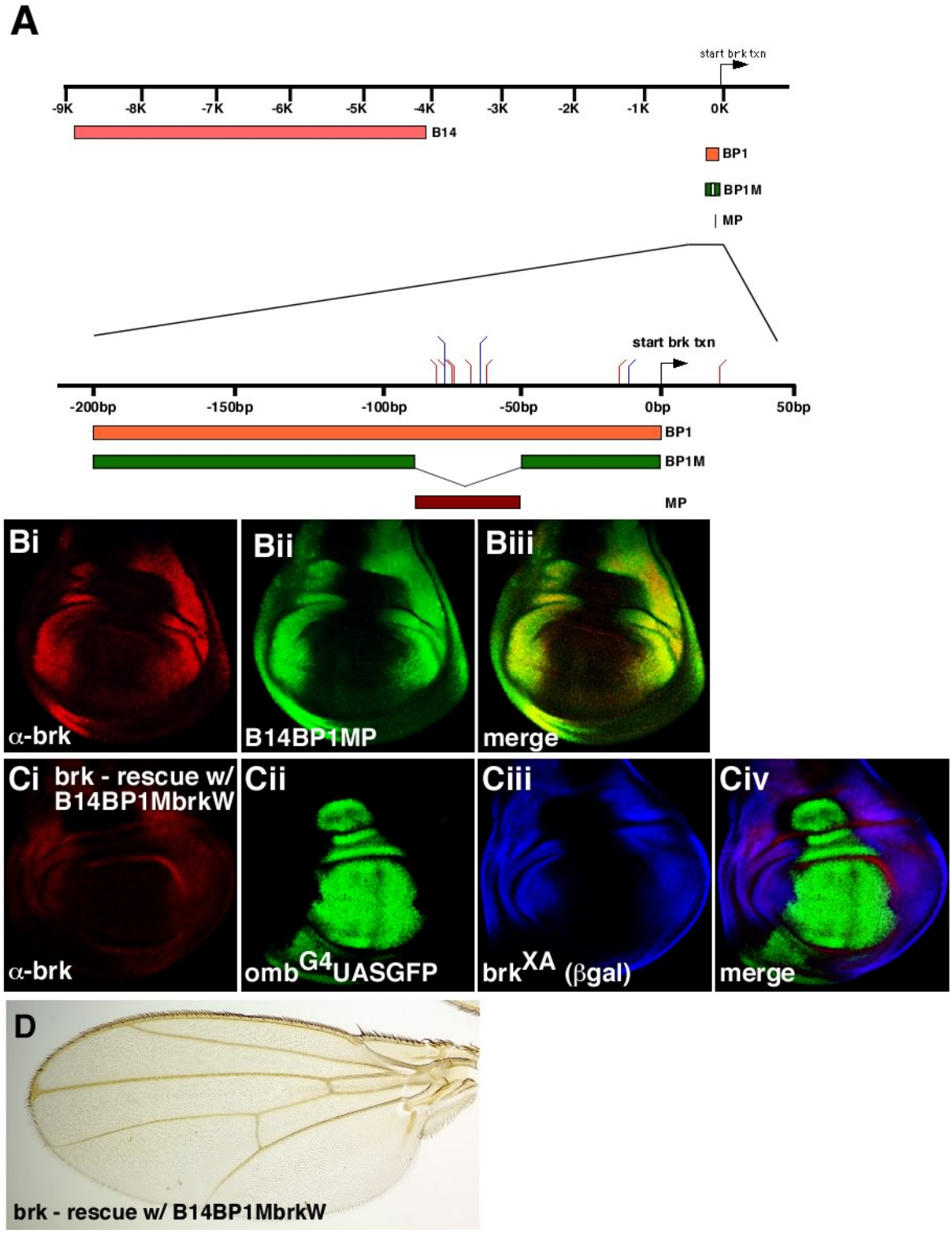

Figure 3-15: BP1 without MP still rescues *brk*^{XA} lethality.
(A) Schematic of the genomic region included in the constructs. The red lines represent putative Mad binding sites while the blue lines represent putative Brk binding sites. (B) Reporter gene expression from B14BP1MP looks identical to anti-brk. (C) B14BP1MbrkW allows rescue of the *brk*^{XA} mutation. Anti-brk staining looks normal, as does that of *omb*, while *brk*^{XA} expression looks wildtype. (D) A *brk*^{XA} mutant adult wing rescued with B14BP1MbrkW is correctly sized and patterned when compared to wildtype.

3.8.2 The BPB element can substitute for the MP element and allow rescue of a *brk*^{XA} mutant

To examine the possibility that other elements redundant to MP exist within BP1, the BP1 response element was first divided into two portions, BPA and BPB. The BPA element contains 83 basepairs of the BP1 element beginning at the MP element and extending to the end of BP1. The BPB element begins just after the MP element and extends for 50 basepairs to the end of the BP1 element (Fig. 3-16). The BPA element was designed to test the removal of the first 109 basepairs of BP1. The activating Mad binding sites contained within the BPA element include those located within the MP element, which was previously been tested on its own, plus one additional site (that is not included in MP). This one additional activating Mad binding site is the only one included within BPB; the B14BPBbrkW construct thus tests the ability of this one activating Mad binding site to combine with B14 and rescue a *brk* mutant. Because Mad binding sites are difficult to predict, it is possible that the BPB element contains more than one site.

The B14BPAbrkW construct rescued a *brk* mutant, as predicted (Fig. 3-16). The B14BPBbrkW construct also rescued a *brk* mutant (Fig. 3-16). These results suggest that the BPB element and the MP element are redundant in their abilities to recruit activating pMad to the *brk* response element region. Additional redundant response elements to MP and BPB may exist that were not identified in this study.

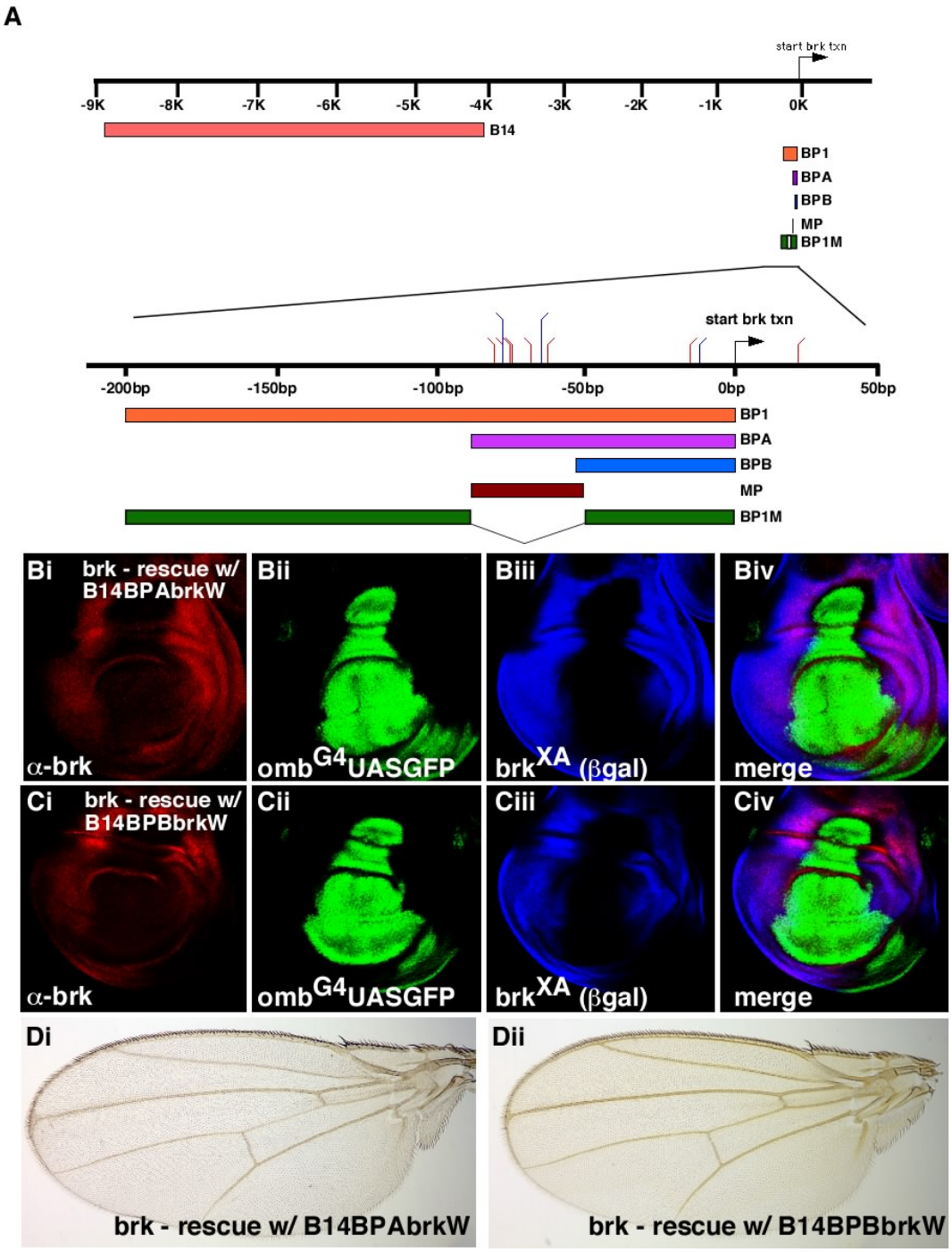

Figure 3-16: B14BPAbrkW and B14BPBbrkW rescue a *brk^{XA}* mutant.
(A) Schematic of the genomic region included in the constructs. The red lines represent putative Mad binding sites while the blue lines represent putative Brk binding sites. (B) B14BPAbrkW rescues the *brk^{XA}* mutation. Anti-brk staining looks normal as does that of *omb* while *brk^{XA}* expression looks like wildtype. (C) B14BPBbrkW rescues the *brk^{XA}* mutation. Anti-brk staining looks normal as does that of *omb* while *brk^{XA}* expression looks like wildtype. (Di) A *brk^{XA}* mutant adult wing rescued with B14BPAbrkW is correctly sized and patterned when compared to wildtype. (Dii) A *brk^{XA}* mutant adult wing rescued with B14BPBbrkW is correctly sized and patterned when compared to wildtype.

3.8.3 B14BPB70brkW can rescue a *brk*^{XA} mutant

To test the hypothesis that the BPB element and the MP element are redundant in their abilities to recruit activating pMad to the *brk* response element region and activate transcription, B14BPB70brkW was created (Fig. 3-17); this construct, much like the B14MP70brkW construct, was designed to test the ability of B14, the BPB response element (which contains one activating Mad binding site), and the *hsp70* promoter (with putative Brk binding sites mutated) to drive the *brk* cDNA and rescue a *brk* mutant. If rescue occured, then the result would lead to the conclusion that BPB is a redundant element to MP. It would also demonstrate that the *brk* rescue phenotype exhibited by B14BPB70brkW is due to its response element composition and not to the use of the endogenous *brk* promoter.

The B14BPB70brkW construct rescued the *brk*^{XA} mutation to adulthood, where normally sized and patterned wings developed compared to wildtype (Fig. 3-17). This result supported the notion that the BPB element is redundant to the MP element in its ability to recruit activating pMad to the *brk* response element region. This also demonstrated that rescue of the *brk* mutant by B14BPBbrkW is due to its response elements, not its use of the endogenous *brk* promoter as B14BPB70brkW rescues a *brk* mutant utilizing a non-endogenous (*hsp70*) promoter.

While my thesis focused my efforts on the ability of pMad to bind and activate at the MP response element, I performed one additional experiment on the BPB response element to test the ability of this region to recruit activating pMad and drive reporter gene expression in the VgQ enhancer study. To this end, VgQBPBB was created; it contains the one putative activating Mad binding site contained within BPB in place of the activating Mad binding sites originally found in VgQB. Upon assaying for reporter gene expression, none was detected (Fig. 3-17). This was unexpected, but it is possible that one activating Mad binding site, while sufficient to

rescue the *brk*^{XA} mutant, is not sufficient in the VgQ enhancer experiment. The VgQB construct has two activating Mad binding sites (see Fig. 3-14), and it is possible that the one site contained within BPB is not sufficient to drive reporter gene expression in this assay; more than one may be necessary.

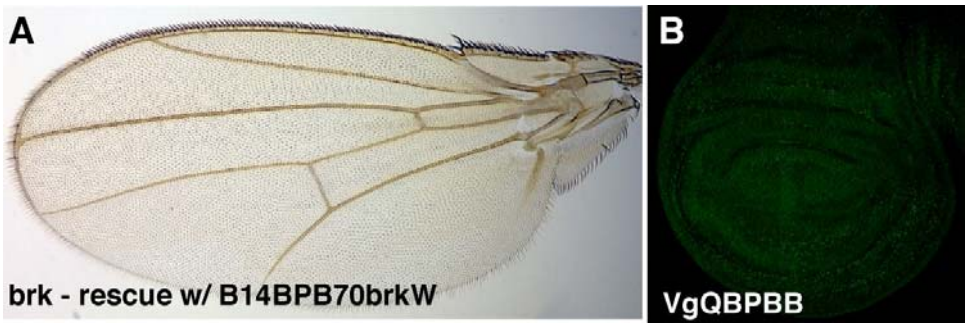

Figure 3-17: B14BPB70brkW rescues *brk*^{XA} but VgQBPBB demonstrates no reporter gene expression.
(A) *brk*^{XA} mutant rescued with B14BPB70brkW transgene was correctly sized and patterned. (B) VgQBPBB drove no expression in the wing disc (photographed at same levels as Fig. 3-14 B-D) (anti-GFP).

3.9 DISCUSSION

3.9.1 Reporter constructs and recapitulating the expression patterns of endogenous genes

The *brk* gene is expressed in lateral to medial gradients along the A/P axis of the wing imaginal disc. Although previous experiments demonstrated that this pattern of expression is established by Dpp signaling repressing *brk* expression through a Mad/Med/Shn complex, the experiments described in Chapter 2 clearly demonstrated that Brk negative autoregulation is essential to establish the normal pattern of *brk* expression. The experiments in this chapter have addressed

whether these inputs are sufficient to establish the normal pattern of *brk* expression and have demonstrated that they are not; an additional input necessary for proper *brk* expression is activation of *brk* by pMad/Med.

The experiments conducted in this chapter stemmed from our initial observations of the pattern of reporter gene expression driven by the B14 response element, an element that was originally stated to drive expression in a pattern identical to that of endogenous *brk*. However, as shown above, this is incorrect—although B14-driven expression superficially resembles that of endogenous *brk*, upon closer inspection, expression does not extend as far medially as that of Brk protein. This is a common failing of many studies using reporter constructs as researchers often overlook slight differences between reporter gene expression and that of the endogenous gene, probably surmising that a minor element is missing from the regulatory sequences in the reporter constructs they are using, but that it is not important because their study has revealed an important element necessary for the control of the expression of a particular gene. This is the case for the previous *brk* study in which the key element under study was the silencer element to which pMad/Med/Shn bind.

However, in regard to *brk*, it is essential to understand how the exact pattern of expression is established because the pattern of gene expression in the wing is dependent upon precise levels of Brk protein: if the gradient profile would be modified in any way, then the development of the wing would be totally disrupted because the pattern of expression of genes such as *sal* and *omb*, which control positioning of structures like wing veins, would be altered. Consequently, the fact that the B14 response element does not drive reporter gene expression in a pattern identical to that of *brk* can not be ignored and, as demonstrated by the experiments in this chapter, the devil is in the details.

3.9.2 B14 and B315 response elements

Upon examination of the reporter gene expression driven by the B14 and B315 response elements in different vectors with different enhancers and reporters, it was determined that the reporter gene used as well as the type of promoter utilized by the vector (or presence of putative *brk* binding sites within that promoter) does not explain why neither response element drives reporter gene expression exactly like *brk.*

B14 and B315 also do not have the same expression patterns as each other when tested with multiple promoters and reporter genes. One possible explanation as to why reporter gene expression driven by the B14 and B315 response elements are different could be that the silencer and enhancer portions of B315 are too close to one another (Small et al., 1992). Additionally, I was able to identify that B14 contains at least two more Dpp-responsive silencer elements in addition to the one (contained in B315) previously described (Pyrowolakis et al., 2004). It is likely that these elements also contribute to the differences in reporter gene expression patterns driven by B14 and B315.

B14 was refuted as the minimal response element necessary to create *brk* expression as it fails, upon combination with an *hsp70* promoter, to drive a *brk* cDNA to rescue a *brk* mutant.

3.9.3 BP1, an additional regulatory element located immediately upstream of the promoter

The failure of B14 to drive reporter gene expression in a precise *brk* pattern and to rescue a *brk* mutant when used to drive Brk suggests that an additional regulatory element is absent from the

B1470brkW construct. This is the BP1 response element. BP1 is necessary, in combination with B14, to drive a *brk* cDNA and rescue a *brk* mutant. This rescue is promoter independent.

3.9.3.1 Additional constructs will confirm that no Brk protein is produced by B1470brkW when it fails to rescue brk^{XA} mutants

The B14B construct, as well as B14 in all response element testing vectors tested (Fig. 3-4), predicted that B1470brkW would drive Brk protein in the lateral regions of the wing disc; the B1470brkW non-rescue discs, however, contain no detectable Brk protein (Fig. 3-7 D). The construct was confirmed to be cloned correctly. Therefore, several possible explanations exist for this observation: (1) The anti-brk antibody is not of a good enough quality to recognize a small amount of Brk protein. (2) The reporter construct responds differently to Brk than the endogenous gene in that B1470brkW construct is more sensitive to autoregulation so that essentially the *brk* in the transgene is keeping its own levels down to undetectable levels. To examine these possibilities, two additional constructs are currently being generated: B1470brkHAW and B1470F124HAW (Fig. 3-6).

B1470brkHAW is exactly the same as B1470brkW except that the *brk* cDNA contains a C-terminal HA tag. The HA tag does not interfere with rescue (B14BP1brkHAW, created by GC, rescues brk^{XA} (personal observation)). The B1470brkHAW construct is expected to not rescue brk^{XA} (as B1470brkW did not) and will be examined for staining with an anti-HA antibody in order to rule out the possibility that low levels of Brk protein are driven by B1470brkW that are just undetectable by the anti-brk antibodies, although this would be unexpected and it is predicted that HA expression will also be undetected.

B1470F124HAW will contain the brk^{F124} point mutation in the recognition helix of the DNA binding domain previously identified from an EMS screen to produce Brk with little or no

activity as *brk*^{F124} mutant clones in wing discs are indistinguishable from null allele clones (Winter and Campbell, 2004). The B1470F124HAW construct is also expected not to rescue as it will produce a protein with little or no activity, but one that can be detected with an anti-HA antibody as well as the anti-brk antibody. The ability of this protein to autoregulate will be examined by staining *brk*^{XA} non-rescue discs with anti-brk and anti-HA. If the B1470F124HAW construct is more sensitive to autoregulation (as predicted from the B1470brkW construct), then no protein expression is expected in *brk*^{XA}/+ heterozygous animals as the endogenous Brk protein is expected to repress the transgene. In the *brk*^{XA} mutant, expression is expected from the B1470F124HAW construct as no endogenous Brk protein will be present to autoregulate and, if our hypothesis is correct, it will be expected that staining with anti-brk and anti-HA will demonstrate expression in only the lateral regions of the wing disc.

3.9.4 pMad binds sequences in MP and positively regulates expression

The BP1 response element contains putative activating Mad binding sites. The cluster of putative activating Mad binding sites contained within BP1, known as the MP response element, binds pMad protein *in vitro* and possesses the ability to be combined with B14, an *hsp70* promoter, and the *brk* cDNA to rescue a *brk* mutant (B14MP70brkW) (Fig. 3-13). Furthermore, the sites are activating Mad sites. Similarly, it was previously demonstrated that the Mad binding sites within the VgQ enhancer were also activating. To test the function of MP, the MP response element was substituted in place of the activating Mad binding sites within the VgQ enhancer and it was demonstrated that the activating Mad binding sites contained within the MP response element could drive reporter gene expression that is dependent on pMad and could also recapitulate VgQB expression. This result confirmed that activation by pMad, in addition to the repression

by pMad at the silencer element as previously noted and discussed in Chapter 2, are necessary to generate the correct *brk* expression pattern/gradient.

3.9.5 The BPB element also appears to positively regulate expression

The MP response element can be removed from BP1 without disrupting rescue; this result identified a response element, BPB, which acts redundantly to MP and can also be combined with B14, an *hsp70* promoter, and the *brk* cDNA (B14BPB70brkW) to rescue a *brk* mutant. While BPB, and presumably its activating Mad binding site, retains the ability to rescue a *brk* mutant, BPB is not able to drive reporter gene expression in the VgQ enhancer. This may be due to the fact that BPB contains one activating Mad binding site. This one site may be sufficient in the context of *brk* mutant rescue but may not be sufficient to drive reporter gene expression in the VgQ enhancer experiment as the VgQB construct reporter gene expression was activated by two Mad binding sites. Further experiments to assess this hypothesis would include placing multiple BPB response elements in the VgQ enhancer and assessing their ability to activate reporter gene expression, removing one of the two activating Mad binding sites from the VgQ enhancer and assessing its ability to active expression when only one site is present, and EMSA experiments to determine if pMad binds to BPB *in vitro* as it does to MP.

Additionally, a synthetic construct could be created where the activating Mad binding sites contained within the VgQ enhancer are added to the B1470brkW construct. If this new construct allows rescue of a *brk* mutant, it would confirm that the rescue demonstrated by B14BP1brkW, B14MPbrkW, B14BPAbrkW, and B14BPBbrkW is due to activation by pMad.

Activating Mad binding sites are difficult to identify; to test if the one Mad binding site contained within BPB is the factor that permits rescue of a *brk* mutant, a construct could be

generated where that Mad binding site is removed from BPB and the ability of the construct to rescue a *brk* mutant evaluated. While the construct is predicted to not rescue, if it would, this result would suggest that other cryptic activating Mad binding sites are contained within BPB.

The one activating Mad binding site contained within BPB is located very close to the *brk* transcriptional start site raising the possibility that it may not be functional *in vivo* due to the masking of the DNA sequences necessary for the recruitment of basal transcription factors or due to the masking of its binding site by the binding of the basal transcriptional machinery. TFIID is the factor that binds to the Inr and DPE elements of DPE-driven promoters like *brk*; DNase I footprinting analyses have been conducted and the region of DNA contacted by TFIID is not predicted to mask the single activating Mad binding site contained within the BPB response element, indicating that it could still be functional (Smale and Kadonaga, 2003). Additionally, the B14BPBbrkW construct utilizes only this one activating Mad binding site within its endogenous promoter to rescue a *brk* mutant, indicating that the Mad binding site must be active while still not inhibiting the binding of the basal transcriptional machinery. The identification of the BPB element may actually suggest that MP and BPB direct short-range activation that may be required for transcription of *brk*.

3.9.6 Redundancy in response elements

The redundancy in response elements (e.g. MP and BPB) possessed by *brk* is not unusual—*brk* itself contains multiple Dpp-responsive silencer elements; most likely they are utilized either combinatorially or to direct repression of expression at various times/places throughout development (Pyrowolakis et al., 2004). Multiple Brk binding sites are present within the *zen* promoter and function in a redundant manner (Rushlow et al., 2001). Recent data from the

Rahul Warrior lab suggests that *brk* contains multiple "B14-like" response elements, each containing a Dpp-responsive silencer element, which can all individually drive reporter gene expression in a pattern similar to *brk* (Yao et al., 2008). Additionally, *kruppel* expression in the central domain of the embryo is directed by two adjacent response elements that each can function independently to drive the correct pattern of *kruppel* expression (Hoch et al., 1990).

It is redundancy that appears to allow brk^{XA}-*lacZ* expression to be identical to *brk* expression in a wildtype/ heterozygote situation—while BPB (with its one activating Mad binding site) is not influencing brk^{XA} expression (as BPB is downstream of the enhancer trap insertion site and, therefore, likely not used by the enhancer trap to drive reporter gene expression), the MP element remains upstream of the brk^{XA} insertion site and appears to direct *lacZ* expression mediolaterally.

3.9.7 Counteracting the repressive forces

The results of my experiments in this chapter have provided possibly the first example of both activation and repression of a gene (*brk*) by the same transcription factor (pMad) in the same cell via different regulatory elements. The activation of the BP1 response element by pMad is necessary to activate *brk* transcription in its graded mediolateral region where Brk also negatively autoregulates (as discussed in Chapter 2). Without activation by pMad, *brk* expression is repressed to the lateral regions of the disc and has a very steep gradient due to repression by pMad and the MMS complex. Activation by pMad is required at the BP1 element near the endogenous *brk* promoter to extend the steep *brk* expression profile into a more shallow, graded expression.

3.9.8 Why both activation and repression by pMad?

Why are both activation and repression of *brk* expression by pMad necessary? There are at least two possible reasons: (1) Both activation and repression by pMad would be useful to act as a mechanism to stabilize the response of *brk* to Dpp and buffer against random fluctuations in pMad levels. (2) pMad both activating and repressing *brk* could (and appears to) serve to modify the *brk* expression gradient. With only repression of *brk* by pMad, the *brk* gradient is steep and confined more laterally (as demonstrated by reporter gene expression in B14B). pMad activation (in addition to repression) serves to make the *brk* expression gradient more shallow and extended further medially. A shallow gradient is more useful for *brk* in that it allows for the generation of more separation of thresholds of responses spatially across the A/P axis; a steep expression gradient would not allow for many thresholds. This spatial separation of thresholds of responses allows targets like *sal* and *omb* to respond more specifically to the amount of Brk present (Fig. 3-18).

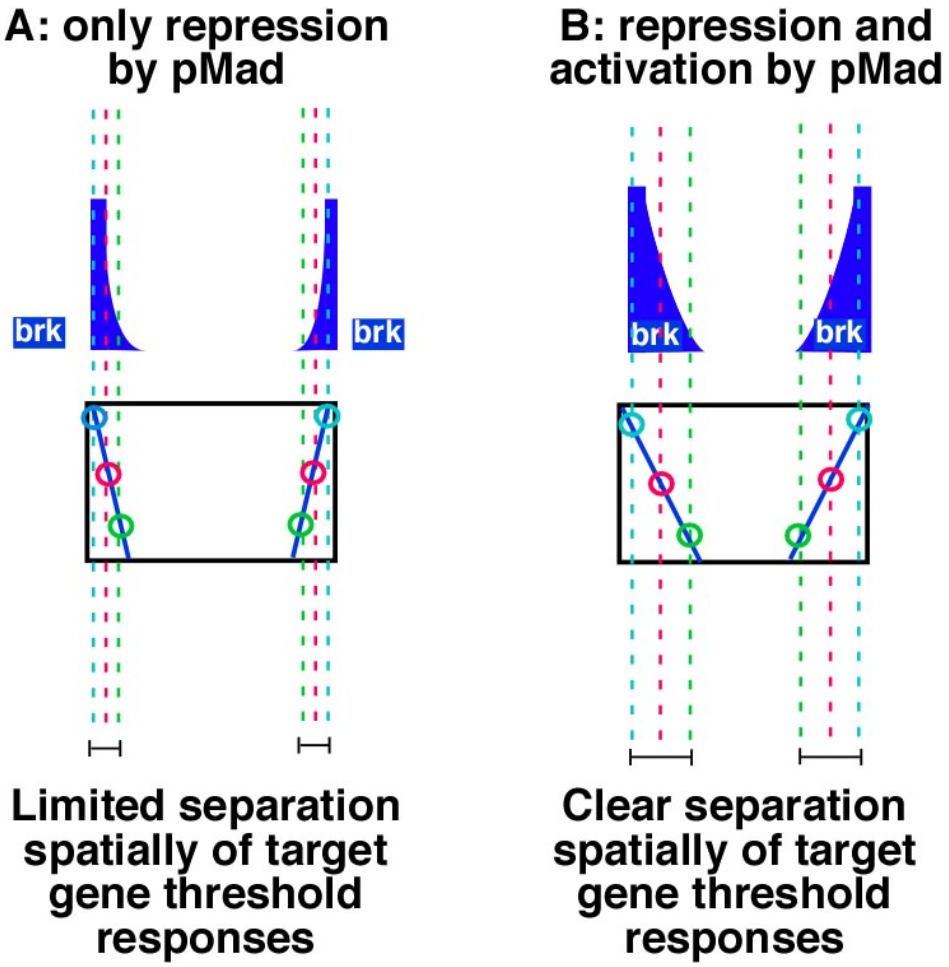

Figure 3-18: Both repression and activation by pMad allow many thresholds of target gene responses.
(A) When only repression by pMad contributes to the *brk* expression profile, the *brk* gradient is steep and confined to lateral regions. Only a limited spatial separation of threshold responses to Brk concentration can be sensed by *brk* target genes. (B) When repression and activation by pMad contribute to the *brk* expression profile, the *brk* gradient is shallower and extends further medially. A shallow gradient allows for a clear spatial separation of threshold of responses sensed by *brk* target genes.

4.0 CONCLUSIONS AND FUTURE DIRECTIONS

The goal of the research presented here was to understand how a *brk* expression gradient is established from a complementary gradient of BMP signaling. Here, I have demonstrated that (1) Brk does not repress through binding standard sites and that it acts at the *brk* S element through interactions with members of the MMS complex to autoregulate, and (2) correct *brk* gradient profile and position requires not only sequences found in B14 but also a region near the endogenous promoter where pMad binds and activates expression. This rescue adds support to the notion that the *brk* expression gradient must be properly placed to allow *brk* to influence spatial patterns of gene expression necessary for correct patterning.

4.1 CAN A UNIFORM ACTIVATOR AND A GRADIENT OF REPRESSOR GENERATE A GRADED REPRESSIVE OUTPUT?

Prior to my studies, it was thought that the *brk* repression gradient is the output established from two inputs: a uniformly expressed activator (the identity of which is still unknown) and a gradient of a repressor (pMad as a member of the MMS complex). In theory, it may be thought that a proportional transcriptional response from a graded input (pMad repression gradient) would be generated quite easily (Fig. 4-1 A); however, very few examples exist in the literature of an input repression gradient resulting in an inverse expression gradient (most examples of

input repression gradients generate threshold output responses) indicating that utilizing a repression gradient to generate an inverse expression gradient may be quite difficult or has not yet been explored in great detail due to its high degree of complexity.

The reporter gene expression pattern of the brk^{XA} mutant is an example of an expression pattern generated by only two inputs: a pMad/MMS complex repression gradient as well as ubiquitous activation by the activator (Fig. 4-1 B). This mutant exhibits a threshold output of expression, not a graded expression output; there is not a proportional response of *brk* expression to the graded MMS input, rather, *brk* expression switches from completely off to completely on at a specific level of pMad. This supports the hypothesis that it may be difficult to generate an inverse output expression gradient output from an input repression gradient. Additionally, many gap genes important for early *Drosophila* embryonic development are repression gradients; however, their outputs are threshold responses, not graded responses. This observation again supports the hypothesis that activation by the activator and graded repression by pMad/MMS complex are unable to generate a graded *brk* output on their own as no extended region of expression levels that can be sensed by the two inputs exists; the decision of whether or not *brk* is expressed is a simple threshold response: on or off.

I would like to propose that for the *brk* gene (and probably for other genes as well), if only a single activator input and a single repressor input exits, it is difficult to establish a situation where there are intermediate levels of output expression; expression is either off or it is on at full levels. In the case of *brk* expression, I believe that Brk negative autoregulation serves several purposes, as discussed in the next section, but the most important purpose of Brk negative autoregulation is that it is required to generate a graded repression output through its

interactions with pMad thereby modulating the activity of the MMS complex (Fig. 4-1 C). This permits the generation of intermediate levels of expression, as will be discussed in detail below.

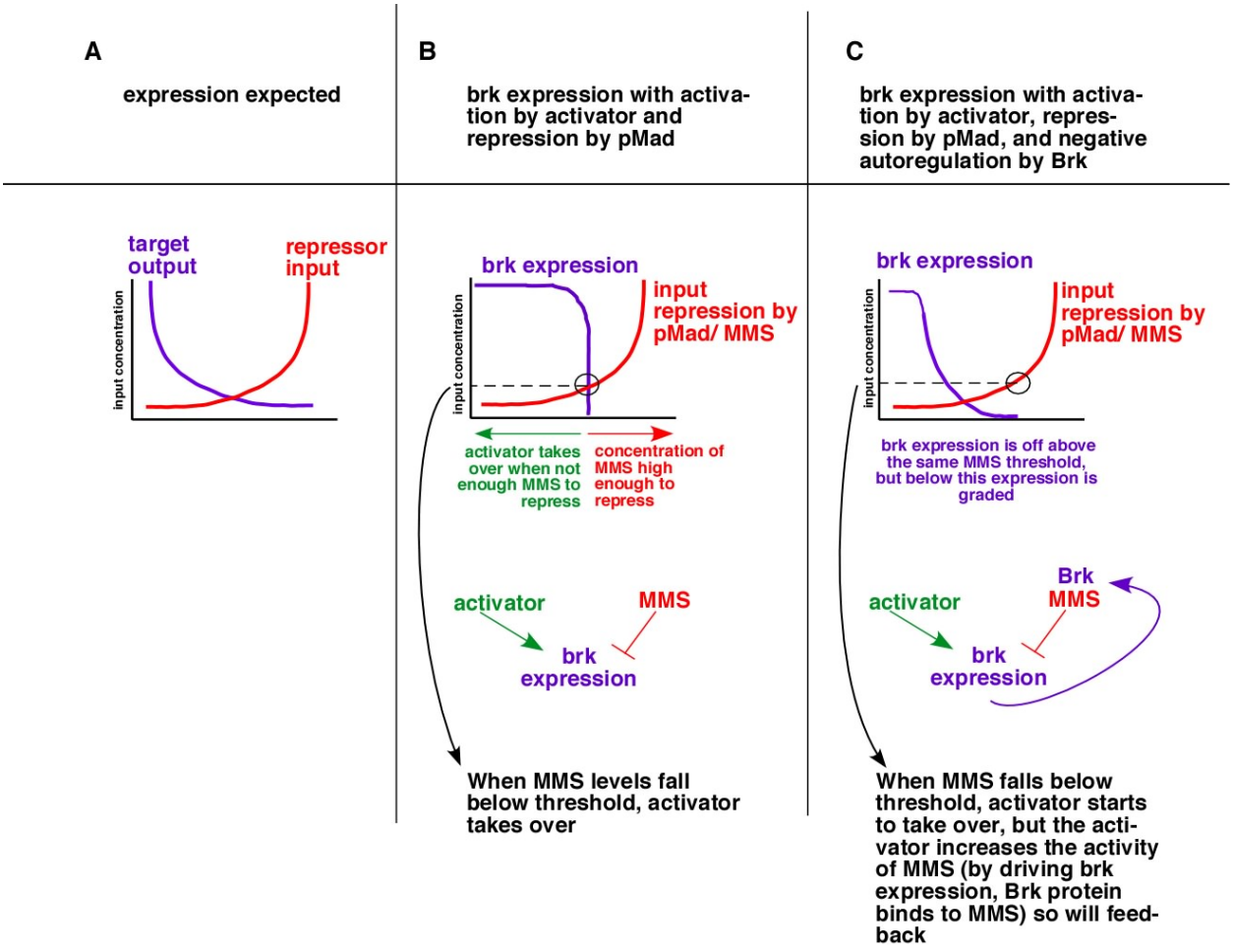

Figure 4-1: Model for the establishment of graded *brk* expression.
(A) Predicted expression model resulting from ubiquitously expressed activator and graded repressor inputs. (B) *brk* expression resulting from ubiquitously expressed activator and graded repressor inputs is not graded, rather a threshold response is achieved; *brk* expression is either on or off. (C) Brk negative autoregulation as a third input allows graded *brk* output expression due to the establishment of a feedback loop.

4.2 WHY DOES BRK NEGATIVELY AUTOREGULATE?

There are at least three reasons why *brk* negatively autoregulates, as discussed in Moser and Campbell, 2005: first, to stabilize the *brk* expression gradient, second, to increase the sensitivity of cells to Dpp, and third, to ensure that the response to pMad is graded and not all-or-none.

First, Brk negative autoregulation may act as a feedback mechanism to ensure that the *brk* expression gradient is stable. This would buffer against random fluctuations in *brk* levels and, importantly, against gross changes in levels that would be found, for example, in *brk* mutant heterozygotes compared to wildtype animals.

Second, as already noted, pMad levels appear to be the same in the presence or absence of Brk, but in the mediolateral region, cells lacking Brk are unable to repress *brk*. Consequently, if *brk* expression is used as a read-out of BMP signaling, these cells now fail to respond to the low levels of Dpp present in this region. When Brk is present, these cells can now repress *brk* and this effectively allows cells to respond to these low levels of Dpp to which they cannot respond in the absence of Brk. Thus, Brk negative autoregulation may act as a novel mechanism to extend the range of Dpp signaling in this tissue.

Third, and most importantly, Brk negative autoregulation appears to be necessary in order to establish graded expression of *brk* from the pMad gradient. It is essential that there is not a simple threshold response of *brk* to pMad, but that the response is continuous from low to high. In the absence of Brk there is a threshold response, i.e. below a certain concentration of pMad there is no effect upon *brk* expression, but slightly higher levels result in complete repression, as demonstrated by the brk^{XA} mutant: *lacZ* expression is either on or off. However, Brk negatively autoregulates by interacting with the MMS complex at the *brk* S. This repression from Brk changes the MMS activity gradient to non-constant, extending/modulating the activity of the

MMS complex and altering the two threshold responses described above into a series of equilibrium responses (to repression by Brk/MMS and activation by the activator) to ultimately create the *brk* expression pattern. It is possible that the activator induces *brk* expression thereby increasing the activity of the MMS complex because more Brk protein can bind to it. Brk protein binding to the MMS complex modulates the activity/effective range of the MMS complex laterally, but the amount of MMS complex is also decreasing laterally as pMad is expressed in a medial to lateral gradient. Therefore, the *brk* expression pattern is an equilibrium response to the amount of each input present at each position in the gradient. There is not a single repressive input of the MMS complex only; the input repression utilizes Brk to contribute to the overall pattern of *brk* output expression.

4.3 THE MECHANISM OF BRK AUTOREGULATION DIFFERS FROM THAT USED FOR TARGET GENE REPRESSION

Brk possesses a DNA-binding domain that it uses to interact directly with the *cis*-regulatory response elements of a number of Dpp target genes to repress their expression (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001; Sivasankaran et al., 2000; Zhang et al., 2001). Target gene repression by Brk requires this DNA-binding domain plus a repression domain/motif (to which it recruits co-repressors) in order for Brk to act as a transcriptional repressor of its target genes such as *sal* and *omb* (Winter and Campbell, 2004).

Brk does not function in its typical transcriptional repressor role when it is autoregulating its own expression. Negative autoregulation by Brk appears to function by Brk not binding to its binding sites on DNA but rather by interacting with pMad and promoting the activity of the

MMS repressive complex. Other examples exist describing transcription factors that bind other complexes of proteins instead of their own binding sites. The Teashirt (Tsh) protein functions as a transcriptional repressor of *UbxB* in the embryonic midgut by acting through a response element in *UbxB* by promoting the activity of another repressive complex, not by binding to the element; in this case, Tsh interacts with Brk, which binds its own binding sites directly on the *UbxB* response element (Saller et al., 2002). It may be important for the function of Brk in autoregulation that it behaves in this previously un-described (for Brk), non-traditional manner—most likely, the two forms of repression exhibited by Brk (binding its binding sites and recruiting co-repressors versus modulating the activity of another repressive complex) are different and the form of repression utilized is specific to the function Brk is performing. Experiments to test this hypothesis will be described below.

4.4 DEFINING THE CORRECT SLOPE AND POSITION OF THE BRK GRADIENT REQUIRES ACTIVATION BY MAD

The studies I have performed indicate that both transcriptional activation and repression by pMad are necessary to generate the *brk* expression profile. pMad activation at the BP1 element is necessary for proper graded *brk* expression. Is it important that the activating Mad binding sites contained within BP1 (like those found in MP and BPB) are near the promoter? The localization of the sites suggests that they act over a short range, possibly to recruit basal transcriptional machinery or to recruit co-factors important for opening the chromatin to allow the transcriptional machinery access to initiate transcription. Although this has not yet been determined, potential experiments to test this hypothesis are described below.

How does pMad both activate and repress the same gene in the same cells at the same time? And why does the repressive power of pMad in the MMS complex win over the activating power of the activator in the medial portions of the disc and not in the mediolateral regions? At least two possible explanations exist: (1) There is a difference in the number or affinity of pMad binding sites between the S and BP1. The repressive sites may be high response targets meaning that they respond to high amounts of pMad while the activating sites contained within BP1 may be low response targets that respond only to low amounts of pMad. Thus, the repressive power of pMad may be stronger than its activating power indicating that when the repressive sites at the S are occupied, they over-come any activation by pMad bound at activating sites. These repressive sites preferentially loose pMad binding when pMad levels begin to decrease mediolaterally such that more pMad is bound at the activating sites in this region of the wing disc. (2) Alternatively, the activating pMad binding sites at BP1 near the endogenous *brk* promoter may not be accessible when pMad is bound at repressive sites at the *brk* S in the medial region of the wing disc. In order for the activating sites at BP1 to be functional, they may need the *brk* activator to initiate transcription mediolaterally for the chromatin to be opened enough for these binding sites to be available for pMad binding. This possibility would help explain why the activating pMad binding sites contained within BP1 are located so close to the *brk* promoter. Regardless of the mechanism of action, this type of response would not be possible if pMad was exerting both transcriptional activation and repression at the same response element.

pMad is not the only example of a transcription factor that can act as both a transcriptional activator and a transcriptional repressor of the same gene: Tcf/Lef and Su(H), as described above, can both activate and repress their target genes (Barolo et al., 2002; Brantjes et al., 2001). However, this activation and repression differs dramatically from that which I am

describing for the actions of pMad on Brk. Tcf/Lef and Su(H) activate and repress transcription through interactions at the same response element of their target genes. The decision to activate or repress is controlled by the partners with which these transcription factors bind at the response element. Conversely, pMad functions to activate and repress the transcription of Brk at the same time by binding to two different response elements. This may be key to understanding how graded *brk* expression is achieved: if pMad were bound to the same response element, transcriptional activation may never occur because it appears that pMad is much more active as a transcriptional repressor. The ability of pMad to activate and repress at separate sequence elements within the same cell to control the same gene appears to allow both transcriptional activities (activation and repression) to be present at the same time in order to regulate the expression of *brk*.

4.5 MUTLIPLE RESPONSE ELEMENTS ARE NECESSARY TO CREATE THE BRK EXPRESSION PATTERN

I have demonstrated that repressive and activating forces at multiple response elements are necessary for proper *brk* expression. Without all the inputs, *brk* expression is not graded and is not located in the correct position. It is not unusual that the *brk* response element would be derived of so many separate response element units; genes important in development often contain response elements with separate specificity (S portion of B14, MP, BPB) and signal amplification (enhancer portion of B14) portions (Arnosti, 2003). Alternatively, response elements like MP and BPB may also be serving in certain contexts for signal amplification; they

appear to be required for *brk* expression to be activated in mediolateral regions of the wing disc as demonstrated by the studies of reporter and rescue constructs with and without BP1.

It is predicted that each separate response element of *brk* can independently affect gene expression. Some of the response element units appear to recruit complexes of proteins (e.g. the silencer) while others appear to function as single or small groups of binding sites to direct function (e.g. MP or BPB). While it appears for *brk* that each response element is independently undergoing interactions with its binding proteins to influence transcription, the total end–product of transcription necessary to generate the complex *brk* pattern of expression during development probably requires the activity of all the response elements functioning combinatorially (Arnosti, 2003; Arnosti and Kulkarni, 2005).

4.6 MODEL OF BRK AUTOREGULATION AND GRADIENT FORMATION IN THE *DROSOPHILA* WING

Based on the research described above, we have generated a model to describe the mechanism by which the *brk* expression gradient is established (Fig. 4-2). Our model proposes that there are four primary inputs necessary for *brk* gradient formation: (1) A yet-unknown activator binds to sequences in the enhancer portion of B14 to drive expression throughout the wing pouch. (2) A MMS repression complex binds to the S, with pMad becoming limiting the further from the Dpp source, repressing the expression of *brk* to lateral regions of the wing disc. With just these two inputs alone, a competition exists between activation by the activator and repression by the MMS complex. (3) Brk negatively autoregulates by interacting with the MMS complex at the *brk* S. (4) Activation by Mad occurs at elements contained within the BP1 response element near the

endogenous *brk* promoter. The combination of all these inputs results in the full recapitulation of *brk* expression.

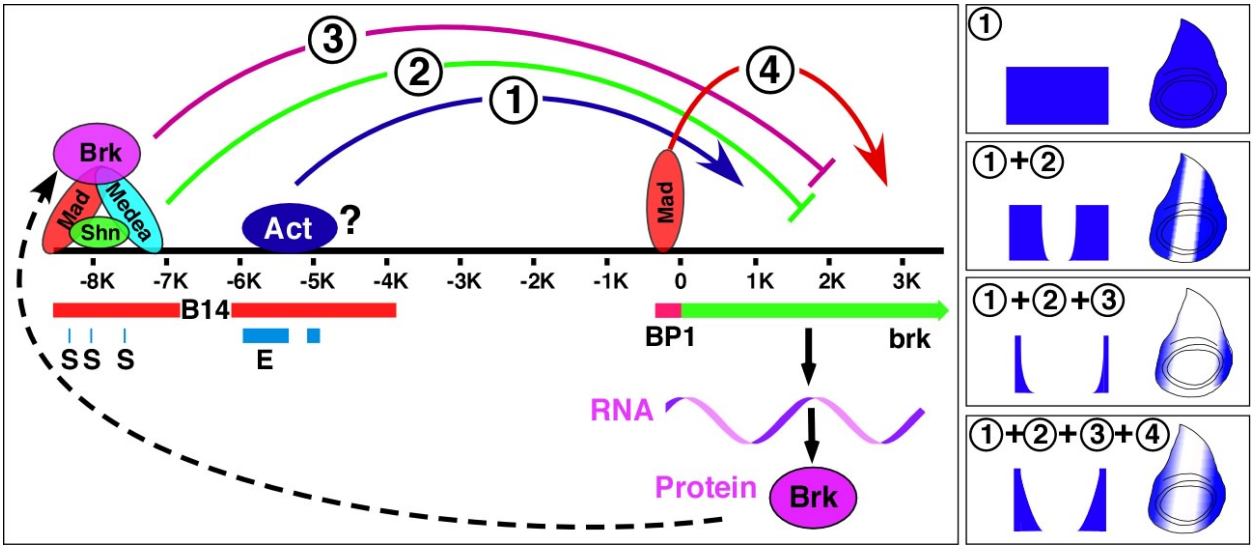

Figure 4-2: Model for the generation of the *brk* expression gradient.
LEFT: The data presented in this thesis supports a model that suggests there are four primary inputs necessary to generate graded *brk* expression: (1) A unidentified activator binds to sequences in the enhancer, (2) A pMad/Med/Shn repression complex binds to the silencer with pMad levels becoming limiting further from the Dpp source, (3) Brk negatively autoregulates, probably by interacting with the MMS complex at the S, (4) Additional activating elements present close to the promoter recruit pMad as an activator. RIGHT: The *brk* expression pattern is described graphically and schematically on wing imaginal discs for each of the represented sets of inputs.

4.7 FUTURE EXPERIMENTS TO STUDY *BRK* AUTOREGULATION AND GRADIENT FORMATION

4.7.1 Interactions between Brk and pMad

Immunoprecipitation experiments indicated Brk binds to pMad and MBP-PD experiments indicated Brk interacts directly with pMad and Med in the absence of DNA. Other transcription

factors that interact with pMad do so via discrete domains. To be confident that interactions between Brk and pMad are specific, we would expect to identify such a discrete pMad interaction domain in Brk by conducting MBP-pull down experiments to further narrow down which portion of Brk is necessary for this interaction. N-terminal deletions of Brk have been created and could be tested for their ability to interact with Mad and Med as C-terminal truncations do not disrupt complex formation.

Additionally, to identify the region or domain/motif of Brk necessary for autoregulation, studies could be conducted in embryos where modified forms of Brk containing different domain/motifs would be driven with en^{G4} and B14 expression evaluated. Modified forms of Brk unable to repress B14 would help to narrow down the domain/motif necessary for Brk autoregulation.

4.7.2 Interactions of Brk at the silencer

4.7.2.1 EMSA studies

Preliminary studies suggested that the N-terminal region of Brk may bind weakly to an EMSA probe covering the *brk* S, but we were unsure if this binding was physiologically relevant as the Brk protein utilized contained only the DNA binding domain (data not shown). The Brk DNA binding domain was not evaluated for its ability to bind weakly to random sequences of DNA that did not contain a cognate Brk site. Additionally, MBP-MadNL and the N-terminal region of Brk could not simultaneously bind to the S (data not shown). To determine if full-length Brk could bind the *brk* S in EMSA studies, *in vitro* translated full-length Brk protein could be tested on EMSA. Similar experiments using full-length Brk could test the ability of Brk to simultaneously bind the S with MBP-MadNL.

The existence of the MMS complex was originally revealed by using S2 cells transfected with constructs encoding tagged forms of Mad, Med, and Shn as well as a constitutively active form of Tkv, utilized to upregulate BMP signaling (Muller et al., 2003; Pyrowolakis et al., 2004). In EMSAs, proteins from cells transfected with these constructs could shift the S element and specificity was demonstrated with supershifts using antibodies against the tags (Pyrowolakis et al., 2004). To determine if Brk binds together with the MMS construct to the S, these experiments could be repeated with co-transfection of a construct encoding a tagged Brk protein. If Brk binds together with the MMS complex, it would result in a shifted band with a slower mobility than that of the MMS complex alone; the band should also supershift with an antibody against Brk or its tag.

4.7.2.2 ChIP experiments

Because the ChIP experiments described in Chapter 2 utilizing embryo extracts failed to indicate an enrichment of Brk at UbxB or the *brk* S, these experiments could be attempted utilizing wing imaginal disc tissue. Driving a tagged Brk protein in half of the tissue utilized in the generation of chromatin (with en^{G4}) will significantly enrich the population of cells in which Brk is autoregulating. Additionally, ChIPs in discs are advantageous as our lab has conducted detailed studies on *sal* and *omb*, two Brk targets in the wing that could be used as positive controls (GC, unpublished results). If ChIP experiments are successful in demonstrating that Brk is found at the *brk* S, this would help to rule out the possibility that Brk autoregulates by regulating the activity of an unknown activator of an unknown repressor of Brk.

If these studies with imaginal discs were successful, ChIP experiments that could be conducted in the future that would include IPs utilizing antibodies against the myc-tagged Mad protein. These experiments would indicate which regions of the *brk* regulatory elements are

bound by pMad. We would expect to find pMad at the S element as well as at the activating Mad binding sites contained within the BP1 element. pMad may also be found at other Mad binding sites present in the *brk* regulatory region. Depending on the specificity of the region Brk was determined to bind, interactions between pMad and Brk could be supported by comparing ChIP results if they indicate that both proteins interacted at the same region, particularly the S element.

ChIP experiments could also be conducted in embryos where UAS-Dad was driven in addition to UAS-Brk. My studies already indicated that UAS-Dad (utilized to reduce the levels of pMad) caused Brk to no longer autoregulate the B14 response element indicating that autoregulation by Brk is dependent on pMad. If Brk was demonstrated by ChIP to interact at the S, then a ChIP experiment in these embryos could be utilized to demonstrate where (or if) Brk binds in the absence of pMad. It would be predicted that if pMad and Brk interact together at the S to allow Brk to autoregulate, driving UAS-Dad in embryos should cause Brk to no longer be found at the S.

4.7.3 Method of Brk negative autoregulation

To determine if Brk could autoregulate by binding its own binding sites rather than interacting with the MMS complex, a form of Brk could be generated that cannot interact with the MMS complex. This could be determined by MBP-pull down experiments. This protein would then be used to replace the endogenous form of Brk. The ability of the modified Brk to rescue the brk^{XA} mutant would then be evaluated. If rescue was still possible, this would indicate that Brk could autoregulate without its interactions at the MMS complex, presumably by binding its own binding sites contained within its regulatory regions.

4.7.4 Studies contrasting the activity of pMad at the S and BP1 elements

To determine what causes pMad to both activate and repress *brk* through the BP1 and S elements, as described above, EMSA studies could be conducted to determine how many pMad protein molecules are bound to both the S and MP elements. Presumably, more than one pMad binds to the BP1 element. EMSA studies could also be conducted to determine if the binding sites at the S are higher affinity Mad binding sites than those contained at the MP element to shed light on the mechanisms utilized by pMad to activate and repress at the same time in the same cells.

4.7.5 Studies to determine how pMad activates transcription

To begin to address the hypothesis that pMad activates at the BP1 element by promoting the chromatin modification or the recruitment of the basal transcriptional machinery, ChIP studies could be performed on *Drosophila* S2 cells transfected with a construct similar to the VgQMPB construct. This S2 cell construct would be transcriptionally activated in response to pMad. ChIP would be performed on cells transfected with this construct using antibodies that detect chromatin modification (specifically against acetylated histones that promote transcriptional activation) and antibodies that detect members of the basal transcriptional machinery. This experiment would indicate what proteins are recruited to pMad when it activates transcription.

4.8 GRADIENTS ARE USED THROUGHOUT DEVELOPMENT TO PATTERN ORGANISMS

BMPs and other TGF-ßs act as morphogens in many developmental systems (Chen and Schier, 2001; McDowell and Gurdon, 1999) and, although a direct vertebrate homolog of Brk has not been identified, the results described here pose a number of questions about how BMP/TGF-ß signaling functions to regulate gene expression. For example, are analogous transcriptional mechanisms required to boost the response of cells to low levels of external signaling molecules? Also, in regard to Smad activity, are transcriptional responses to Smads more likely to be largely all-or-none as with *brk* expression in the absence of Brk so that special mechanisms are required to modulate this to a more graded response when required?

The studies I have performed have added two additional layers to the information known about the establishment of the *brk* expression gradient. While it was previously thought that one positive input (activation by a yet un-identified activator) and one negative input (repression by the MMS complex on the S) created graded *brk* expression, my studies demonstrated that an additional negative and positive influence were necessary: negative autoregulation by Brk and activation by Mad at a previously un-identified element. This information greatly contributed to the understanding of how a *brk* expression gradient is established in the correct location so that it can properly generate spatial patterns of gene expression.

The research I have conducted will be useful and applicable to other gradients of factors established in response to morphogen gradients. Gradients of transcription factors play essential roles in virtually all developmental systems. In addition to *brk* and the other *Drosophila* examples I have described, gradients of transcription factors pattern vertebrates where examples include a gradient of Pax6 that appears to be negatively autoregulated and is important for

corticogenesis, a gradient of thyroid hormone that is responsible for establishing graded cone opsin expression in the developing mouse retina, and an inverse expression gradient of Irx5 to graded potassium channel expression where Irx5 recruits a co-repressor, m-Bop, to repress potassium channel expression in mouse cardiac ventricular repolarization (Costantini et al., 2005; Flanagan, 2006; Manuel et al., 2007; Roberts et al., 2006). Understanding how these gradients are established and how they are used to establish spatial patterns of gene expression is essential to understanding how these developmental systems operate. The generation of the *brk* expression gradient and the use of the Brk protein gradient to define limits of gene expression provides an excellent model system to examine the basic mechanisms involved in these processes and should provide novel insights into how other transcription factor gradients are established and utilized.

5.0 METHODS

5.1 FLY STRAINS UTILIZED FOR STUDIES

w^{1118} (w[1118]), w;Δ2-3 (w[*]; ry[506]P(ry[+t7.2]=Delta2-3)99B), brk^{M68}, brk^{XA}, brk^{X47}, brk^{F124}, UAS-3PF3-HA (Dmel\brk$^{Scer\UAS.T:Ivir\HA1}$), tkv^{QD} (tkv$^{QD.Act5C}$), B14X (Ecol\lacZ$^{brk.B14}$), B315X (Ecol\lacZ$^{brk.B315}$), dppE-brkS (Ecol\lacZ$^{dpp.DB271}$), omb^{G4} (bi$^{omb1-GAL4}$), UASGFP (*avic*\GFP$^{ScerFRT.RnorCD2:\alpha Tub84B}$), en^{G4} (Scer\GAL4$^{en-e16E}$), hs-GFP (avic\GFP$^{hs.T:HsapMYC}$), hs-flp (P(hsFLP)22), FRT18 ((P(ry[+t7.2]=neoFRT)18), Ubiq-mycmad (Mad$^{Ubi-p63E.T:Hsap\MYC}$), UAS-Dad (Dad$^{Scer\UAS.cTa}$), Mad$^{1-2}$ (w[*]; Mad[1-2] P(ry[+t7.2]=neoFRT)40A/CyO), Med^{2} (ru[1] h[1] P(ry[+t7.2]=neoFRT)82B sr[1] e[s] Med[2]/TM3, Sb[1])

5.2 GENERATION OF REPORTER CONSTRUCTS

5.2.1 Reporter constructs created to test B14 and B315

Constructs created to test the regulatory regions of *brk* were cloned into GFP or *lacZ* reporter vectors. pH-Stinger, pStinger, and pH-Pelican vectors are as described (Barolo et al., 2000). pHSB is a modified version of the pH-Stinger vector in which the two Brk binding sites in the *hsp70* promoter have been mutated by inverse PCR (Campbell, unpublished). pHSG4 is a

modified version of pHSB where Gal4 replaces GFP (Campbell, unpublished). C4PLZ is a *lacZ* reporter enhancer-testing vector as described (Wharton and Crews, 1993). pX27 is a *lacZ* reporter enhancer-testing vector as described (Segalat et al., 1994). pUAST is an over-expression vector as described (Brand, 1993).

All transgenic lines were generated in the following manner: plasmid DNA (final concentration 0.8μg/μL) containing the transgene were injected into the posterior of w^{1118} or w;Δ2-3 embryos. Injected embryos were incubated at 18°C in humidity chambers until larvae emerged from vitelline membranes. Larvae were collected and placed in food vials at 25°C until adults emerged. Each adult G_0 fly was mated to three w^{1118} flies of the opposite sex and the F1 generation from each vial was screened for flies containing w^+ eye color indicative of transformation.

5.2.1.1 B14 enhancer-testing constructs

B14B was created by cloning the 6.8 kb EcoRV fragment of lambda genomic clone 347 (Campbell and Tomlinson, 1999) into the EcoRV sites of pBluescript to create 347RVS. The AvrII/NotI fragment from 347RVS was cloned into the AvrII/NotI sites of pBluescript to create B14S. The Asp718/SacII fragment of B14S was cloned into the Asp718/SacII sites of pHSB to create B14B. B14Z was created by cloning the Asp718/NotI fragment of B14S into the Asp718/NotI sites of C4PLZ. B14G was created by cloning the Asp718/SacII fragment of B14S into the Asp718/SacII sites of pHSG4.

5.2.1.2 B14 with deletions of putative Brk binding sites

B14ΔBB was created by first cloning the XbaI fragment of lambda genomic clone x4795 (Campbell and Tomlinson, 1999) into the XbaI site of pBluescript creating 347B. The

Asp718/BglII fragment of 347B was then cloned into the Asp718/BamHI sites of pBluescript creating 347ABgS. Inverse PCR was performed on 347ABgS using primers ΔBrk7A and ΔBrk7B (primer sequences reported in Table 5-1). The PCR product was digested with BsiWI (restriction site provided by primers) and ligated to create 347ΔBaBgS. The AvrII/PmeI fragment of 347ΔBaBgS was cloned into the SpeI/PmeI sites of B14S to create B14ΔBS. The Asp718/SacII fragment of B14ΔBS was cloned into the Asp718/SacII sites of pHSB creating B14ΔBB. B14Δ2BB was created by initially performing inverse PCR on B14S using primers ΔBrk14A and ΔBrk14B. The PCR product was digested with AgeI (restriction site provided by primers) and ligated to create B14Δ2BS. The Asp718/SacII fragment of B14Δ2BS was cloned into the Asp718/SacII sites of pHSB creating B14Δ2BB. B14Δ2M15B was created by initially performing inverse PCR on B14Δ2BS using primers brk15MA and brk15MB. The PCR product was digested with AfeI (restriction site provided by primers) and ligated to create B14Δ2M15S. The Asp718/SacII fragment of B14Δ2M15S was cloned into Asp718/SacII digested pHSB creating B14Δ2M15B.

5.2.1.3 B315 enhancer-testing constructs

B315X was obtained from Bruno Muller (Muller et al., 2003). B315B was created by first cloning the Asp718/XbaI fragment of B315X into the Asp718/XbaI sites of pBluescript to create B315S. The Asp718/SacII fragment of B315S was then cloned into the Asp718/SacII sites of pHSB to create B315B. B315R was created by cloning the Asp718/SacII fragment of B315S into the Asp718/SacII sites of pH-Stinger. B315N was created by cloning the Asp718/SacII fragment of B315S into the Asp718/SacII sites of pH-Pelican.

5.2.1.4 B315 with mutations of putative Brk binding sites

B315M15B was created by initially performing inverse PCR on B315S with brk14MA and brk15MB. The PCR product was digested with AfeI (restriction site provided by primers) and ligated to create B315M15S. The Asp718/SacII fragment of B315M15S was cloned into the Asp718/SacII sites of pHSB to create B315M15B. B315M15X was created by cloning the Asp718/XbaI fragment of B315M15S into the Asp718/XbaI sites of B315X.

5.2.2 Reporter constructs created to test regions near the endogenous promoter of *brk* in the fly

B14BP1P was created by initially performing PCR on 347RVS template DNA with primers B14F2 and B14R1. The PCR product was digested with AvrII and cloned into the XbaI site of pStinger to create B14P. PCR was then performed on lambda genomic clone 366 (Campbell and Tomlinson, 1999) template DNA with primers BP1F1 and BP1R1. The PCR product was digested with Asp718/BsiWI and cloned into the Asp718 site of B14P creating B14BP1P. B14BP2P was created by performing PCR on 366 template DNA with primers BP2F2 and BP1R1. The PCR product was digested with Asp718/BsiWI and cloned into the Asp718 site of B14P creating B14BP2P. B14BP1MP was created by initially performing PCR on 366 template DNA with primers BP1F1 and BP1R1. The PCR product was digested with Asp718/BsiWI and cloned into the Asp718/BsiWI sites of the pLitmus29 vector to create BP1L. Inverse PCR was then performed on BP1L using PCR primers brkMPF2 and brkMPR2. The PCR product was digested with AgeI (restriction site provided by primers) and ligated to create BP1ML. The Asp718/BsiWI fragment of BP1ML was cloned into the Asp718 site of B14P creating B14BP1MP.

B14BPAP was created by initially performing PCR on BP1L template DNA with primers brkMPF4b and pLitseqR. The PCR product was digested with Asp718/BglII and cloned into the Asp718/BglII sites of pLitmus29 to create BPAL. The Asp718/BsiWI fragment of BPAL was then cloned into the Asp718 site of B14P creating B14BPAP. B14BPBP was created by initially performing PCR on BP1L template DNA with primers brkMPF3 and pLitseqR. The PCR product was digested with Asp718/BglII and cloned into the Asp718/BglII sites of pLitmus29 to create BPBL. The Asp718/BsiWI fragment of BPBL was then cloned into the Asp718 site of B14P creating B14BPBP. B14MPB was created by initially annealing oligos brkMPds1 and brkMPds2 and cloning into the BamHI site of pHSB to create MPB. The Asp718/SacII fragment of B14S was then cloned into the Asp718/SacII sites of MPB to create B14MPB.

5.2.3 Reporter constructs created to test for activation by Mad

To create VgQB, VgQS (also known as VgQBsk) was obtained from the Carroll Lab. The Asp718/SacII fragment of VgQS was cloned into the Asp718/SacII sites of pHSB creating VgQB. VgQnomadB was created by initially performing inverse PCR on VgQS with primers VgQF1 and VgQR1. The PCR product was digested with BamHI (restriction site provided by primers) and ligated to create VgQnomadS. The Asp718/SacII fragment of VgQnomadS was cloned into the Asp718/SacII sites of pHSB to create VgQnomadB. To create VgQMPB, annealed oligos brkMPds1 and brkMPds2 were cloned into the BamHI site of VgQnoMadS to create VgQMPS. The Asp718/SacII fragment of VgQMPS was cloned into the Asp718/SacII sites of pHSB to create VgQMPB. To create VgQBPBB, annealed oligos BPB2dsF and BPB2dsR were cloned into the BamHI site of VgQnoMadS to create VgQBPBS. The

Asp718/SacII fragment of VgQBPBS was cloned into the Asp718/SacII sites of pHSB to create VgQBPBB.

5.2.4 Miscellaneous reporter constructs created

B216X was created by Asp718/SpeI digestion of B315X. The product was Klenow treated and re-ligated to create B216X. HA3PF3HAU was created by initially performing PCR on template DNA Flu3PRX (Winter and Campbell, 2004) with primers FluF and FluR. The PCR product was digested with Asp718/XhoI and cloned into the Asp718/XhoI sites of pUAST to create FluU. PCR was then performed on template DNA brk3PF2 (Winter and Campbell, 2004) with primers 3PFAspF and M13R. The PCR product was digested with Asp718/XbaI and cloned into the Asp718/XbaI sites of FluU to create HA3PF3HAU. Flag3PF3HAU was created by initially performing PCR on template vector DNA pCMV-3FLAG-1a (Sigma-Aldrich) with primers FlagF and FlagR. The PCR product was digested with Asp718/XhoI and cloned into the Asp718/XhoI sites of pUAST to create FlagU. PCR was then performed on template DNA brk3PF2 with primers 3PFAspF and M13R. The PCR product was digested with Asp718/XbaI and cloned into the Asp718/XbaI sites of FlagU to create Flag3PF3HAU.

5.3 GENERATION OF RESUCE CONSTRUCTS

B1470brkW was created by initially performing PCR on template DNA pHSB with primers pHSBA1 and pHSBA2. The PCR product was digested with KpnI/BsiWI and cloned into the KpnI/BsiWI sites of pLitmus29 creating hsp70L. The KpnI/BsiWI fragment of hsp70L as well

as the BsiWI/XbaI fragment of lambda genomic clone 366 (Campbell and Tomlinson, 1999) were cloned into the KpnI/XbaI sites of pLitmus29 creating hsp70brkL. The KpnI/XbaI fragment of hs70brkL was then cloned into the KpnI/SpeI sites of B14BP1P creating B1470brkW. B14BP1brkW was created by cloning the XbaI fragment of 366 into the XbaI/SpeI sites of B14BP1P. B14BP2brkW was created by cloning the XbaI fragment of 366 into the XbaI/SpeI sites of B14BP2P. B14BP1MbrkW was created by cloning the XbaI fragment of 366 into the XbaI/SpeI sites of B14BP1MP.

B14BPAbrkW was created by cloning the XbaI fragment of 366 into the XbaI/SpeI sites of B14BPAP. B14BPBbrkW was created by cloning the XbaI fragment of 366 into the XbaI/SpeI sites of B14BPBP. B14MP70brkW was created by initially cloning the XhoI/XbaI fragment of hsp70brkL into the XhoI/SpeI sites of MPB creating MP70brkW. PCR was then performed on B14S template DNA with primers B14FAsc and B14RAsp. The PCR product was digested with Asp718/AscI and cloned into Asp718/AscI cut MP70brkW to create B14MP70brkW. B14BPB70brkW was created by initially performing PCR on BP1L template DNA with primers BPBBamF and BPBBamR. The PCR product was digested with BamHI and cloned into the BamHI site of pBluescript creating BPBS. The Asp718/BamHI fragment from BPBS was then cloned into the Asp718/BamHI sites of pHSB creating BPBB. The XhoI/XbaI fragment of hsp70brkL was then cloned into the XhoI/SpeI sites of BPBB creating BPB70brkW. PCR was then performed on B14S template DNA with primers B14FAsc and B14RAsp. The PCR product was digested with Asp718/AscI and cloned into Asp718/AscI cut BPB70brkW to create B14BPB70brkW.

Table 5-1: Primers used to generate transgenic lines.

Primer Name	5'-Sequence-3'
3PFAspF	gatcggtaccATGGATAGCAGCAGCGAACAG
B14F2	AGGTCGCACCACAAAGGG
B14FAsc	gatcggcgcgccCCTAGGGAGACCTAAAACTACAG
B14R1	gatccctaggCGGCCGCACACTCATGTC
B14RAsp	gatcggtaccTACGGCCGCACACTCATGTCG
BP1F1	gatcggtaccCTGCACGCACACGAACAC
BP1R1	ATGTCTGCGTGCTGTTGC
BP2F2	gatcggtaccTGTAGCGCAGCCAGAAAC
BPB2dsF	gatcAACTAGAACGCCGAGGGCAGAACAATTGTGTTTTGGATTCCTTGCCGTGC
BPB2dsR	gatcGCACGGCAAGGAATCCAAAACACAATTGTTCTGCCCTCGGCGTTCTAGTT
BPBBamF	gatcggatccGGCGAGGCAGTCTAGAAC
BPBBamR	gatcggatccGCACGGCAAGGAATCCAAAAC
brk15MA	gatcgatcagcgctTAATGGATAGTTCTTCTC
brk15MB	gatcgatcagcgctTGAGTTCGCTTCGGTTCG
brkMPds1	gatcGTCGACGGCGGCGCCGGCAGCGACGCCGGCCGAGGC
brkMPds2	gatcGCCTCGGCCGGCGTCGCTGCCGGCGCCGCCGTCGAC
brkMPF2	gatcaccggtGGCGAGGCAGTCTAGAAC
brkMPF3	atgcggtaccGGCGAGGCAGTCTAGAAC
brkMPF4b	atgcggtaccGTCGACGGCGGCGCCGGCAGCGAC
brkMPR2	gatcaccggtTGCGGTTCACTCTGAAAT
ΔBrk14A	gatcaccggtAACAGGTACTCGAGGAAC
ΔBrk14B	gatcaccggtGTCGACGCCTGCTAACTA
ΔBrk7A	gatccgtacgAACAGGTACTCGAGGAAC
ΔBrk7B	gatccgtacgCAAAAGAGCTGTAGCCAG
FlagF	gatcctcgagGCCGCCACCATGGATTACAAG
FlagR	gatcggtaccCGCCCGGGCTTTATCGTCATC
FluF	gatcctcgagATGGCTGGGAATATCTACCCAT
FluR	gatcggtaccTCCTGCGTAGTCCGGGACGTC
M13R	GGAAACAGCTATGACCATG
pHSBA1	GCATGCTGCAGCAGATCTG
pHSBA2	gatccgtacgCTCTAGCGCGTACCCTAG
pLitseqR	TGGCGAAAGGGGGATGTG
VgQF1	atgcggatccTTGGCGAGTGTGCCA
VgQR1	atgcggatccCCAAGCACAAACATT

5.4 CLONAL ANALYSIS AND EMBRYONIC ECTOPIC EXPRESSION STUDIES

Homozygous mutant clones were generated in wing imaginal discs by hs-flp/FRT-induced mitotic recombination. Clones were generated in the second or early third instar of larvae by heat shock for 60 minutes at 37°C.

Ectopic expression of UAS-3PF3-HA was performed by crossing to the Gal-4 expression lines en-Gal4 and en-Gal4UAS-GFP; both drive expression in the stripes in the embryo.

5.5 IMMUNOSTAINING AND X-GAL STAINING

Dissection and staining of wing imaginal discs was carried-out by standard techniques. The following primary antibodies were used: anti-Sal (rabbit, 1:50) (Kuhnlein et al., 1997); anti-βgal (rabbit, 1:2000 Cappell) and (mouse, 1:200 Promega); anti-HA (mouse, 1:1000 Covance); anti-GFP (rabbit, 1:1000 Torrey Pines); anti-brkR3 (rat, 1:200) (Campbell and Tomlinson, 1999). Secondary antibodies included Alexa 594 (rabbit), Alexa 488 (rabbit, mouse, rat), Alexa 568 (mouse minX) (Molecular Probes), Cy3 (rat minX), and Cy5 (rabbit, mouse minX, rat minX) (Jackson Immuno Research). Discs containing *lacZ* reporters were stained along side one another following a standard protocol and wings from adult flies were mounted in GMM.

5.6 ELECTROMOBILITY SHIFT ASSAYS

5.6.1 Probe preparation

Equal molar ratios of complementary oligos (Table 5-2) were mixed and annealed by heating to 90°C and slowly cooling to RT to a final concentration of 50pmol/uL. Probes were radiolabeled by Klenow extension of 3' ends using α-^{32}P dCTP and unincorporated nucleotides were removed using Bio-Rad Micro Bio-Spin P30 gel spin columns.

5.6.2 EMSA reactions

Reactions were performed by incubating bacterially expressed, purified fusion proteins (as described in 5.9) with 10,000cpm ^{32}P-labeled probe in 10uL reactions in Mad Binding Buffer (all buffer recipes are in appendix A). For competition experiments, 50X, 100X, or 300X of unlabeled probe was added to the reaction. Reactions were incubated for 30 minutes at room-temperature. The entire reaction was separated on a non-denaturing 4% polyacrylamide gel following standard protocol. The gels were dried and exposed to autoradiographic film.

Table 5-2: EMSA probe sequences.

Primer Name	5'-Sequence-3'
brkMPF	atgGTCGACGGCGGCGCCGGCAGCGACGCCGGCCGAGGC
brkMPR	atgGCCTCGGCCGGCGTCGCTGCCGGCGCCGCCGTCGAC
brkMPM7MadF	atgGTCGACAGCAGCGACAGCAGCGACACAGTCAGAGGCCAT
brkMPM7MadR	atgATGGCCTCTGACTGTGTCGCTGCTGTCGCTGCTGTCGAC
UbxBF	gacTCTGGACTGGCGTCAGCGCCGGCGCTTCCAGCT
UbxBR	GGCAGCTGGAAGCGCCGGCGCTGACGCCAGTCCAGA
UbxBM2F	atgTCTGGACTGATGTCAATAGTACTGCTTCATAT
UbxBM2R	atgATATGAAGCAGTACTATTGACATCAGTCCAGA

5.7 WESTERN BLOTS

All Western blot procedures were performed in a similar manner using standard procedures (Sambrook, 2001). Proteins were separated on SDS-polyacrylamide gels along with Precision Plus Kaleidoscope protein standard markers (Bio-Rad) and transferred to Immobilon-P PVDF membranes (Millipore) for immunoblotting. Immobilon-P PVDF membranes were pre-wet in methanol and soaked in 1X Anode Buffer. Gels were soaked for 30 minutes in 1X Cathode Buffer prior to transfer. Proteins were transferred to the membrane using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell at constant amperage for 30 minutes as suggested by the manufacturer (Bio-Rad). Membranes were blocked in 1X PBS, pH 7.4 with 5% non-fat dry milk overnight at 4°C.

Detection of immunocomplexes was completed essentially as described in the manufacturer's protocol for the ECL Western detection reagent (Amersham). Blots were incubated in 1X TBST, 1% BSA, and primary antibody for one hour at room-temperature with

rocking followed by three five minute washes using 1X TBST. Blots were then incubated in 1X TBST, 5% non-fat dry milk, and secondary antibody for 45 minutes with rocking followed by three five minutes rinses using 1X TBST. After washing, immunocomplexes were detected using the ECL Western blotting kit (Amersham) and exposed to film. Following imaging, if desired, blots were striped using 1X Western Strip Buffer for one hour at 50°C then rinsed five times with 1X TBST and blocked as previously described before probing with a different antibody.

The primary antibodies used for Western blotting are as follows: anti-HA (1:500, rabbit, Santa Cruz Biotechnology); anti-HA (1:200, mouse, Santa Cruz Biotechnology); anti-BrkR3 (1:1200, rat); anti-myc•HRP (1:400, mouse, Santa Cruz Biotechnology). HRP-conjugated secondary antibodies for Western blotting were used at the following concentrations: anti-rat (1:5,000 Jackson Immuno Research); anti-rabbit (1:10,000 Jackson Immuno Research); anti-mouse (1:18,000 Jackson Immuno Research).

5.8 CO-IMMUNOPRECIPITATIONS

5.8.1 Embryo lysate preparation

Embryo collections were 0-20 hours at 25°C for W^{1118} or $\frac{\text{UAS-3PF3-HA}}{+\text{ or }>}$; $\frac{\text{engrailed}^{\text{Gal4}}}{+}$; $\frac{\text{UAS-tkv}^{\text{QD}}}{\text{Ubiq-Myc-Mad}}$ genotypes. Embryos were de-chorionated in 50% bleach for three minutes, rinsed well, and transferred into Eppendorf tubes containing cold 1X RIPA Lysis Buffer with protease inhibitors. Embryos were homogenized with a pestle and the supernatant analyzed for the concentration of total protein as determined by Bradford analysis, according to standard procedure (Bio-Rad).

5.8.2 Immunoprecipitation reactions

Reactions were performed in 500μL volumes in 1X IP Buffer. Typically, 25μL of embryo lysate was used in each reaction with 1μg of commercial antibody or 2μL of anti-brkR3 antibody. The antibody/embryo lysate/buffer solution was mixed for 4 hours at 4°C with rocking after which 20μL of 50% slurry of Protein A/G PLUS Agarose (Santa Cruz Biotechnology) was added to each reaction. The reactions were incubated overnight at 4°C with rocking. To isolate the immunocomplexes, the resin was washed four times with 1mL of IP buffer for two minutes. The resin was then re-suspended in 50μL of 2XSB, boiled for five minutes, cooled, and debris pelleted before loading on an SDS-polyacrylamide gel and subjecting to Western blot.

5.9 GENERATION OF FUSION PROTEINS

5.9.1 Generation of MBP fusion proteins Mad and Med

Initially, small-scale expression and purification of the MBP-fusion proteins was conducted in order to test for solubility. MBP, MBP-MadNL, and MBP-MedNL (gifts from Allen Laughon) were expressed on a large-scale essentially as described in Molecular Cloning (Sambrook, 2001). Plasmids were transformed into BL21 cells using standard procedures and grown on 2XYT plates with 60μg/mL carbecillin. Single colonies were grown in 5mL of 2XYT with 60μg/mL ampicillin for approximately 8 hours at 37°C; the entire culture was then diluted 1:10 and grown for 16 hours at 37°C. The cultures were again diluted 1:20 in media with 60μg/mL ampicillin and grown at 37°C for 60 minutes and then RT until the OD_{A600} readings were between 0.6 and

0.8. The fusion proteins were induced with 0.2mM IPTG and the cultures grown for three hours at RT. Cultures were chilled on ice and centrifuged at 5000 g for 10 minutes at 4°C and pellets resuspended in 20mL 1X Column Loading Buffer (1X CLB) before freezing at -20°C overnight.

The cells were thawed and sonicated on ice for 8 minutes (50% pulse, 2.5 power) and centrifuged at 10,000 g for 20 minutes. The supernatant was incubated with 3mL amylose resin (pre-washed in 1X CLB) and mixed for 30 minutes. The resin was gently pelleted and washed four times with 10 volumes 1X CLB. Protein was eluted in 6mL 1X CLB with 10mM maltose and dialyzed against four changes of Mad Dialysis Buffer. The concentration was then determined by Bradford analysis and aliquots frozen at -80°C.

5.10 MBP-PULL DOWN ASSAYS

5.10.1 Radiolabeled protein construct generation

Luciferase was provided by the TNT *in vitro* transcription/translation rabbit reticulocyte lysate system kit (Promega). ZenAR was provided by the Rushlow Lab (Rushlow et al., 2001). 3PFT was created by initially performing PCR on template DNA brk3PF2 (Winter and Campbell, 2004) with primers brkGFBGL and T3 (primer sequences in Table 5-3). The product was digested with BglII /NotI and cloned into the BamHI/NotI sites of vector pET-21a.

FluT was created by performing PCR on brk3PRX (Winter and Campbell, 2004) template DNA using primers M13F and M13R. The PCR product was digested with EcoRI/NotI and cloned into the EcoRI/NotI sites of vector pET-21a. To create Stop1T, PCR was performed on brk3PF2 with primers brkGFBGL and Stop1RI. The PCR product was digested with

BglII/EcoRI and cloned into the BamHI/EcoRI sites of FluT to create Stop1T. Stop2T and Stop3T were created as described for Stop1T except using forward primer brkGFBGL and reverse primers Stop2RI and Stop3RI, respectively.

RH2DT was created by first performing inverse PCR on brk3PF2 with primers brkA10a and brkA11a. The PCR product was digested with AgeI (restriction site provided by primers) and ligated to create brkRHDS. PCR was then performed using brkRHDS as a template and brkGFBGL and M13R as primers. The PCR product was digested with BglII/NotI and cloned into the BamHI/NotI sites of pET-21a creating RH2DT.

NTAT was created by performing PCR on template DNA 3PF2 with primers Stop1RI and brkNT1BglF. The PCR product was digested with BglII/EcoRI and cloned into the BamHI/EcoRI sites of FluT to create NTAT. NTBT and NTCT were created as described for NTAT except that Stop1RI was used as the forward PCR primer and brkNT2BglF and brkNT3BglF were used as the reverse primers, respectively.

Table 5-3: Primers used to create *in vitro* translated protein.

Primer Name	5'-Sequence-3'
brkA10a	gatcaccggtGTTGTATTTCCTGGCCGT
brkA11a	gatcaccggtGAGTCAAATTTGCGATCA
brkGFBGL	AGTCagatctATGGATAGCAGCAGCGAA
brkNT1BglF	agtcagatctGGCAGCCCCAAAATGGGAAG
brkNT2BglF	agtcagatctAATCAGCAACAGCAGCAG
brkNT3BglF	agtcagatctGGCATGGTTCCACACCCC
M13F	gtaaaacgacggccagt
M13R	GGAAACAGCTATGACCATG
Stop1RI	atgcgaattcGGCTGGTGTGGCAACCGC
Stop2RI	atgcgaattcCAGCTTGTGCTCCTCCGG
Stop3RI	atgcgaattcTTTGCTGGAGCTGCAGTT
T3	ATTAACCCTCACTAAAGGGA

5.10.2 *In vitro* transcription/translation reactions

The coupled TNT *in vitro* transcription/translation rabbit reticulocyte lysate system (Promega) was used in the presence of ^{35}S-methionine to generate both full-length and truncated radiolabeled Brk fusion proteins following the manufacturer's protocol using 1µg of plasmid DNA in a 50µL final reaction volume.

5.10.3 Immunoprecipitations

Immunoprecipitation experiments utilizing radiolabeled *in vitro* translated proteins were performed as described in section 5.8.2 substituting 20µL *in vitro* translated protein for the embryonic lysate and with no pre-absorption.

5.10.4 Pull-down assays

Pull-down reactions were performed essentially as described (Rushlow et al., 2001). Briefly, 20µL of radiolabeled *in vitro* translated fusion protein was pre-absorbed on amylose resin (New England Biologicals) for 45 minutes on ice. The pre-absorbed protein was then mixed with 200µL DNAP Buffer and 250ng MBP, MBP-MadNL, or MBP-MedNL (as described above) and incubated at 37°C for 60 minutes. Following incubation, 20µL of 50% slurry of amylose resin in 1X DNAP Buffer was added and the reactions incubated for 60 minutes at RT on a nutator. Resin was washed five times with 1mL DNAP Buffer and the pellet re-suspended in 60µL 2XSB. The samples were boiled for five minutes and 15µL of supernatant run on SDS-

polyacrylamide gels alongside 10% input radiolabeled protein. The gels were fixed, dried, and exposed to film following standard procedures.

5.11 GENERATION AND PURIFICAITON OF ANTIBODIES

GST-Pro6 antigen was previously purified in the lab and subsequently used for antibody production. The fusion protein was injected into three rats and three guinea pigs. The animals were boosted at days 21, 42, and 63 followed by a terminal bleed on day 73.

5.11.1 Preparation of affinity column containing GST

GST was coupled to CNBr-Sepharose 4B (cat# 17-0430-01, Amersham) as described by the manufacturer. Approximately 10 mg (4 mL) of the protein, dialyzed in 1X Coupling buffer (Appendix A), was mixed with the prepared resin and incubated overnight with rocking at 4°C. The mixture was centrifuged at 1500 g for 4 minutes, and the supernatant discarded. The CNBr resin was washed twice in 10 bed volumes of 1X Coupling buffer before the active groups on the resin were blocked with 10 bed volumes 1X Active group blocking buffer (0.1M Tris-HCl, pH 8) at room-temperature for 3 hours with rocking. The resin was then washed twice in 10 bed volumes of 1X Coupling buffer, and transferred to a gravity-flow column (Novagen). The column drained completely to pack with a final bed volume of 2.5mL and contained approximately 10mg of antigen.

To remove all loosely bound antigen, the column was washed through the following series of buffers, checking that the pH of the last few drops of eluate reached the pH values of

the respective buffers (all listed in Appendix A): (1) 10 bed volumes of 1X Coupling buffer (pH 8.3), (2) 10 bed volumes of Low pH elute buffer (pH 2.5), (3) 10 bed volumes of 1X Coupling buffer (pH 8.3), (4) 10 bed volumes of High pH elute buffer (pH 11.5), (5) 10 bed volumes of 1X Coupling buffer (pH 8.3). The five washes were then repeated to ensure that all loosely bound proteins were removed.

5.11.2 Removal of anti-GST antibodies from antisera

Approximately 3mL of the anti-brkGP10 antisera was diluted 1:3 in 0.1 M Tris-HCl, pH 8, and filtered through a 0.22 μm filter disc before loading onto the affinity purification CNBr-coupled column. To eliminate GST-specific antibodies the antisera was loaded onto a CNBr-GST column. The entire eluate from this column was collected.

5.11.3 Final antibody purification

For final antibody purification, a Protein A Sepharose column was prepared following manufacturer directions (Amersham). Briefly, the Protein A Sepharose was expanded in deionized water, washed for 3 washes of 10 mL 1 mM HCl, with 10 minutes rocking followed by centrifugation at 5000 g for 5 minutes. The Protein A Sepharose resin was loaded onto a gravity-flow column (Novagen) and allowed to drain completely, packing the column with a final bed volume of 1.5 mL Protein A Sepharose resin. The column was washed with 10 bed volumes 1X PBS, pH 7.4 (Appendix A) to equilibrate the column.

To concentrate the antibody, the eluates obtained above were loaded onto the Protein A Sepharose column. The column was washed with 10 bed volumes of 1X PBS buffer and the pH

of the last few drops of the wash buffer was checked to have reached pH 7.4. The antibodies were eluted by washing with 5 volumes low pH elute buffer, pH 2.5. The eluate was collected in 0.5mL fractions into microfuge tubes containing 100µL of 1 M Tris, pH 8. Upon completion, the pH of the eluate was checked to be sure it was approximately pH 7.5.

The fractions that contained the antibodies were determined by spot testing each fraction for the presence of protein. The antibodies were centrifuged at 1500 g for 5 minutes and the supernatant was dialyzed against 1X PBS, pH 7.4, for 4 hours at 4°C, then dialyzed twice overnight against 1X PBS, pH 7.4 at 4°C. Upon completing dialysis, the dialyzed antibody was stored in 1X PBS, pH 7.4 with 1% BSA added as a stabilizer. Antibodies were sterile filtered through a 0.22 µm filter disc, aliquoted, and the purified antibodies were stored at 4°C and -80°C.

5.12 CHROMATIN IMMUNOPRECIPITATIONS

5.12.1 Chromatin preparation

All buffers for ChIP are described in Appendix B. Overnight collections of embryos of the genotype $\frac{\text{UAS-3PF3-HA}}{\text{+ or >}}$; $\frac{\text{engrailed}^{\text{Gal4}}}{\text{+}}$; $\frac{\text{UAS-tkv}^{\text{QD}}}{\text{Ubiq-Myc-Mad}}$ were collected on plates and dechorionated for three minutes in a solution of 50% bleach. They were washed well with distilled water and 0.5g of embryos was transferred into a 50mL Falcon tube. The embryos were washed with 50mL PBT, and then cross-linked by adding 10mL Cross-linking Solution, 649µL 37% formaldehyde (Fisher Scientific), and 30mL n-Heptane. The embryos were vigorously shaken for 15 minutes. After crosslinking, the embryos were resuspended in 30mL PBT/Glycine Solution. The embryos were

washed in PBT and from this point on, processed at 4ºC. The embryos were resuspended in ice-cold PBT+PIs and dounced 20X with a loose pestle in a Wheaton homogenizer before spinning 1 minute at 400g. The supernatant was saved and spun again at 1100g for 10 minutes. The pellet was resuspended in 15mL ice-cold Cell Lysis Buffer+PIs and dounced 20X with a tight pestle in a Wheaton homogenizer. The nuclei were pelleted by centrifugation at 2000g and resuspended in 1mL ice-cold Nuclear Lysis Buffer+PIs. To two 50mL Falcon tubes, 2mL ice-cold Nuclear Lysis Buffer+PIs and 0.3g acid-washed 212-300 micron glass beads (Sigma) were added. After 20 minutes, the re-suspended nuclei was split and added to this mixture for sonication. Sonication was carried out to produce chromatin fragments with an average size of 500bp. Each batch of chromatin was sonicated 15 times with 25 seconds on, 25 seconds off at power 5. The glass beads were pelleted by centrifugation at 5000g and the supernatant transferred to microfuge tubes where any remaining glass beads were pelleted. The supernatant (containing fixed sheared chromatin) was then pooled, 200µL aliquots were generated, and the chromatin flash frozen and stored at -80C.

5.12.2 Immunoprecipitation

Chromatin was thawed on ice and reactions conducted at 4ºC. Varied amounts of chromatin (20µL-400µL) were tested in IP reactions by combining the appropriate amount of chromatin in 400µL minimal reactions with IP Dilution Buffer+PIs. Chromatin was pre-absorbed by incubating with 50µL 50% Protein A/G PLUS Agarose (Santa Cruz) for 4 hours. Reactions were then conducted following condition A or B as follows:

A: Pre-absorbed chromatin was mixed with varied amounts of antibody (0.5µL-10µL) overnight with rotation. 50µL of a 50% slurry of Protein A/G Plus Agarose (Santa Cruz) pre-absorbed with BSA and Salmon Sperm DNA was then added to the IP for 3 hours with rotation.

B: Varied amounts of antibody were mixed with 50µL of a 50% slurry of Protein A/G Plus Agarose (Santa Cruz) pre-absorbed with BSA and Salmon Sperm DNA for 4 hours with rotation. The resin was washed well with IP Dilution Buffer+PIs. Pre-absorbed chromatin was then added to the reaction in 400µL minimal reaction volumes and rotated overnight.

The chromatin/antibody/resin complex was pelleted and washed 2 times for 5 minutes with 1mL IP Dilution Buffer, 2 times for 5 minutes with 1mL High Salt Wash Buffer, four times for 5 minutes with 1mL Wash Buffer, and two times for 5 minutes with 1mL TE Wash Buffer. The reactions were then brought to room temperature and the resin re-suspended in 150µL IP Elution Buffer, rotated for 15 minutes, and the supernatant saved to a clean tube. This elution was repeated one more time.

The appropriate amount of input chromatin was brought up to 300µL with Nuclear Lysis buffer. The input and eluted chromatin were then treated with 3µL of 5mg/mL RNaseA for 30 minutes at 37ºC. Proteins were removed by adding 6µL of 50mg/mL ProteinaseK to each reaction and incubated 90-120 minutes at 45ºC. The crosslinking was then reversed by incubation at 65ºC overnight. The immuno-complexes were then purified using the QIAquick PCR purification kit (Qiagen) and eluted in 50µL Buffer EB (Qiagen).

5.12.3 PCR analysis

PCR was carried out using Platinum Taq DNA polymerase (Invitrogen). Reaction conditions and cycling parameters were optimized for each primer pair. 25µL reactions were utilized and

half of each reaction was run on a 2% TBE gel, stained with ethidium bromide, and imaged on a Kodak Image Station 440CF.

Table 5-4: Primers used in ChIP analysis.

Primer Name	5'-Sequence-3'
brkSF1	GGGAAGAATTGAATGAGTTGTGGTAAC
brkSR1	ACAAGCGAGAGACAGAGAGCGAGCGGGC
DNApolF4	GAATCGGAGGACATTAGTAGGTTGC
DNApolR4	GCAGTGGTTTCTTTCCATCGTAGTG
UbxBF1	ACCATCTAACAGGTGCCGAGC
UbxBR1	GCCACTTACGCAGCTAATAAAGCAG
UbxBF5	AATGCCGAAAGACACAGCCG
UbxBR5	ATGCCATCCGTATGCGTGCG

APPENDIX A

GENERAL BUFFER RECIPES

Mad Binding Buffer:
50mM KCl
20mM HEPES, pH 7.6
1mM DTT
1mM EDTA
50μg/mL poly[d(A-T)]
4% Ficoll
0.25mg/mL BSA

1X PBS, pH 7.4:
10mM Na_2HPO_4O
2mM KH_2PO_4O
137mM NaCl
2.7mM KCl

1X Anode Buffer, pH 9.6:
60mM Tris
40mM CAPS
15% Methanol

1X Cathode Buffer, pH 9.6:
60mM Tris
40mM CAPS

1X TBST, pH 7.5:
20mM Tris-HCl, pH 7.5
150mM NaCl
0.05% Tween-20

1X Western Strip Buffer:
62.5mM Tris-HCl, pH 6.8
100mM 2-β-mercaptoethanol
2% SDS

1X RIPA Lysis Buffer:
150mM NaCl
1% NP-40
0.5% Sodium deoxycholate
0.1% SDS
50mM Tris, pH 8.0
1mM PMSF
2μg/mL Aprotinin
1μg/mL Leupeptin
0.7μg/mL Pepstatin

1X IP Buffer:
150mM NaCl
0.5% NP-40
5mM EDTA, pH 8.0
50mM Tris, pH 7.5
2ug/mL Leupeptin
1ug/mL Pepstatin A

2XSB:
100mM Tris, pH 6.8
20% glycerol
4% SDS
0.01% Bromophenol Blue
200mM DTT

Mad Dialysis Buffer:
50mM KCl
20mM HEPES, pH 7.6
1mM DTT
1mM EDTA

1X CLB:
20mM Tris, pH 7.5
0.2M NaCl
1mM EDTA
1mM DTT

1X DNAP Buffer:
80mM NaCl
35mM KCl
5mM $MgCl_2$
25mM Tris, pH 7.5
10% glycerol
0.1% NP-40
1mM DTT

1X Coupling Buffer:
0.1M $NaHCO_3$, pH 8.3
0.5M NaCl
1mM EDTA, pH 8.0

Low pH Elute Buffer, pH 2.5:
0.1M glycine, pH 2.5
20mM NaCl

High pH Elute Buffer, pH 11.5:
20mM Tris-HCl, pH 11.5
0.5M NaCl
1mM EDTA, pH 8.0

APPENDIX B

CHROMATIN IP BUFFER RECIPES

Protease Inhibitor Concentrations:
3.3µg/mL Aprotinin
10µg/mL Leupeptin
4µg/mL Pepstatin
1mM AEBSF or PMSF

10X PBS, pH 7.4:
80g Sodium chloride
2g Potassium chloride
14.4g Disodium hydrogen orthophosphate
2.4g Sodium dihydrogen orthophosphate

PBT:
1X PBS
1% Triton-X100

PBT/Glycine Solution:
4.69g Glycine
5mL 10% Triton-X100
50mL 10X PBS
dH_2O to 500mL

Crosslinking Solution:
50mM HEPES pH 8.0
1mM EDTA
0.5mM EGTA
100mM NaCl

Cell Lysis Buffer:
5mM HEPES pH 8.0
85mM KCl
0.5% NP-40

Nuclear Lysis Buffer:
50mM Tris pH 8.0
10mM EDTA
1% SDS

IP Dilution Buffer:
0.01% SDS
1.1% Triton X-100
1.2mM EDTA
16.7mM Tris pH 8.0
167mM NaCl

High Salt Wash Buffer:
0.1% SDS
1% Triton X-100
2mM EDTA
20mM Tris pH 8.0
500mM NaCl

Wash Buffer:
100mM Tris pH 8.0
500mM LiCl
1% NP-40
1% Sodium Deoxycholate

TE Wash Buffer:
10mM Tris pH 8.0
1mM EDTA

Elution Buffer:
1% SDS
0.1M $NaHCO_3$

BIBLIOGRAPHY

Adkins, M. W. and Tyler, J. K. (2006). Transcriptional activators are dispensable for transcription in the absence of Spt6-mediated chromatin reassembly of promoter regions. *Mol Cell* **21**, 405-16.

Arnosti, D. N. (2003). Analysis and function of transcriptional regulatory elements: insights from Drosophila. *Annu Rev Entomol* **48**, 579-602.

Arnosti, D. N. and Kulkarni, M. M. (2005). Transriptional enhancers: intelligen enhanceosomes or fleixble billboards? *JCB* **94**.

Ashe, H. L. and Briscoe, J. (2006). The interpretation of morphogen gradients. *Development* **133**, 385-94.

Bangi, E. and Wharton, K. (2006). Dpp and Gbb exhibit different effective ranges in the establishment of the BMP activity gradient critical for Drosophila wing patterning. *Dev Biol* **295**, 178-93.

Barolo, S., Carver, L. A. and Posakony, J. W. (2000). GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. *Biotechniques* **29**, 726, 728, 730, 732.

Barolo, S. and Posakony, J. W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev* **16**, 1167-81.

Barolo, S., Stone, T., Bang, A. G. and Posakony, J. W. (2002). Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. *Genes Dev* **16**, 1964-76.

Barrio, R. and de Celis, J. F. (2004). Regulation of spalt expression in the Drosophila wing blade in response to the Decapentaplegic signaling pathway. *Proc Natl Acad Sci U S A* **101**, 6021-6.

Bhaumik, S. R., Smith, E. and Shilatifard, A. (2007). Covalent modifications of histones during development and disease pathogenesis. *Nat Struct Mol Biol* **14**, 1008-16.

Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackerman, L., Carretto, R., Uemura, T., Grell, E. et al. (1989). Searching for pattern and mutation in the Drosophila genome with a P-lacZ vector. *Genes Dev* **3**, 1273-87.

Blackwood, E. M. and Kadonaga, J. T. (1998). Going the distance: a current view of enhancer action. *Science* **281**, 60-3.

Blair, S. S. (1995). Compartments and appendage development in Drosophila. *Bioessays* **17**, 299-309.

Brand, A. H. a. P., N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-15.

Brantjes, H., Roose, J., van De Wetering, M. and Clevers, H. (2001). All Tcf HMG box transcription factors interact with Groucho-related co-repressors. *Nucleic Acids Res* **29**, 1410-9.

Bray, S. (1999). DPP on the brinker. *Trends Genet* **15**, 140.
Brook, W. J., Diaz-Benjumea, F. J. and Cohen, S. M. (1996). Organizing spatial pattern in limb development. *Annu Rev Cell Dev Biol* **12**, 161-80.
Burke, T. W. and Kadonaga, J. T. (1996). Drosophila TFIID binds to a conserved downstream basal promoter element that is present in many TATA-box-deficient promoters. *Genes Dev* **10**, 711-24.
Burley, S. K. and Roeder, R. G. (1996). Biochemistry and structural biology of transcription factor IID (TFIID). *Annu Rev Biochem* **65**, 769-99.
Burz, D. S., Rivera-Pomar, R., Jackle, H. and Hanes, S. D. (1998). Cooperative DNA-binding by Bicoid provides a mechanism for threshold-dependent gene activation in the Drosophila embryo. *Embo J* **17**, 5998-6009.
Campbell, G. and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of Drosophila: regulation of Dpp targets by brinker. *Cell* **96**, 553-62.
Carneiro, K., Fontenele, M., Negreiros, E., Lopes, E., Bier, E. and Araujo, H. (2006). Graded maternal short gastrulation protein contributes to embryonic dorsal-ventral patterning by delayed induction. *Dev Biol* **296**, 203-18.
Certel, K., Hudson, A., Carroll, S. B. and Johnson, W. A. (2000). Restricted patterning of vestigial expression in Drosophila wing imaginal discs requires synergistic activation by both Mad and the drifter POU domain transcription factor. *Development* **127**, 3173-83.
Chen, D. and McKearin, D. (2003a). Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. *Curr Biol* **13**, 1786-91.
Chen, D. and McKearin, D. M. (2003b). A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell. *Development* **130**, 1159-70.
Chen, G. and Courey, A. J. (2000). Groucho/TLE family proteins and transcriptional repression. *Gene* **249**, 1-16.
Chen, Y. and Schier, A. F. (2001). The zebrafish Nodal signal Squint functions as a morphogen. *Nature* **411**, 607-10.
Chinnadurai, G. (2007). Transcriptional regulation by C-terminal binding proteins. *Int J Biochem Cell Biol* **39**, 1593-607.
Cohen, S. M. (1993). Imaginal disc development. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
Costantini, D. L., Arruda, E. P., Agarwal, P., Kim, K. H., Zhu, Y., Zhu, W., Lebel, M., Cheng, C. W., Park, C. Y., Pierce, S. A. et al. (2005). The homeodomain transcription factor Irx5 establishes the mouse cardiac ventricular repolarization gradient. *Cell* **123**, 347-58.
Courey, A. J. and Jia, S. (2001). Transcriptional repression: the long and the short of it. *Genes Dev* **15**, 2786-96.
Cowell, I. (1994). Repression versus activation in the control of gene transcription. *Trends Biochem Sci.* **19**, 38-42.
Crauk, O. and Dostatni, N. (2005). Bicoid determines sharp and precise target gene expression in the Drosophila embryo. *Curr Biol* **15**, 1888-98.
Dreiver, W. a. N.-V., C. (1988). The bicoid Protein Determines Position in the Drosophila Embryo in a Concentration-Dependent Manner. *Cell* **54**, 95-104.
Flanagan, J. G. (2006). Neural map specification by gradients. *Curr Opin Neurobiol* **16**, 59-66.
Gao, S. and Laughon, A. (2006). Decapentaplegic-responsive silencers contain overlapping mad-binding sites. *J Biol Chem* **281**, 25781-90.

Gao, S., Steffen, J. and Laughon, A. (2005). Dpp-responsive silencers are bound by a trimeric Mad-Medea complex. *J Biol Chem* **280**, 36158-64.
Gazzerro, E. and Canalis, E. (2006). Bone morphogenetic proteins and their antagonists. *Rev Endocr Metab Disord* **7**, 51-65.
Gelbart, W. M. (1989). The decapentaplegic gene: a TGF-beta homologue controlling pattern formation in Drosophila. *Development* **107 Suppl**, 65-74.
Gray, S. and Levine, M. (1996). Transcriptional repression in development. *Curr Opin Cell Biol* **8**, 358-64.
Gurdon, J. B. and Bourillot, P. Y. (2001). Morphogen gradient interpretation. *Nature* **413**, 797-803.
Hanna-Rose, W. and Hansen, U. (1996). Active repression mechanisms of eukaryotic transcription repressors. *Trends Genet* **12**, 229-34.
Hasson, P., Muller, B., Basler, K. and Paroush, Z. (2001). Brinker requires two corepressors for maximal and versatile repression in Dpp signalling. *Embo J* **20**, 5725-36.
Hewitt, G. F., Strunk, B. S., Margulies, C., Priputin, T., Wang, X. D., Amey, R., Pabst, B. A., Kosman, D., Reinitz, J. and Arnosti, D. N. (1999). Transcriptional repression by the Drosophila giant protein: cis element positioning provides an alternative means of interpreting an effector gradient. *Development* **126**, 1201-10.
Hoch, M., Schroder, C., Seifert, E. and Jackle, H. (1990). Cis-acting control elements for Kruppel expression in the Drosophila embryo. *Embo J* **9**, 2587-95.
Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S. and Rushlow, C. (1999a). The Drosophila gene brinker reveals a novel mechanism of Dpp target gene regulation. *Cell* **96**, 563-73.
Jazwinska, A., Rushlow, C. and Roth, S. (1999b). The role of brinker in mediating the graded response to Dpp in early Drosophila embryos. *Development* **126**, 3323-34.
Jiang, J., Rushlow, C. A., Zhou, Q., Small, S. and Levine, M. (1992). Individual dorsal morphogen binding sites mediate activation and repression in the Drosophila embryo. *Embo J* **11**, 3147-54.
Johnson, A. D. (1995). The price of repression. *Cell* **81**, 655-8.
Juven-Gershon, T., Hsu, J. Y. and Kadonaga, J. T. (2006). Perspectives on the RNA polymerase II core promoter. *Biochem Soc Trans* **34**, 1047-50.
Kaufman, P. D. and Rio, D. C. (1991). Drosophila P-element transposase is a transcriptional repressor in vitro. *Proc Natl Acad Sci U S A* **88**, 2613-7.
Kim, J., Johnson, K., Chen, H. J., Carroll, S. and Laughon, A. (1997). Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. *Nature* **388**, 304-8.
Kirkpatrick, H., Johnson, K. and Laughon, A. (2001). Repression of dpp targets by binding of brinker to mad sites. *J Biol Chem* **276**, 18216-22.
Kokavec, J., Podskocova, J., Zavadil, J. and Stopka, T. (2008). Chromatin remodeling and SWI/SNF2 factors in human disease. *Front Biosci* **13**, 6126-34.
Kornberg, R. D. (1999). Eukaryotic transcriptional control. *Trends Cell Biol* **9**, M46-9.
Kornberg, R. D. (2005). Mediator and the mechanism of transcriptional activation. *Trends Biochem Sci* **30**, 235-9.
Kuhnlein, R. P., Bronner, G., Taubert, H. and Schuh, R. (1997). Regulation of Drosophila spalt gene expression. *Mech Dev* **66**, 107-18.
Kutach, A. K. and Kadonaga, J. T. (2000). The downstream promoter element DPE appears to be as widely used as the TATA box in Drosophila core promoters. *Mol Cell Biol* **20**, 4754-64.

Lagrange, T., Kapanidis, A. N., Tang, H., Reinberg, D. and Ebright, R. H. (1998). New core promoter element in RNA polymerase II-dependent transcription: sequence-specific DNA binding by transcription factor IIB. *Genes Dev* **12**, 34-44.
Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from drosophila? *Cell* **85**, 951-61.
Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 387-93.
Lewis, E. B. (1998). The bithorax complex: the first fifty years. *Int J Dev Biol* **42**, 403-15.
Lim, C. Y., Santoso, B., Boulay, T., Dong, E., Ohler, U. and Kadonaga, J. T. (2004). The MTE, a new core promoter element for transcription by RNA polymerase II. *Genes Dev* **18**, 1606-17.
Llimargas, M. (2000). Wingless and its signalling pathway have common and separable functions during tracheal development. *Development* **127**, 4407-17.
Manuel, M., Georgala, P. A., Carr, C. B., Chanas, S., Kleinjan, D. A., Martynoga, B., Mason, J. O., Molinek, M., Pinson, J., Pratt, T. et al. (2007). Controlled overexpression of Pax6 in vivo negatively autoregulates the Pax6 locus, causing cell-autonomous defects of late cortical progenitor proliferation with little effect on cortical arealization. *Development* **134**, 545-55.
Massague, J., Seoane, J. and Wotton, D. (2005). Smad transcription factors. *Genes Dev* **19**, 2783-810.
McDowell, N. and Gurdon, J. B. (1999). Activin as a morphogen in Xenopus mesoderm induction. *Semin Cell Dev Biol* **10**, 311-7.
Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H. and Tabata, T. (1999). brinker is a target of Dpp in Drosophila that negatively regulates Dpp-dependent genes. *Nature* **398**, 242-6.
Moser, M. and Campbell, G. (2005). Generating and interpreting the Brinker gradient in the Drosophila wing. *Dev Biol* **286**, 647-58.
Muller, B., Hartmann, B., Pyrowolakis, G., Affolter, M. and Basler, K. (2003). Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. *Cell* **113**, 221-33.
Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-68.
O'Kane, C. J. and Gehring, W. J. (1987). Detection in situ of genomic regulatory elements in Drosophila. *Proc Natl Acad Sci U S A* **84**, 9123-7.
Ochoa-Espinosa, A., Yucel, G., Kaplan, L., Pare, A., Pura, N., Oberstein, A., Papatsenko, D. and Small, S. (2005). The role of binding site cluster strength in Bicoid-dependent patterning in Drosophila. *Proc Natl Acad Sci U S A* **102**, 4960-5.
Padgett, R. W., Das, P., Krishna, S. (1998). TGFBeta signaling, Smads, and tumor suppressors. *Bioessays* **20**, 382-391.
Papatsenko, D. and Levine, M. (2005). Quantitative analysis of binding motifs mediating diverse spatial readouts of the Dorsal gradient in the Drosophila embryo. *Proc Natl Acad Sci U S A* **102**, 4966-71.
Pyrowolakis, G., Hartmann, B., Muller, B., Basler, K. and Affolter, M. (2004). A simple molecular complex mediates widespread BMP-induced repression during Drosophila development. *Dev Cell* **7**, 229-40.

Raftery, L. A. and Sutherland, D. J. (1999). TGF-beta family signal transduction in Drosophila development: from Mad to Smads. *Dev Biol* **210**, 251-68.
Roberts, D. B. (1998). Drosophila: A Practial Approach. Oxford: Oxford University Press.
Roberts, M. R., Srinivas, M., Forrest, D., Morreale de Escobar, G. and Reh, T. A. (2006). Making the gradient: thyroid hormone regulates cone opsin expression in the developing mouse retina. *Proc Natl Acad Sci U S A* **103**, 6218-23.
Rushlow, C., Colosimo, P. F., Lin, M. C., Xu, M. and Kirov, N. (2001). Transcriptional regulation of the Drosophila gene zen by competing Smad and Brinker inputs. *Genes Dev* **15**, 340-51.
Saller, E. and Bienz, M. (2001). Direct competition between Brinker and Drosophila Mad in Dpp target gene transcription. *EMBO Rep* **2**, 298-305.
Saller, E., Kelley, A. and Bienz, M. (2002). The transcriptional repressor Brinker antagonizes Wingless signaling. *Genes Dev* **16**, 1828-38.
Sambrook, J. a. R., D.W. (2001). Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Schmierer, B. and Hill, C. S. (2007). TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* **8**, 970-82.
Segalat, L., Berger, G. and Lepesant, J. A. (1994). Dissection of the Drosophila pourquoi-pas? promoter: complex ovarian expression is driven by distinct follicle cell- and germ line-specific enhancers. *Mech Dev* **47**, 241-51.
Sekelsky, J. J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H. and Gelbart, W. M. (1995). Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. *Genetics* **139**, 1347-58.
Sekiya, T. and Zaret, K. S. (2007). Repression by Groucho/TLE/Grg proteins: genomic site recruitment generates compacted chromatin in vitro and impairs activator binding in vivo. *Mol Cell* **28**, 291-303.
Shi, Y., Sawada, J., Sui, G., Affar el, B., Whetstine, J. R., Lan, F., Ogawa, H., Luke, M. P. and Nakatani, Y. (2003). Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* **422**, 735-8.
Sivasankaran, R., Vigano, M. A., Muller, B., Affolter, M. and Basler, K. (2000). Direct transcriptional control of the Dpp target omb by the DNA binding protein Brinker. *Embo J* **19**, 6162-72.
Smale, S. T. and Kadonaga, J. T. (2003). The RNA polymerase II core promoter. *Annu Rev Biochem* **72**, 449-79.
Small, S., Arnosti, D. N. and Levine, M. (1992). Regulation of even-skipped stripe 2 in the Drosophila embryo. *Embo J* **11**, 4047-57.
Spiegelman, B. M. and Heinrich, R. (2004). Biological control through regulated transcriptional coactivators. *Cell* **119**, 157-67.
St Johnston, R. D. a. N.-V., C. (1992). The origin of pattern and polarity in the Drosophila embryo. *Cell* **68**, 201-19.
Stathopoulos, A. and Levine, M. (2005). Genomic regulatory networks and animal development. *Dev Cell* **9**, 449-62.
Strigini, M. and Cohen, S. M. (1999). Formation of morphogen gradients in the Drosophila wing. *Semin Cell Dev Biol* **10**, 335-44.

Suzuki, Y., Tsunoda, T., Sese, J., Taira, H., Mizushima-Sugano, J., Hata, H., Ota, T., Isogai, T., Tanaka, T., Nakamura, Y. et al. (2001). Identification and characterization of the potential promoter regions of 1031 kinds of human genes. *Genome Res* **11**, 677-84.
Tabata, T. (2001). Genetics of morphogen gradients. *Nat Rev Genet* **2**, 620-30.
Teleman, A. A. and Cohen, S. M. (2000). Dpp gradient formation in the Drosophila wing imaginal disc. *Cell* **103**, 971-80.
ten Dijke, P., Miyazono, K. and Heldin, C. H. (2000). Signaling inputs converge on nuclear effectors in TGF-beta signaling. *Trends Biochem Sci* **25**, 64-70.
Vincent, J. P. and Briscoe, J. (2001). Morphogens. *Curr Biol* **11**, R851-4.
Vincent, S. and Perrimon, N. (2001). Developmental biology. Fishing for morphogens. *Nature* **411**, 533, 535-6.
Wharton, K. A., Jr. and Crews, S. T. (1993). CNS midline enhancers of the Drosophila slit and Toll genes. *Mech Dev* **40**, 141-54.
Willy, P. J., Kobayashi, R. and Kadonaga, J. T. (2000). A basal transcription factor that activates or represses transcription. *Science* **290**, 982-5.
Winter, S. E. and Campbell, G. (2004). Repression of Dpp targets in the Drosophila wing by Brinker. *Development* **131**, 6071-81.
Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J Theor Biol* **25**, 1-47.
Wolpert, L. (2002). Principles of Development. New York: Oxford University Press.
Xu, M., Kirov, N. and Rushlow, C. (2005). Peak levels of BMP in the Drosophila embryo control target genes by a feed-forward mechanism. *Development* **132**, 1637-47.
Xu, X., Yin, Z., Hudson, J. B., Ferguson, E. L. and Frasch, M. (1998). Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the Drosophila mesoderm. *Genes Dev* **12**, 2354-70.
Yamamoto, Y. and Oelgeschlager, M. (2004). Regulation of bone morphogenetic proteins in early embryonic development. *Naturwissenschaften* **91**, 519-34.
Yamasaki, Y. and Nishida, Y. (2006). Mi-2 chromatin remodeling factor functions in sensory organ development through proneural gene repression in Drosophila. *Dev Growth Differ* **48**, 411-8.
Yanagita, M. (2005). BMP antagonists: their roles in development and involvement in pathophysiology. *Cytokine Growth Factor Rev* **16**, 309-17.
Yao, L. C., Blitz, I. L., Peiffer, D. A., Phin, S., Wang, Y., Ogata, S., Cho, K. W., Arora, K. and Warrior, R. (2006). Schnurri transcription factors from Drosophila and vertebrates can mediate Bmp signaling through a phylogenetically conserved mechanism. *Development* **133**, 4025-34.
Yao, L. C., Phin, S., Cho, J. Y., Rushlow, C., Arora, K. and Warrior, R. (2008). Multiple modular promoter elements drive graded brinker expression in response to the Dpp morphogen gradient. *Development* **135**, 2183-2192.
Zecca, M., Basler, K. and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the Drosophila wing. *Development* **121**, 2265-78.
Zhang, H., Levine, M. and Ashe, H. L. (2001). Brinker is a sequence-specific transcriptional repressor in the Drosophila embryo. *Genes Dev* **15**, 261-6.