Repression of Dpp targets in the *Drosophila* wing by Brinker

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During development, gradients of transcription factors regulate patterning along a body axis by activation or repression of target genes above distinct threshold levels. In the *Drosophila* wing, expression of genes such as *spalt (sal)* and *optomotor-blind (omb)* is restricted along the A/P axis by lateral-to-medial gradients of the transcriptional repressor Brinker (Brk). *sal* is more sensitive to repression by Brk than *omb*, and thus has a narrower expression domain compared to *omb*. The Brk expression pattern is established by Decapentaplegic (Dpp), a TGF-β superfamily member, which forms a complementary medial-to-lateral morphogen gradient along the (A/P) axis of the wing. We have investigated the mechanisms Brk uses to repress gene expression to gain an understanding of why some targets such as *sal* are more sensitive to Brk than others such as *omb*. Previous studies have suggested that Brk represses gene expression simply by competing with activators, but we show that Brk requires an active repression domain along with the DNA binding domain for repressor activity. Brk possesses four repression domains, but these domains are not equivalent; for example, 3R is sufficient to repress *omb* but not *sal*. Consequently, although *sal* and *omb* show quantitative differences in their response to Brk, there are qualitative differences in the mechanisms that Brk uses to repress each of these genes.

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PREFACE

My thesis contains work completed in two different laboratories: Chapter 2 describes the research completed in the laboratory of Dr. Gerard Campbell and Chapter 3 describes the research completed in the laboratory of Dr. Richard Carthew.

1 Chapter 1: Introduction

One of the fundamental questions in developmental biology is: How does a single, undifferentiated cell produce millions of cells that give rise to structures such as arms, legs, organs and ultimately the entire organism? How do these dividing cells, derived from a single cell, become different from each other? What influences direct these cells to form organized structures? What controls the behavior of these cells and allows complex pattern formation within each tissue? The ability of cells to become different from their neighbors relies on differential gene expression in which genes are turned on and off according to temporal and spatial cues received from their environment. The cues in the environment correspond predominantly to different types of secreted signaling polypeptides, and these molecules control gene expression via signaling pathways that regulate the activity of transcription factors. However, a given signal can be interpreted and elicit different responses depending on the particular cell type or the position of a cell within a cellular field (Wolpert, 1969). Furthermore, it has been determined that there are only a limited number of signaling pathways utilized for setting up the body pattern during development (Gerhart, 1999).

1.1 Morphogens/Secreted signaling molecules

Several of these secreted signaling molecules function as morphogens or "form-generating" substances that diffuse through a tissue, their distribution directing the development of cells in a tissue (Turing, 1952). Wolpert (1969) proposed a model of positional information, where the position of the cell within a cellular field is defined by a coordinate system. Inherent

in the model is the presence of a positional signaling factor or morphogen produced at a localized source that diffuses across the cellular field producing an extracellular gradient which can define the position along the ordinate or abscissa (x, y, if we are dealing with 2D Cartesian coordinates). Cells will adopt different fates if they can measure different levels of this factor along one developing axis within a tissue, and can respond in a concentration dependent manner (Fig. 1) (Wolpert, 1969). In molecular terms, this translates into different genes being activated above distinct threshold levels of signaling within a cell. The ability of a morphogen to activate gene expression decreases as a function of its distance from the source (Vincent and Perrimon, 2001). Genes receptive to low levels of signal should be expressed by cells at a farther distance from the source of the secreted ligand than those requiring higher levels of activity (Strigini and Cohen, 1999). Consequently, the intracellular signal transduction pathway receiving the signal must be able to discriminate the concentration of the morphogen and only activate target genes above corresponding threshold levels. For example, threshold levels can be thought of as the amount of morphogen required to bind receptor molecules to elicit activation of intracellular signaling pathways to an appropriate level. Therefore, cells will take on different fates depending on their position in a cellular field relative to the source of the morphogen (Vincent and Perrimon, 2001).

1.2 Regulation of Eukaryotic gene expression

In addition to the regions coding for a particular protein, a eukaryotic gene also contains control regions consisting of promoter and response elements that regulate the genes expression (Maniatis et al., 1987). The promoter region is the sequence specific site where RNA Polymerase II and other proteins known as general transcription factors bind DNA to form the

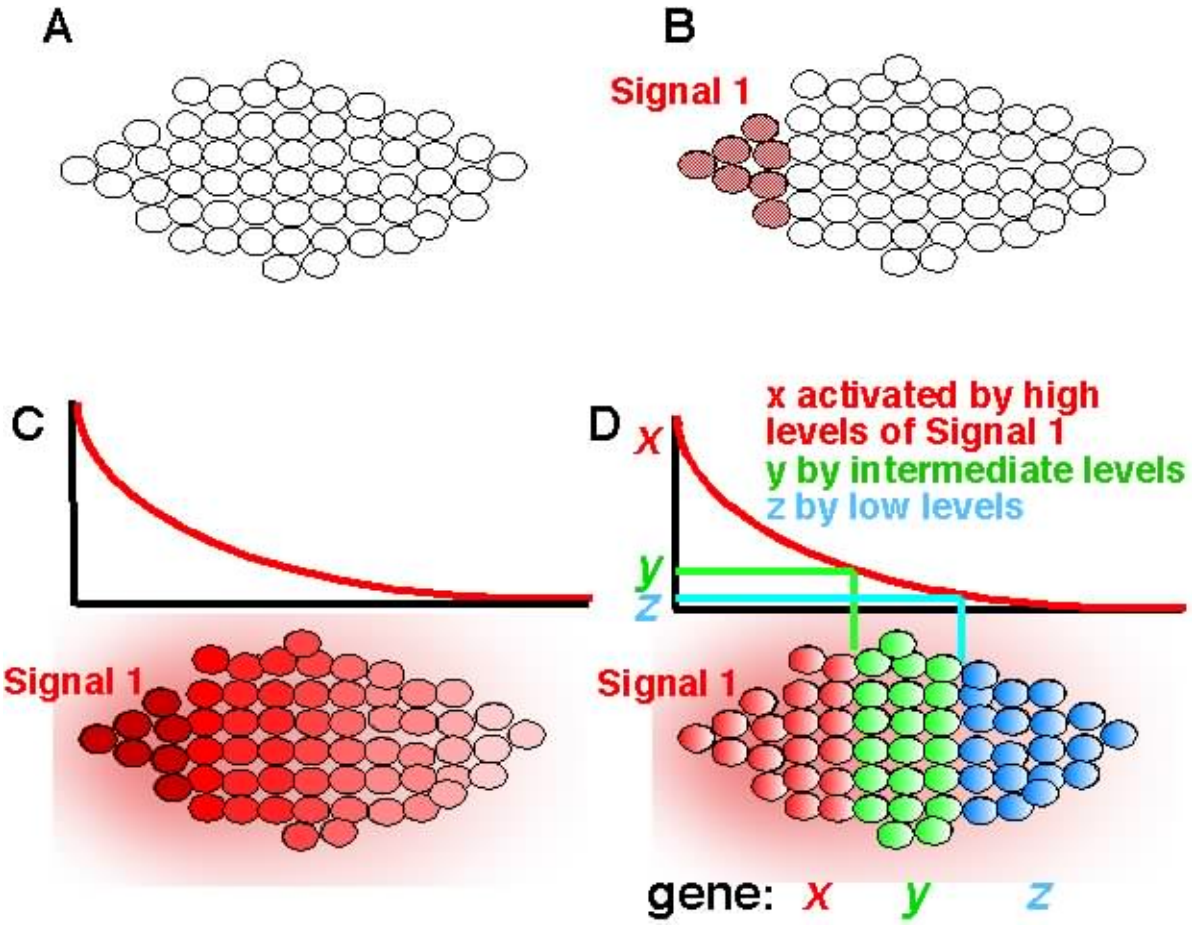

Figure 1. Morphogen Model.

(A) Cellular field. (B) Signal 1 represents a localized source of a diffusible signaling molecule with high levels near the source (red hatched circles). (C) Diffusion will generate a gradient across the cellular field with the highest level of morphogen nearest the source and the lowest levels furthest from the source (dark red circles, high levels and light pink circles, low levels). (D) Different target genes will be activated above distinct threshold levels; gene x (red) will be activated by high levels, gene y (green) by intermediate levels and gene z (blue) by low levels of the morphogen.

pre-initiation complex (Gaston and Jayaraman, 2003). This region can be either the TATA box or a Downstream Promoter Element (DPE). Transcriptional regulation in eukaryotes differs from prokaryotes because RNA polymerase II cannot initiate transcription without other general transcription factors, which must be assembled at the promoter for transcription to begin (Novina and Roy, 1996). Response elements may be adjacent to the promoter or thousands of base pairs away from the promoter, and can activate (enhancer) or repress (silencer) transcription at a specific promoter (Maniatis et al., 1987). Promoters and enhancers contain binding sites for regulatory proteins known as regulatory transcription factors, and transcription of a gene can be activated or inhibited by transcription factors binding at these sites (Maniatis et al., 1987). Enhancer regions are crucial in regulating the expression of genes during development, and the interaction of transcription factors with transcriptional machinery (RNA Polymerase II and general transcription factors) regulates transcription initiation (Gaston and Jayaraman, 2003; Hanna-Rose and Hansen, 1996; Johnson, 1995). Eukaryotic genes contain extremely complex regulatory regions (response elements) with binding sites for many different regulatory transcription factors, allowing for differential gene expression. Regulatory transcription factors can be either activator or repressor proteins.

1.2.1 Transcriptional Activation

Transcriptional activation is mediated by activator proteins, sequence specific DNA-binding proteins that stimulate transcription at a promoter (Gaston and Jayaraman, 2003). Activator proteins are usually modular, containing a DNA binding domain (DBD) that recognizes a specific regulatory sequence and an activation domain that interacts with transcriptional machinery to initiate transcription (Kornberg, 1999). Activator proteins

frequently interact with another group of proteins known as co-activators, that cannot bind DNA directly, but rather they assist the activator in the stimulation of pre-initiation complex formation (Gaston and Jayaraman, 2003). Activators and co-activators can also stimulate transcription by promoting changes in chromatin (complex of DNA and associated proteins that make up chromosomes) structure near the promoter by recruitment of histone modification enzymes, chromatin binding proteins and ATP-dependent nucleosome remodeling proteins (Gaston and Jayaraman, 2003; Kornberg, 1999). Co-activators may recruit histone acetyltransferases or possess histone acetyltransferase activity, leading to acetylation of histone proteins and remodeling of chromatin structure (Kornberg, 1999). One of the best known co-activators is CREB binding protein (CRB), which contains a histone acetyltransferase activity implicated in chromatin decondensation, allowing the transcriptional machinery to bind the promoter (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). These changes in chromatin structure regulate transcription by altering the accessibility of DNA to transcription factors or the transcriptional machinery (Kornberg, 1999; Kornberg and Lorch, 2002).

1.2.2 Transcriptional Repression

Transcriptional repression also plays a critical role in gene regulation. During development, gene expression boundaries can be established by spatially restricted localization or activity of transcriptional repressors (Mannervik et al., 1999). In addition, gene expression is controlled by extracellular signaling molecules, and signal responsive genes are maintained in the ‘off’ state by repressor proteins until an appropriate signal is transduced resulting in de-repression (Roose and Clevers, 1999). Transcription factors that function as repressors bind to specific sequences in control regions of genes they regulate, and prevent the initiation of

transcription by RNA polymerase II (Cowell, 1994). Similar to transcriptional activators, repressors can also recruit additional proteins, known as co-repressors, which assist the repressor in inhibiting transcription initiation (Gaston and Jayaraman, 2003). Transcriptional repressors can function in a number of ways including passive repression or active repression which is broken down into two classes, direct repression and quenching (Hanna-Rose and Hansen, 1996). In addition, transcriptional repressors can be grouped by their ability to mediate long-range or short-range repression (Gray and Levine, 1996).

1.2.2.1 Passive Repression

Passive repression can involve a repressor blocking transcription by competing for DNA binding sites with an activator protein. If the repressor protein is present at a higher concentration, it will out compete the activator (Cowell, 1994). In addition, passive repression could also involve competing for something besides a binding site that is needed for transcription initiation (Johnson, 1995). However, passive repression appears to be used infrequently as a mechanism for repression in eukaryotes, possibly due to the complex organization of regulatory regions in eukaryotic genes (Johnson, 1995).

1.2.2.2 Active Repression

In order for DNA binding proteins to repress by active mechanisms, they require additional domains or motifs in addition to the DNA binding domain (Hanna-Rose and Hansen, 1996). These domains/motifs may function autonomously, or they may function by recruiting

additional proteins or co-repressors which are able to mediate direct repression or quenching, usually by remodeling chromatin structure (Hanna-Rose and Hansen, 1996).

1.2.2.2.1 Direct Repression

Direct repression refers to the ability of a repressor to interfere with the activity of the basal transcriptional machinery at the promoter. This type of repression can be very specific for a single component of the transcriptional machinery such as binding to a general transcription factor or RNA Polymerase II (Hanna-Rose and Hansen, 1996). Interactions of repressors with co-repressors that interfere with the transcriptional machinery are also classified under direct repression. Ultimately, the interactions between repressor/co-repressor and the transcriptional machinery prevent pre-initiation complex formation or further interactions with other activator proteins required for transcription initiation (Hanna-Rose and Hansen, 1996).

1.2.2.2.2 Quenching

The complexity of regulatory regions allows both activators and repressors to bind DNA, but the repressor may interfere with the activity of an activator, known as quenching (Hanna-Rose and Hansen, 1996). Repression via quenching can potentially be specific for individual promoters or activators, or non-specific with general blocking of any activator protein (Hanna-Rose and Hansen, 1996). Quenching can occur by direct interaction of a repressor protein with an activator, with a co-activator or by interaction with a general transcription factor that is receptive to specific activators (Hanna-Rose and Hansen, 1996).

1.2.2.2.3 Chromatin Modification

Adding to the complexity of active mechanisms of repression, there are the mechanisms that modify chromatin structure to make genes less accessible to RNA polymerase (Gaston and Jayaraman, 2003). If a repressor/co-repressor complex can modify chromatin structure when bound close to the promoter, then it could appear to be directly repressing or quenching, and may appear as a non-specific quencher. Therefore, chromatin modification and some mechanisms of active repression are interconnected.

1.2.3 Long and Short Range Repression

For long-range repression, a repressor protein renders a promoter inactive to all enhancers, even when the enhancers are located thousands of base pairs from where the repressor protein is bound (Courey and Jia, 2001). The co-repressor Groucho (Gro), a protein that does not bind DNA directly, but is recruited to DNA by a transcriptional repressor, mediates long-range repression and acts over 1 kb of DNA (Cai et al., 1996; Chen and Courey, 2000; Fisher and Caudy, 1998; Mannervik et al., 1999; Parkhurst, 1998). The interaction of Gro with repressors functions to actively silence transcription of promoters in a global manner (Barolo and Levine, 1997). Gro was originally identified as a transcriptional co-repressor via its role in repression by the Hairy family of repressor proteins that contain a characteristic WRPW motif, and this motif is necessary and sufficient for recruitment of Gro (Chen and Courey, 2000; Fisher et al., 1996; Grbavec and Stifani, 1996; Paroush et al., 1994). Variations of the WRPW motif such as WRPY in *Drosophila* have also been shown to recruit Gro (Aronson et al., 1997).

There are several ways in which repressor proteins can mediate long range repression including: a) regulation of chromatin structure, by modifying the acetylation state of histones

(hypoacetylated) via recruitment of deacetylases (Chen et al., 1999; Choi et al., 1999) and b) interference with basal transcriptional machinery. Long-range repression may also involve the formation of a DNA loop that allows a silencer element to be in close proximity with the promoter (Yu et al., 2001).

In contrast to long-range repression, short-range repression involves repressors blocking activators bound close to repressor binding sites, while not blocking activation by more distantly bound activators (Courey and Jia, 2001). It appears that the distance over which a short-range repressor can exert its action is dependent upon the repressor concentration, enabling a sensitive way of responding to a transcription factor gradient (Hewitt et al., 1999). In the *Drosophila* embryo, the *even-skipped* (*eve*) gene provides a prime example of an autonomous enhancer regulated by short-range repression (Akam, 1989). Moreover, many short-range repressors in the embryo including Giant, Krüppel, Knirps and Snail are partially dependent on C-terminal Binding Protein (CtBP) as a co-repressor (Mannervik et al., 1999; Nibu et al., 2001). CtBP, like Gro, is a protein that does not bind DNA directly, but is recruited to DNA by a repressor protein and mediates short-range repression, acting over a distance of up to 150 bp (Gray and Levine, 1996; Nibu et al., 1998a; Nibu et al., 1998b; Poortinga et al., 1998; Zhang and Levine, 1999). CtBP is recruited by a motif having similarity to a PxDLS consensus sequence (Schaeper et al., 1995; Schaeper et al., 1998). There are several possible mechanisms CtBP uses to exert short-range repression including: a) CtBP may recruit histone deacetylases (Criqui-Filipe et al., 1999; Sundqvist et al., 1998; Zhang et al., 2001a) and b) CtBP may act by quenching, which involves interactions of repressor/co-repressor complex with activators bound to nearby sites (Gray and Levine, 1996).

The major advantage for recruitment of CtBP is that it allows repression of one enhancer without interfering with activity of a nearby enhancer, which would be repressed with Gro recruitment (Chen and Courey, 2000; Chinnadurai, 2002; Nibu et al., 2001). Therefore, most transcriptional repressors recruit only one of these co-repressors. There are two transcription factors that recruit CtBP and Gro, Hairy and Hairless. But in the case of Hairy, CtBP functions to antagonize Gro activity rather than its typical role as a co-repressor (Phippen et al., 2000; Zhang and Levine, 1999). In contrast, both CtBP and Gro confer repressor activity for Hairless (Barolo et al., 2002). In addition to Hairy and Hairless, Brinker (Brk) is another transcriptional repressor containing interaction motifs for CtBP and Gro and has been shown to interact with both of these co-repressors (Hasson et al., 2001).

1.3 Transcriptional regulators and signaling pathways

Some transcription factors are always active and available to bind at regulatory elements, but the activity of other transcription factors are regulated by signaling pathways. Upon pathway activation, transcription factors can be modified (e.g. phosphorylated), resulting in changes of their activity. Consequently, for these signal regulated factors to be activated and exert their action, they require input from a signaling pathway which modifies the activity of a particular transcription factor (Barolo and Posakony, 2002). To analyze the activity of the input of a signaling pathway that controls regulatory transcription factors, reporter constructs can be generated in which a LacZ or GFP reporter can be fused to the enhancer/promoter of a gene and transcriptional output can be assessed based on the level of reporter gene expression (Barolo et al., 2000). Binding sites contained within enhancer/promoter regions can be altered to analyze

the difference in reporter gene expression compared to normal regions to determine the importance of these sites for regulation of gene expression.

1.4 Signal Transduction Pathways

Although there are multiple developmental outputs, there are a limited number of signaling pathways controlling cell fate decisions, including Wnt, TGF-β, Hedgehog, receptor tyrosine kinase, nuclear receptor, Jak/STAT and Notch pathways (Barolo and Posakony, 2002; Gerhart, 1999). Each of these signaling pathways are used over and over again throughout the development of an organism to activate target gene expression corresponding to various developmental contexts via signal regulated transcription factors. Mechanisms of signal transduction utilized by each of these pathways are very different from one another, but the output is the same, activation of target genes (Barolo and Posakony, 2002). The seven signaling pathways listed above utilize different mechanisms of signal transduction from direct transcriptional regulation by nuclear receptor proteins to the phosphorylation cascades of receptor tyrosine kinase pathways (Barolo and Posakony, 2002).

A primary role of signaling pathways is to regulate transcription of a subset of target genes following binding of a ligand to its corresponding receptor, but the ultimate function of the pathways is to regulate the activity of one or more transcription factors specific to that pathway (Barolo and Posakony, 2002). In most cases, signaling results in activation of a transcription factor to promote transcription of specific target genes containing binding sites for this transcription factor. However, transcriptional regulation via signaling pathways is more complex than this simple picture, and three basic properties have been described for this process during

development: ‘activator insufficiency’, ‘cooperative activation’ and ‘default repression’ (Barolo and Posakony, 2002).

Activator insufficiency describes the observation that activation of a single signaling pathway in a cell, and consequently activation or one or more transcription factors by this pathway, is not usually sufficient on its own to induce expression of a known ‘target’ gene (Barolo and Posakony, 2002). The reason for this is inherent in ‘cooperative activation’, which describes the general requirement for more than one transcription factor for activation of a particular target gene (Barolo and Posakony, 2002). Activation of target genes is not simply dependent upon the positive action of a transcriptional activator, because these targets are often being actively repressed in the absence of signal, a property referred to as ‘default repression’ (Barolo and Posakony, 2002). The Wnt and Notch signaling pathways have the interesting property that the transcription factor responsible for default repression, dTCF/LEF and Su(H), respectively, is also the factor responsible for activating target genes. Thus in the absence of signal, these factors act as repressors, but in the presence of signal, they are converted into activators (Bray and Furriols, 2001; Klein et al., 2000; van de Wetering et al., 1997).

A combination of multiple transcription factors and tissue specific activator binding may provide the synergistic effect required for transcriptional activation (Barolo and Posakony, 2002). Following pathway activation, transcription factors along with tissue specific factors can bind to specific response elements in promoters or enhancer regions of target genes and activate transcription. Although local activators are present, transcriptional repressors keep target genes in an inactive state until an appropriate signal is transduced via the receptor signaling complex (Barolo and Posakony, 2002). A combination of the above mentioned transcriptional controls enable the limited number of signaling pathways to specifically activate target gene expression

only under the proper conditions, otherwise transcription at these genes remains inactive (Barolo and Posakony, 2002). Furthermore, the ability of a limited number of signaling pathways to elicit a multitude of cellular responses demonstrates the exceptional specificity in both repression and activation of target genes leading to such responses.

1.5 Patterning in the *Drosophila* embryo by transcription factor gradients

During development, a single transcription factor can provide the information to establish different spatial patterns of gene expression along a body axis (either A/P or D/V) if the transcription factor concentration forms a gradient along the axis and if target genes are differentially sensitive to different concentrations of the transcription factor. In the *Drosophila* egg, *bcd* mRNA is localized at the anterior of the egg, but following fertilization, it is translated and Bcd protein diffuses posteriorly producing a gradient along the anterioposterior axis (St Johnston and Nusslein-Volhard, 1992). The Bcd gradient sets up segmentation of anterior structures including the head and thorax (Driever and Nusslein-Volhard, 1988a; Driever and Nusslein-Volhard, 1988b; Frohnhofer et al., 1986). In addition, Bcd activates high levels of *hunchback*, another transcription factor that forms a gradient along the A/P axis and establishes specific domains of gap (*giant*, *Krüppel* and *knirps*) and pair rule (*even-skipped*, *fushi tarazu* and *hairy*) genes along this axis (Rivera-Pomar and Jackle, 1996). For example, high levels of Bcd in the anterior activate expression of *buttonhead* and *orthodenticle*, whereas lower levels activate *hunchback* and *Krüppel*, and even lower levels activate *knirps* (Burz et al., 1998; Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990). The proteins produced by the gap and pair rule genes in turn form concentration gradients controlling genes that further define segmental

compartments of the embryo including the segment polarity genes and homeotic selector genes (Lewis, 1978; Nusslein-Volhard and Wieschaus, 1980).

Patterning of the embryonic dorsoventral (D/V) axis is dependent upon a nuclear-cytoplasmic gradient of the transcription factor Dorsal. Dorsal protein is localized in the nucleus in the ventral region of the embryo and in the cytoplasm in dorsal regions of the embryo (Stathopoulos and Levine, 2002). The expression patterns of genes along the D/V axis such as *rhomboid*, *tolloid*, *decapentaplegic* (*dpp*), *zerknullt*, *twist*, *snail* and *single-minded* are determined by their sensitivity to either activation or repression by Dorsal protein, and these gradient thresholds established along the D/V axis initiate differentiation of mesoderm, neurogenic ectoderm and dorsal ectoderm (Stathopoulos and Levine, 2002). For example, high levels of Dorsal activate *twist* and *snail*, and low levels activate *rhomboid, short gastrulation* and *single minded*, to name a few.

In the early *Drosophila* embryo, gradients of transcription factors such as Bcd and Hb can be established along the A/P axis from a localized source of RNA because the embryo is a syncytium and the factors can diffuse in the cytoplasm (St Johnston and Nusslein-Volhard, 1992). In other systems gradients of transcription factors, or more correctly, gradients of activated transcription factors (in D/V this corresponds to a gradient of nuclear Dorsal) are also established along developing axes. However, the gradients cannot be established in the same way as for the A/P axis in the embryo because there are membranes between the cells, so gradients are usually established by a gradient of an extracellular signal. For example, in D/V patterning, a gradient is produced by a processed form of the Spatzle protein, which is present in the perivitelline space surrounding the embryo, where it is processed in the ventral region producing a ventral to lateral to dorsal gradient (Morisato and Anderson, 1994). Activation of

the Toll receptor by Spatzle promotes nuclear localization of Dorsal (Morisato and Anderson, 1994; Stathopoulos and Levine, 2002). In other systems including the *Drosophila* wing, gradients of extracellular signaling proteins result in a similar gradient of the activated form of the transcription factor, in which modification of the transcription factor by the signaling pathway occurs downstream of the signaling protein. One of these signaling proteins is Decapentaplegic (Dpp), a TGF-β homolog, whose gradient controls patterning along the antero-posterior axis of the developing *Drosophila* wing.

1.6 Imaginal discs and the adult wing

Imaginal disc transplantation studies revealed that the primordia of adult structures, such as wing blade or notum, originate at specific regions within the disc and allowed fate maps to be developed (Cohen, 1993). For example, the center of the wing pouch corresponds to the distal tip of the adult wing blade and the line delineating the A/P axis of the wing disc corresponds to the center of the wing blade just anterior to wing vein IV (Fig. 2A,B) (Blair, 1995; Cohen, 1993). When the wing forms at metamorphosis, the ventral surface folds under the dorsal surface in the distal region to form the adult wing (Cohen, 1993). Consequently, the adult wing is composed of two sheets of cuticle, previously secreted by epidermal cells, which are fused at the margin (Fig. 2B) (Cohen, 1993). Each wing has several distinct invariant features including veins and bristles that can be used to identify abnormalities.

We are interested in understanding regulation of patterning during animal development and how these intricate patterns of differentiated cells produce functioning structures such as appendages. The *Drosophila* wing is an excellent system to study pattern formation due to the vast knowledge of the regulation of gene function and the numerous techniques developed in

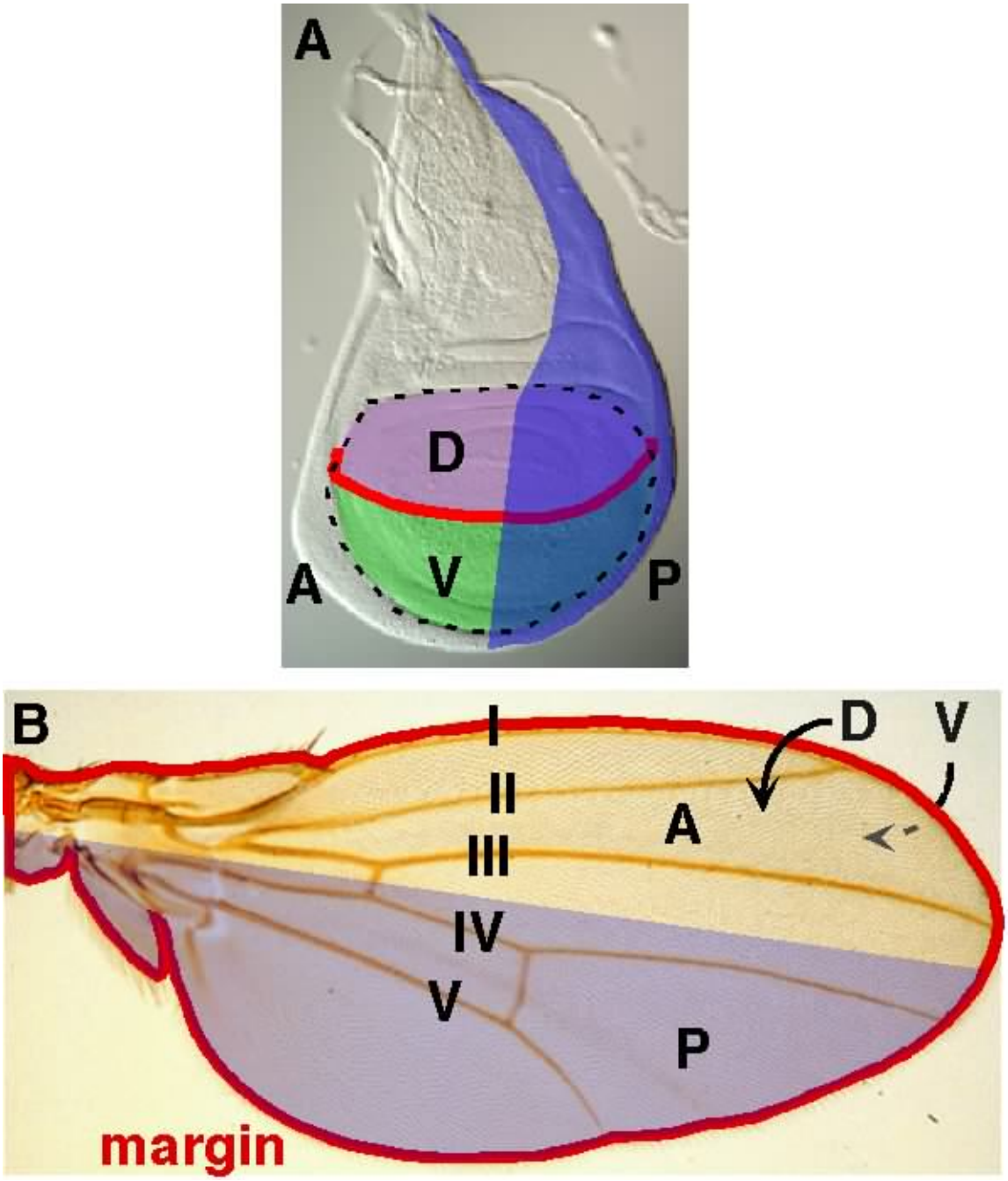

Figure 2. Fate map of wing imaginal disc and projection onto adult wing.

(A) Wing imaginal disc showing regions that correspond to adult wing regions. Only a portion of the wing disc gives rise to the future wing blade (dashed region). The wing disc is divided into anterior (A) and posterior (P) compartments, as well as dorsal (D) and ventral (V) compartments. (B) The adult wing is composed of dorsal and ventral sheets fused at the margin. Designation of the anterior and posterior regions of the wing and positioning of the five invariant wing veins.

Drosophila, allowing genetic and molecular analysis of regulation of differential gene expression.

In *Drosophila*, adult appendages derive from larval imaginal discs. An imaginal disc is a single-layered sac composed of columnar epithelial cells that originate in the embryo and give rise to adult structures such as legs and wings (Cohen, 1993). During early larval development the fate of imaginal disc cells are uncommitted, but by the third instar, the discs are fully patterned and divided into distinct regions or compartments (Blair, 1995). The wing imaginal disc is divided into anterior (A) and posterior (P) compartments along the A/P axis and into dorsal (D) and ventral (V) compartments along the D/V axis (Fig. 2A), and cells within one compartment do not intermix with cells from another compartment, as compartments are regions of lineage restriction (Garcia-Bellido et al., 1973; Garcia-Bellido et al., 1976). Expression of the selector gene *engrailed* (*en*), by cells in the posterior compartment but not the anterior compartment, provides these cells with their posterior identity (Guillen et al., 1995; Simmonds et al., 1995; Tabata et al., 1995).

1.7 Control of *Drosophila* wing development by TGF-β homolog Dpp

The morphogen patterning an axis lies at the boundary between compartments. In the wing disc, *dpp* RNA is expressed as a narrow stripe along the center of the A/P axis just anterior to the interface between the anterior and posterior compartments (Fig. 3) (Blackman et al., 1991; Masucci et al., 1990). Following synthesis, the secreted Dpp protein forms an almost symmetrical medial to lateral gradient in the anterior and posterior compartments (Fig. 3) (Entchev et al., 2000; Teleman and Cohen, 2000). Genes requiring high levels of Dpp are

activated nearest to the Dpp source, whereas genes requiring lower levels of activity are transcribed in a wider expression domain, producing nested expression domains of target genes around the Dpp source (Lecuit et al., 1996; Nellen et al., 1996; Zecca et al., 1995). For example, the *spalt* (*sal*) gene is transcribed in regions where Dpp levels are high, while the *optomotor blind* (*omb*) gene is transcribed in a wider domain responding to both higher and lower levels of Dpp and the vestigial quadrant enhancer (vg-QE, an enhancer recapitulating a portion of the expression of the *vestigial* gene) responds to even lower levels of Dpp (Fig. 3) (Kim et al., 1996; Lecuit and Cohen, 1998; Nellen et al., 1996; Zecca et al., 1995). Consequently, the corresponding expression patterns of these genes along the A/P axis relates to their response to Dpp signaling: the *sal* expression domain is more narrow than *omb* which is more narrow than *vg-QE* (Fig. 3). Although *sal* and *omb* are each regulated by distinct levels of Dpp, it remains to be determined how Dpp regulates gene expression in a concentration dependent manner.

1.8 Transforming Growth Factor-β Signaling Pathway

Members of the Transforming Growth Factor-β (TGF-β) superfamily of secreted signaling molecules/growth factors influence a plethora of developmental processes in multicellular organisms, by controlling the transcription of target genes in responding cells (Shi and Massague, 2003; ten Dijke et al., 2000; Whitman, 1998; Wozney, 1998). Some of the cellular responses controlled by TGF-β superfamily members include changes in cell shape, proliferation, cell cycle regulation, apoptosis and specification of cell fate (Raftery and Sutherland, 1999).

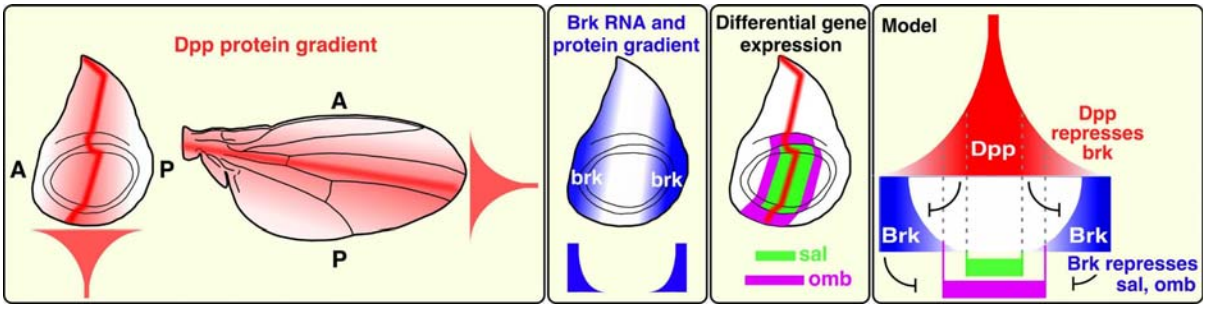

Figure 3. Regulation of gene expression in the *Drosophila* wing by Dpp and Brk.

dpp RNA is expressed in the center of the anteroposterior (A/P) axis of the wing disc. Following secretion, the protein becomes distributed in a medial-to-lateral gradient in the anterior and posterior halves. How this would project onto the adult wings is also shown, although Dpp is not expressed there at this stage. Brk is expressed at high levels in the lateral regions of the wing disc and shows graded expression towards the center; its expression is directly regulated by Dpp. Dpp target genes, *sal* and *omb* are expressed in a nested pattern centered on the stripe of Dpp expression with *sal* requiring higher levels of Dpp than *omb*. *sal* and *omb* are repressed by Brk and the model proposes that they are differentially sensitive to Brk so that *sal* is repressed by lower levels than *omb* and this explains why the *sal* expression domain is narrower.

1.8.1 Receptor Complex and signaling

The effectors of TGF-β signaling are transmembrane serine-threonine kinases designated type I and type II receptors (Massague, 1998; ten Dijke et al., 1996) . Type I receptors possess a glycine-serine rich region (GS domain) located N-terminal to the kinase domain. Phosphorylation of the GS domain by the type II receptor is critical for signaling by the receptor complex (Padgett et al., 1998). In contrast, the type II receptor is a constitutively active kinase that activates the type I receptor upon phosphorylation (Padgett et al., 1998). Following ligand binding, the type I and type II receptors dimerize forming a heteromeric complex in which the type II receptor will then phosphorylate the type I receptor (ten Dijke et al., 2000). In order for signaling to occur, both the type I and type II receptors must be present, otherwise signaling is blocked (Luo and Lodish, 1996). Moreover, the type I receptor is required to phosphorylate its substrates, the Smad protein, for transmission of the signal (Kretzschmar et al., 1997; Macias-Silva et al., 1996; Souchelnytskyi et al., 1997).

1.8.2 Smad Family of signal transducers

The Smad family of proteins is classified into three groups: the receptor-regulated Smads (R-Smad), the Co-mediator Smads (Co-Smad) and the inhibitory Smads (I-Smad) (Mehra and Wrana, 2002; Shi and Massague, 2003). Smads are predominantly cytoplasmic in the absence of ligand, but upon ligand binding, R-Smads (vertebrate Smad 1, 2, 3, 5, 6, 7, 8 and *Drosophila* Mad) are phosphorylated by activated type I receptors leading to formation of a heteromeric complex with a Co-Smad (Kretzschmar et al., 1997; Lagna et al., 1996; Macias-Silva et al., 1996). Association of R-Smads with the activated receptor complex may be facilitated by an

anchor protein, SARA, allowing for increased recruitment to the receptor (Tsukazaki et al., 1998). The Smad complex is then translocated into the nucleus where it regulates the transcription of target genes (Kretzschmar et al., 1997; Lagna et al., 1996; Macias-Silva et al., 1996). The inhibitory Smads, on the other hand, compete with R-Smads for binding to activated receptors or for association with Co-Smads and target receptors for degradation (Shi and Massague, 2003). However, the I-Smads are only expressed in the presence of TGF-β ligand compared to the ubiquitous expression of the other Smads, implicating the I-Smads as a component of a negative feedback mechanism to downregulate TGF-β signaling (Christian and Nakayama, 1999). Ultimately, the Smad-Co-Smad complex regulates transcription as a sequence specific transcription factor by binding to cis-regulatory elements in TGF-β responsive genes and through interaction with tissue specific transcription factors (Massague and Wotton, 2000).

1.9 Decapentaplegic (Dpp) Signaling Pathway

The Decapentaplegic (Dpp) protein of *Drosophila* is a member of the TGF-β superfamily of signaling molecules closely related to vertebrate Bone Morphogenic Proteins (BMPs). Dpp is involved in numerous developmental processes including specification of the embryonic dorsoventral axis (Ferguson and Anderson, 1992; Irish and Gelbart, 1987; Podos and Ferguson, 1999), endoderm and mesoderm induction (Bienz, 1997; Frasch, 1995), and tracheal cell migration (Vincent et al., 1997). Dpp acts as a long-range morphogen, providing positional information to pattern tissues, most notably the embryonic ectoderm and the wing imaginal disc (Gelbart, 1989; Padgett et al., 1998; Spencer et al., 1982). As with other TGF-β superfamily

members, extracellular Dpp ligand binds to the type I transmembrane serine-threonine kinase receptors, Thickveins (Tkv) or Saxophone (Sax), that in turn recruit the type II receptor, Punt (Put), forming a heteromeric receptor complex (Fig. 4) (Brummel et al., 1994; Letsou et al., 1995; Nellen et al., 1996; Penton and Hoffmann, 1996; Ruberte et al., 1995). Punt, a constitutively active kinase, phosphorylates Tkv, which in turn recruits and phosphorylates the founding member of the Smad family of signal transducers, Mothers against Dpp (Mad) (Newfeld et al., 1996; Sekelsky et al., 1995). Phosphorylated Mad (P-Mad) interacts with a Co-Smad, Medea and the heteromeric complex translocates to the nucleus where it can bind cis-regulatory elements in Dpp target genes to activate or repress transcription (Fig 4) (Das et al., 1998; Hudson et al., 1998; Xu et al., 1998).

1.10 Dpp activates or represses gene expression directly

As stated previously, Dpp signaling follows the canonical TGF-β signal transduction pathway resulting in Mad (R-Smad) and Medea (Co-Smad) binding to cis-regulatory elements of target genes in various tissues to drive reporter gene expression. This includes the *vestigial* enhancer expressed across the entire wing blade and a *tinman* enhancer with expression in the dorsal mesoderm of embryos (Kim et al., 1997; Xu et al., 1998). In contrast, Dpp has also been shown to repress gene expression, again via Mad and Medea (Pyrowolakis et al., 2004). In the larval wing disc, *brk* is expressed in a lateral to medial gradient with highest levels of Brk protein at the marginal region, forming a complementary gradient to the medial to lateral Dpp gradient (Fig. 4) (Muller et al., 2003). The *brk* response elements can be divided into an enhancer and a silencer, in which the enhancer can drive ubiquitous reporter gene expression in the wing disc (Muller et al., 2003). In contrast, the Mad/Medea complex can bind the silencer region, recruit

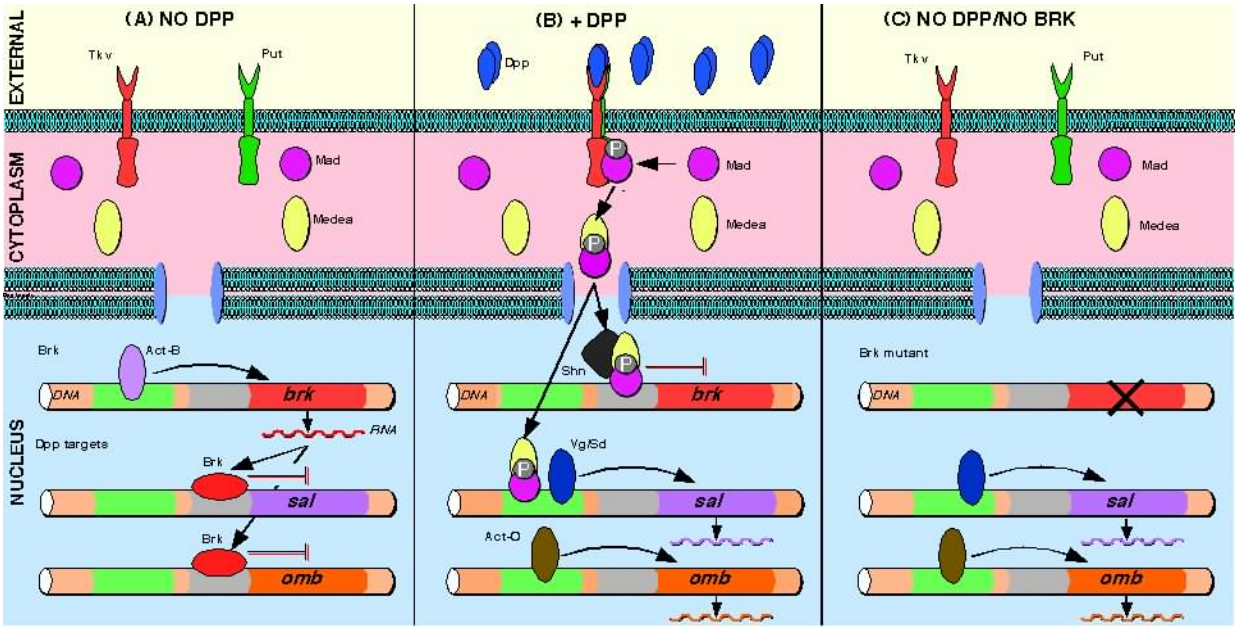

Figure 4. Model for regulation of gene expression by Dpp and Brk in the *Drosophila* wing.

(A) In the absence of Dpp, the R-Smad, Mad, and the Co-Smad, Medea, remain in the cytoplasm and this allows high-level expression of *brk* under the control of an unidentified activator, Act-B. Brk protein binds to response elements at Dpp target genes, such as *sal* and *omb* and represses their expression. (B) In the presence of Dpp, Mad is recruited to the activated receptors, Thickveins and Punt, and is phosphorylated. Mad then translocates to the nucleus in combination with Medea. In the nucleus, Mad/Medea bind to response elements in the *brk* gene and in combination with Shn, represses its expression. The absence of Brk relieves repression of Dpp target genes, which are then activated by tissue specific transcriptional activators, possibly Vg/Sd in the case of *sal*; the activator for *omb*, Act-O is unknown. Mad/Medea may also bind to Dpp targets such as *sal* and enhance activation. (C) In the absence of Dpp and Brk, Dpp targets are expressed because the tissue specific activators are sufficient, although those targets whose activation is enhanced by Mad/Medea, may be expressed at lower levels than in the presence of Dpp.

the zinc finger protein Schnurri (Shn) and act as a transcriptional repressor (Marty et al., 2000; Muller et al., 2003; Pyrowolakis et al., 2004). Experimental evidence suggests the spacing between Mad and Medea sites is critical for recruitment of Shn and is present in cis-regulatory regions of genes repressed by Dpp activity, such as *brk*, but not those genes activated by Dpp signaling (Pyrowolakis et al., 2004). The transcriptional output provided by Dpp signaling can rely solely on direct Mad/Medea activation or repression, but the transcriptional output of some target genes is regulated by repression of the Brinker (Brk) protein (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Kirkpatrick et al., 2001; Minami et al., 1999; Muller et al., 2003; Pyrowolakis et al., 2004).

1.11 Brk protein is a sequence specific transcription factor

The *brk* gene encodes a nuclear, sequence specific DNA binding protein of 704 amino acids, containing an N-terminal helix-turn-helix (HTH) DNA binding motif (Fig. 5) (Campbell and Tomlinson, 1999). Studies have revealed that Brk binds to regulatory elements of numerous TGF-β regulated genes including *omb* in the wing, as well as *zerknullt* (*zen*), *tolloid*, *labial* and *Ultrabithorax* (*Ubx*) in the embryo (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001; Sivasankaran et al., 2000). *In vitro* binding studies demonstrated that amino acids 44-99 contain the DNA binding domain, which corresponds to the predicted helix-turn-helix motif and additional N-terminal region (Saller and Bienz, 2001). DNA binding site selection and DNA footprinting identified a Brk consensus binding site of GGCGYY (Sivasankaran et al., 2000; Zhang et al., 2001b). In some enhancer regions, Brk binding sites overlap with activator sites (UbxB), while others do not (*sal*) (Guss et al., 2001; Kirkpatrick et al., 2001; Kuhnlein et al., 1997; Saller and Bienz, 2001). For the embryo target gene *zerknullt*, mutation of putative

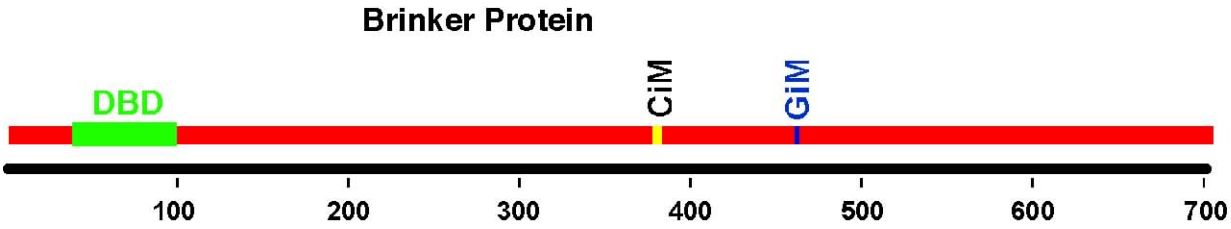

Figure 5. Schematic Representation of Brinker (Brk) Protein.

The *brk* gene encodes a 704 amino acid protein, and contains an N-terminal DNA binding domain. The protein also contains sequence corresponding to interaction motifs for transcriptional co-repressors CtBP and Groucho, CiM and GiM, respectively. Does Brk contain any additional repression domains besides the CiM and GiM? What mechanism(s) does Brk use to repress target genes?

Brk binding sites results in de-repression of reporter gene expression (Rushlow et al., 2001). In contrast, mutation of Brk binding sites in an *omb* enhancer reduces the ability of this enhancer to activate reporter gene expression, indicating the Brk binding sites overlap with those of a transcriptional activator which also fail to bind when Brk sites are mutated (Sivasankaran et al., 2000).

1.12 Dpp indirectly regulates target genes via Brk repression

In the *Drosophila* wing, Brk functions to repress expression of Dpp target genes *sal* and *omb*. In *brk* mutants, the *sal*, *omb* and vg-QE expression domains are expanded laterally (Fig 6B, D, F), whereas ectopic *brk* expression in central regions of the wing disc where *brk* is not normally expressed, results in a loss of both *sal* and *omb* expression (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999; Muller et al., 2003). Therefore, expression of *sal* and *omb* requires that Brk protein must be absent or reduced, which is the case in central portions of the wing disc corresponding to high levels of Dpp causing repression of *brk*. Consequently, does Dpp regulate target gene expression indirectly via repression of *brk*? Double mutant studies demonstrated that if Dpp signaling and *brk* are eliminated, *sal* is expressed, supporting an indirect mechanism of target gene regulation by Dpp (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Marty et al., 2000). However, *brk* mutant clones in lateral regions of the wing disc possess lower levels of *sal* expression compared to its endogenous central region, and loss of both *brk* and Mad in the central region produced a partial reduction in *sal* levels (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Marty et al., 2000). This indicates that even though Dpp signaling acting through Mad may not be needed for *sal* expression, direct Dpp signaling may be required to obtain maximal *sal* expression levels. In

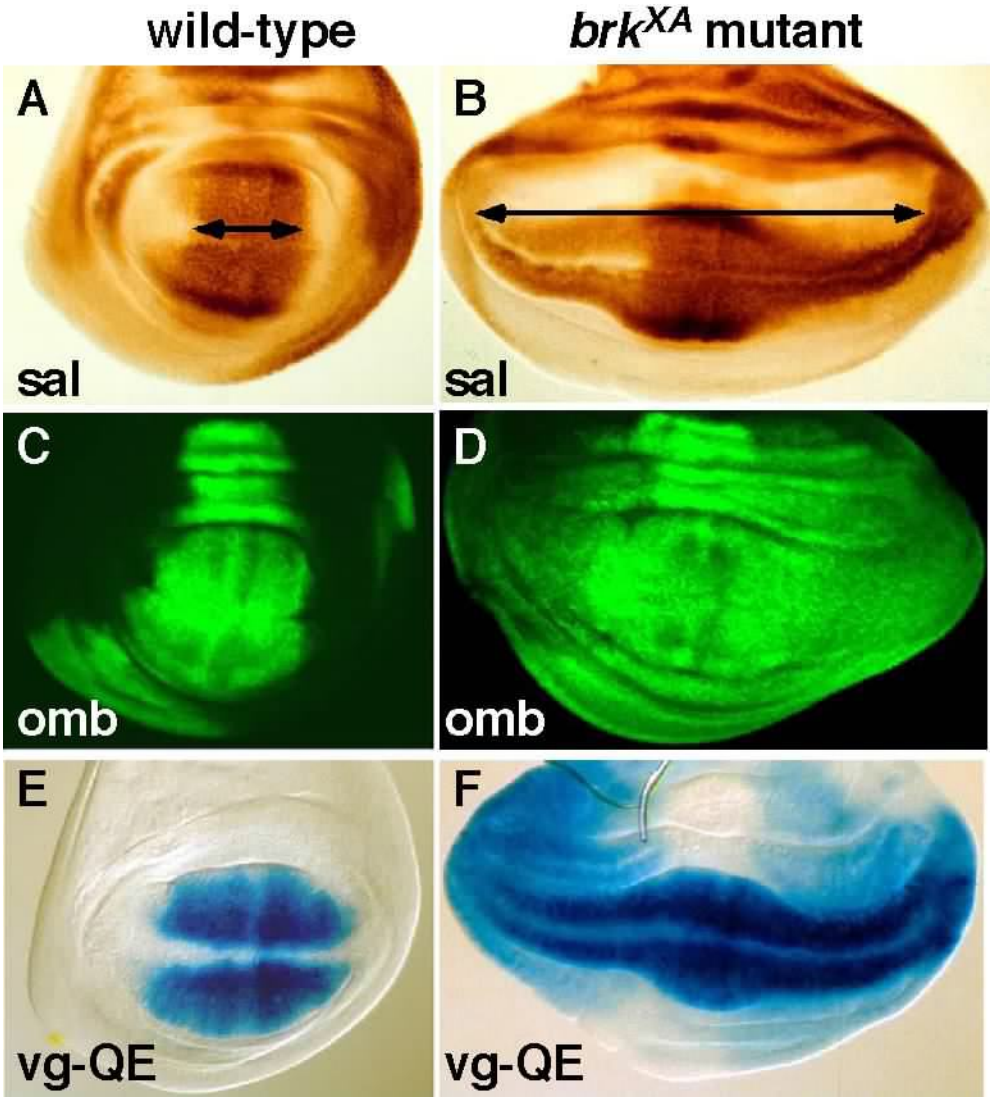

Figure 6. Phenotype of *brk*^{XA} Mutant Wing Imaginal Discs.

Third instar wing discs comparing wild-type to *brk*^{XA} mutant discs. *brk*^{XA} mutant discs have an overgrowth phenotype showing expansion of the A-P axis in the wing pouch. (A & B) *sal* expression (anti-Sal). The arrow designates the *sal* expression domain that widens into the expanded wing pouch in the mutant disc. (C & D) *omb* expression (UAS-GFP; Omb-Gal4). The *brk*^{XA} mutant disc shows misexpression of *omb*. (E & F) β-gal expression driven by vg-QE enhancer (x-gal). vg-QE expression in the mutant disc is expanded into lateral regions of the wing disc. Note: *brk*^{XA} is also an enhancer trap, and staining in (F) reveals expression from both enhancer traps, but vg-QE is stronger than *brk*^{XA}.

comparison, *brk* mutant clones in lateral regions seem to contain similar levels of *omb* expression as central regions, suggesting Dpp may not be directly activating *omb* (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Marty et al., 2000).

1.13 Differential sensitivity of Dpp targets to Brk

Even though Brk represses both *sal* and *omb*, the *sal* expression domain is more narrow compared to the *omb* expression domain. However, Brk forms a lateral to medial gradient and it appears that *sal* has a lower threshold than *omb* to repression by Brk, producing the difference in the width of the expression domains (Fig. 4) (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999). Misexpression studies provide some support for these differences (Muller et al., 2003), but the mechanism of differential sensitivity between *sal* and *omb* is not known. The ability of a single transcription factor to activate or repress a gene at one concentration, but the necessity for increased levels of the factor to have an effect on another gene remains unclear. One of the most probable mechanisms explaining the differences in variability and sensitivity to threshold responses to a transcription factor relates to the number of binding sites or affinity to these sites in enhancers of target genes. The above mechanisms seem to be working in enhancers regulated by transcription factors Dorsal (Dl), Hunchback (Hb), Krüppel (Kr) and Knirps (Kni) (Clyde et al., 2003; Jiang et al., 1992; Langeland et al., 1994). On the other hand, other mechanisms may be used to regulate the sensitivity, for instance, the position of binding sites relative to a promoter can alter the sensitivity to repression by Giant (Hewitt et al., 1999).

1.14 Mechanisms of Brk repression

1.14.1 *In vitro* studies suggest Brk represses by simple competition with activators

Evidence that Brk represses by competition with activators comes from studies of the *omb* wing enhancer in which Brk binding sites appear to overlap with the binding sites of an unknown activator (Sivasankaran et al., 2000). In the embryo, Brk targets *zen* and *Ubx* contain Brk binding sites which frequently overlap with activator (Mad) binding sites and *in vitro* binding studies showed direct competition between Brk and Mad for binding to the same region of DNA (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001).

1.14.2 Brk may repress through recruitment of co-repressors, Gro and CtBP

Sequence analysis of the Brk protein has identified interaction motifs for co-repressors, Groucho (Gro) and C-terminal binding protein (CtBP) and both of these proteins are capable of binding Brk *in vitro* (Hasson et al., 2001; Saller and Bienz, 2001; Zhang et al., 2001b). In the absence of both CtBP and Gro, some Brk targets show ectopic expression such as *brk* itself, which negatively autoregulates, but other targets such as *omb* do not, suggesting Brk may recruit CtBP and Gro to repress some genes, but not others (Hasson et al., 2001). Moreover, in the embryo, ectopic expression of modified/mutated brk transgenes suggest CtBP and Gro interaction motifs are required for maximal Brk activity.

1.15 Brk target genes in the *Drosophila* wing

Brk functions to repress expression of several target genes in the wing that were initially classified as Dpp target genes, including *sal*, *omb* and the vestigial quadrant enhancer (vg-QE) (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Kim et al., 1996; Marty et al., 2000).

The *sal* gene encodes a zinc-finger protein, containing three widely spaced, sequence related zinc finger groups (Kuhnlein et al., 1994). The product of the *sal* gene participates in vein patterning and cell growth in the adult wing (de Celis et al., 1996). Previous studies demonstrated that a 10.2 kb Sal fragment could drive LacZ expression in the wing disc in an almost identical pattern to wild-type *sal* and a smaller 1.8 kb sub-fragment could also drive expression in the wing pouch (Kuhnlein et al., 1997). Another study reported that a 328 bp subfragment of sal1.8 could drive expression in the wing pouch, but with wider expression and absence of expression in the central region (Guss et al., 2001). In the developing wing disc, the *sal* domain is nested within the *omb* domain (Lecuit et al., 1996). The *omb* gene encodes a member of the T-box family of transcription factors (Pflugfelder et al., 1992). Brk has been shown to bind a minimal Dpp-responsive *omb* wing enhancer region (Sivasankaran et al., 2000) and repress *omb* expression. In addition, *omb* is one of the few genes that is positively regulated by Dpp in the wing disc and its expression along the A/P axis seems to be controlled by Brk (Sivasankaran et al., 2000). However, a recent study in the Campbell Lab revealed that upregulation of Mad levels antagonizes the ability of ectoptic Brk to repress *omb*, suggesting *omb* is directly activated by Mad (Moser and Campbell, 2005).

In addition to *sal* and *omb*, Dpp signaling activates expression of the *vestigial* gene. The vestigial quadrant enhancer, (Vg-QE), is expressed in the developing wing disc and its expression domain extends to lateral regions of the wing pouch, overlapping the Brk domain. Dpp signaling activates expression of *vg* in the wing disc via the quadrant enhancer (QE), which is located within the fourth intron of *vg* and contains two Mad binding sites (Kim et al., 1996). The expression of vg-QE is de-repressed in brk mutant clones, indicating that vg-QE is a direct target of Brk repression (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al.,

1999). Studies also demonstrated activated Mad associates with Medea and directly regulates *vg* expression (Kim et al., 1997). Moreover, Dpp response elements of *vg* are dependent on its Mad binding sites to drive Dpp dependent reporter gene expression (Kim et al., 1997). *In vitro* studies revealed that Brk binds to a *vg* Dpp response element and represses reporter expression (Kirkpatrick et al., 2001). Since *vg* is expressed throughout the wing pouch at a distance from the Dpp source, it appears that vg-QE has a low sensitivity to repression by Brk (Kirkpatrick et al., 2001).

1.16 Brk target genes in the *Drosophila* embryo

During embryonic development, Dpp regulates expression of Hox genes *Ultrabithorax* (Ubx) and *labial* (Hursh et al., 1993; Thuringer et al., 1993; Tremml and Bienz, 1992) involved in endoderm patterning. Analysis of the *Ubx* and *labial* midgut enhancers revealed each contains binding sites for Mad (Kim et al., 1997) that mediate the response to Dpp stimulation (Szuts et al., 1998). Dissection of the upstream regions of *Ubx* revealed a 260 bp fragment (UbxB) that drives reporter gene expression in a pattern similar to Ubx, but extends slightly wider than endogenous *Ubx* (Thuringer et al., 1993). The minimal *Ubx* midgut enhancer (UbxB) is controlled by Brk, and *brk* mutant embryos showed de-repression in reporter gene expression in the anterior and posterior midgut (Saller and Bienz, 2001). Further analysis of the UbxB enhancer revealed that Brk binding sites overlap activating Mad/Medea binding sites (GCCGNCGC) in this enhancer, and *in vitro* binding studies demonstrated that Brk competes with Mad for binding to these sites (Kirkpatrick et al., 2001; Saller and Bienz, 2001).

In addition to *Ubx* expression in the embryo, *zen* is another gene that is regulated by Brk protein. During early to mid-cellularization, maintenance of the *zen* pattern becomes dependent

on Dpp because *zen* transcript disappears in *dpp* mutant embryos (Ray et al., 1991; Rushlow and Levine, 1990). The z*en* expression pattern is also dependent on Brk repression because *zen* transcripts expand into the ventral ectoderm in *brk* mutants (Jazwinska et al., 1999b). The broad pattern of *zen* is maintained by Dpp in dorsal region and repressed by Brk in the ventral regions (Rushlow et al., 2001). Analysis of a 1.6 kb zen promoter region revealed 6 putative Brk binding sites and 10 Mad binding sites. Of the 6 Brk binding sites, 5 overlap with Mad binding sites (Rushlow et al., 2001). Mutation or deletion of several combinations of two Brk sites did not effect reporter gene expression, but mutation of four Brk binding sites produced ectopic expression in the ventral ectoderm during late cellularization (Rushlow et al., 2001), reminiscent of expression pattern observed in *brk* mutant embryos (Jazwinska et al., 1999b). These findings demonstrated that Brk binding to *zen* promoter sites are critical for Brk repressor activity and in this tissue it appears that a cumulative effect of Brk binding sites is required, not just the presence of any single site (Rushlow et al., 2001).

1.17 Project Goals

The preceding introduction illustrates the complex transcriptional control of gene expression in eukaryotes. Although Brk represses both *sal* and *omb*, the *omb* expression domain is wider than *sal*, demonstrating that *sal* is more sensitive to Brk than *omb*. This work investigates the mechanisms Brk uses to repress Dpp target gene expression to gain an understanding of why some targets are more sensitive to repression by Brk than others. Does Brk function identically to repress each of its targets? What mechanisms does Brk use to repress transcription? Does Brk use the same mechanism to repress each of its target genes? To answer these questions, several approaches were utilized: 1) Previous work in the Campbell lab

analyzed endogenous *brk* mutants and EMS induced mutations in a *brk* transgene. Due to the limited availability of *brk* mutants, I utilized another approach. 2) *In vitro* mutated UAS-brk transgenes were generated and ectopically expressed in the developing *Drosophila* wing or embryo. Phenotypes of adult wings were analyzed following misexpression of modified/mutated forms of Brk using two Gal4 drivers (C765-ubiquitous expression in wing and en-Gal4-expressed exclusively in the posterior). Expression of Brk target genes *sal, omb,* Vg-QE and salE1 reporter (a *sal* enhancer fragment that drives expression in a pattern similar to *sal*) were analyzed following misexpression of UAS-brk transgenes in the posterior of the wing using en-Gal4. Expression of embryonic midgut mesoderm reporter, UbxB, was analyzed following misexpression of UAS-brk transgenes in the embryonic mesoderm.

Analysis of endogenous *brk* mutants revealed the importance of a functional DNA binding domain for Brk function, as well as that CtBP and Groucho interaction motifs are not required for repression of some targets. To determine whether Brk uses the same mechanism to repress each of its targets, it was necessary to analyze mutants with specific repression domains/motifs mutated or deleted. Initially, I expected that Brk used the same mechanism to repress each of its targets, and the difference in sensitivity between *sal* and *omb* result from the number of Brk binding sites or affinity for the binding sites contained in enhancer/promoter regions. However, analysis of modified/mutated forms of Brk protein revealed that Brk does not repress by simple binding site competition, but requires specific repression domains in combination with its DNA binding domain. Interestingly, although *sal* and *omb* show quantitative differences in their response to Brk, there are qualitative differences in the mechanisms that Brk uses to repress each target.

2 Chapter 2: Repression of Dpp Targets in the *Drosophila* Wing by Brinker

2.1 Introduction

Decapentaplegic (Dpp), a TGF-β superfamily member, plays an important role in many developmental events in *Drosophila* including patterning of the wing imaginal disc, where it forms a medial to lateral morphogen gradient along the anterioposterior (A/P) axis of the wing (Blackman et al., 1991; Entchev et al., 2000; Masucci et al., 1990; Teleman and Cohen, 2000). Dpp signaling regulates gene expression in a dose dependent manner and does this in part, by downregulating the expression of the *brinker* (*brk*) gene, which encodes a transcriptional repressor; *brk* is consequently expressed in a lateral to medial gradient (Muller et al., 2003). We have investigated the mechanisms Brk uses to repress gene expression to gain an understanding of why some targets such as *spalt (sal)* are more sensitive to Brk than others such as *optomotor-blind* (*omb)* that in turn is more sensitive than the vestigial quadrant enhancer (vg-QE) (Kim et al., 1997; Lecuit et al., 1996; Nellen et al., 1996).

It has been suggested that Brk may repress different target genes using different mechanisms. First, Brk binding sites in the cis-regulatory regions of some embryonic Brk targets, including *Ubx* and *zen* overlap with activator sites for Mad, and *in vitro* studies demonstrate that Brk and Mad can compete for binding to the same region of DNA (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001). Second, Brk contains interaction motifs for the co-repressors CtBP (Chinnadurai, 2002) and Gro (Chen and Courey, 2000), indicating Brk may use more active mechanisms to repress target genes (Zhang et al., 2001b). The absence of Gro or CtBP does result in derepression of some Brk targets, such as the vg-QE,

but not others, such as *omb*, suggesting Brk may use alternate mechanisms to repress different genes (Hasson et al., 2001).

To characterize the importance of the DNA-Binding Domain, Groucho interaction motif (GiM), CtBP interaction motif (CiM) or other regions of Brk protein required for its repressor activity, modified/mutated forms of Brk protein were generated in which one or more of these domains/motifs were mutated or deleted. The current study reveals that Brk requires its DNA-binding domain (DBD) plus a repression domain to act as a transcriptional repressor. Moreover, the DBD alone cannot repress target genes, even those shown to possess overlapping Brk and Mad binding sites, suggesting competition may not be a real mechanism *in vivo*. Brk possesses four independent repression domains, Gro and CtBP interaction motifs and two other domains defined as 3R (Winter and Campbell, 2004) and 4R for third and fourth repression domain, respectively. However, these domains are not equivalent: 3R can repress *omb* but not *sal*, and this difference may be related to the spacing of Brk-binding sites relative to activator sites.

2.2 Comparison of Brk homologs in other insect species

Brinker (Brk) is a sequence specific transcription factor that regulates Dpp responsive genes by binding to response elements and repressing expression of these genes. In an attempt to identify the regions of Brk protein required to repress gene expression, a comparison of four insect species, *Drosophila melanogaster*, *Drosophila pseudoobscura*, the mosquito *Anopheles gambiae* and the distantly related silkworm *Bombyx mori* was completed. A 60 amino acid segment corresponding to the helix-turn-helix (HTH) motif (residues 68-89), predicted from the original Brk sequence, plus additional N-terminal sequence (Campbell and Tomlinson, 1999) were similar in all four species (Fig. 7). The region spanning amino acids 44-99 was identified

as the DNA binding domain (DBD) by *in vitro* studies (Rushlow et al., 2001; Saller and Bienz, 2001; Sivasankaran et al., 2000; Zhang et al., 2001b). Based on the size of the Brk DNA binding domain and the positioning of the HTH motif, there are definite similarities between the Brk DNA binding domain and the homeodomain (Jazwinska et al., 1999a), a DNA binding domain found in many important transcription factors, which is composed of three helicies, with the last two forming a helix-turn-helix. The Brk DNA binding domain has weak homology with other homeodomain proteins including Engrailed and Hox proteins (Jazwinska et al., 1999a). Outside the DNA binding domain, only the regions centered on the Groucho Interaction Motif (GiM) and the CtBP Interaction Motif (CiM) are conserved among all four insect species (Fig. 7). The two *Drosophila* species share some identical resides such as poly-glutamine stretches before and after the 3R region, and the region from amino acids 593-629 which are identical in both species, as well as several regions with 2-5 identical amino acids.

2.3 Experimental Approaches

The fact that the DNA binding domain, the CiM and GiM have been conserved through millions of years of evolution indicates these regions are essential for Brk activity, at least in some contexts. However, questions that remain to be answered are as follows. First, is the DNA binding domain required for repression of all Brk target genes? Second, can Brk repress some target genes simply by competing with activators (such as Mad) for overlapping binding sites? If so, this may only require the DBD and not the CiM, GiM or other repression domain. Third, in addition to the functional domains/motifs already identified, does Brk possess additional regions that act as repressor domains? To determine the answers to the above questions, previous work in

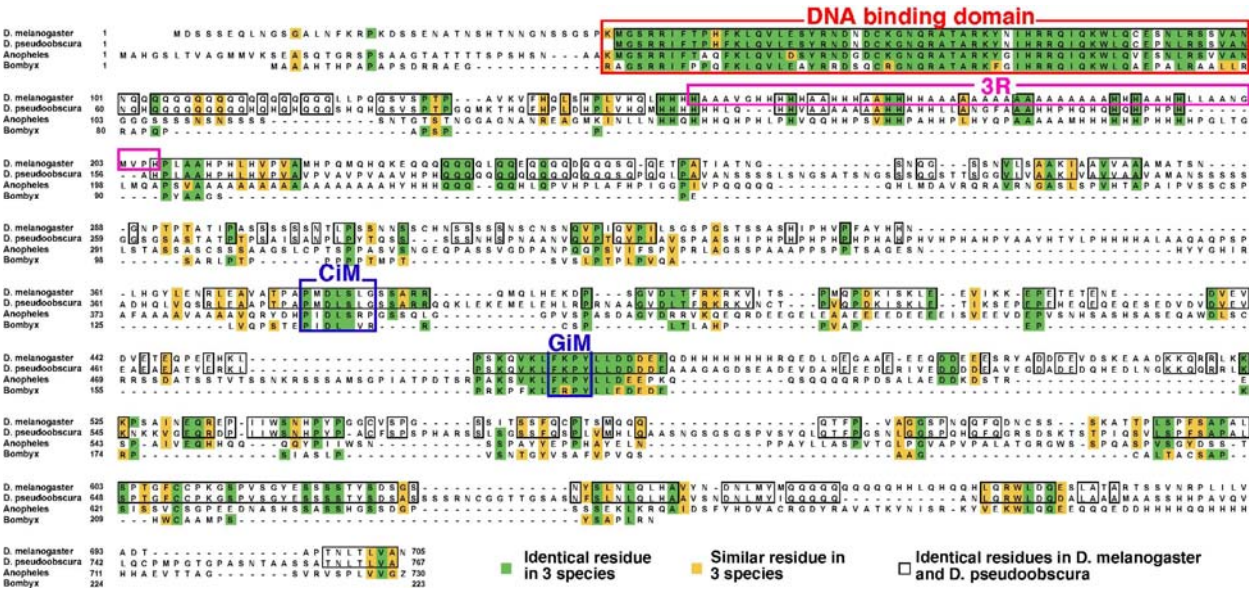

Figure 7. Sequence comparison of Brk from *Drosophila melanogaster* and from three other insects, *D. pseudoobscura, Anopheles gambiae* and *Bombyx mori*.

The DNA-binding domain (boxed in red) is strongly conserved in all four species. Outside of the DNA-binding domain there is only one other region of significant similarity, centered on the Groucho interaction motif (GiM, blue), although the short CtBP interaction motif (CiM, blue) is also conserved. The third repression domain, 3R, of *D. melanogaster*, identified by functional studies described here, is outlined in pink. This domain shares some limited sequence identity with *D. pseudoobscura*, but this does not extend to the other two species, although it is rich in Ala and His residues in *Anopheles*, as in the two *Drosophila* species.

the Campbell lab utilized two approaches: 1) characterize three EMS mutations in the endogenous *brk* gene (Lammel et al., 2000; Lammel and Saumweber, 2000) and 2) generation of additional mutations in a *brk* transgene UAS-brk^{A438}. The second approach screened for EMS induced mutations in the transgene by driving ubiquitous expression of the UAS-brk^{A438} transgene in the wing using Gal4 C765, resulting in an almost complete loss of the wing blade at 25°. An EMS induced lesion in the *brk* transgene would result in reversion of the wing phenotype, producing an increase in wing size. Five point mutants in UAS-brk^{A438} were isolated and characterized. 3) The third approach was the basis of my thesis research, and involved generation of UAS-brk transgenes that produced HA-tagged proteins in which different regions were included, modified, mutated or fused to other domains, including nuclear localization sequences (NLS) and repression motifs. Activity of the modified/mutated Brk proteins was compared to that of wild type Brk protein (Brk^{3PF3}) by misexpression of transgenes in the developing wing and analysis of: a) adult wings using two Gal4 drivers and b) expression of known Brk targets in the imaginal wing disc and embryo.

2.4 Results

2.4.1 Analysis of Brk target genes in the wing imaginal disc and embryo

Previous studies have identified Dpp targets in the wing and embryo that are repressed by Brinker (Brk) protein including *sal*, *omb*, vg-QE, UbxB and salE1. The normal expression pattern for each of these targets (Fig 8A, C, E, G, I) has been analyzed and will be compared to expression following misexpression of wild type Brk (Fig. 8B, D, F, H, J) and modified/mutated forms of Brk protein to determine the repressor activity of altered Brk proteins. A majority of

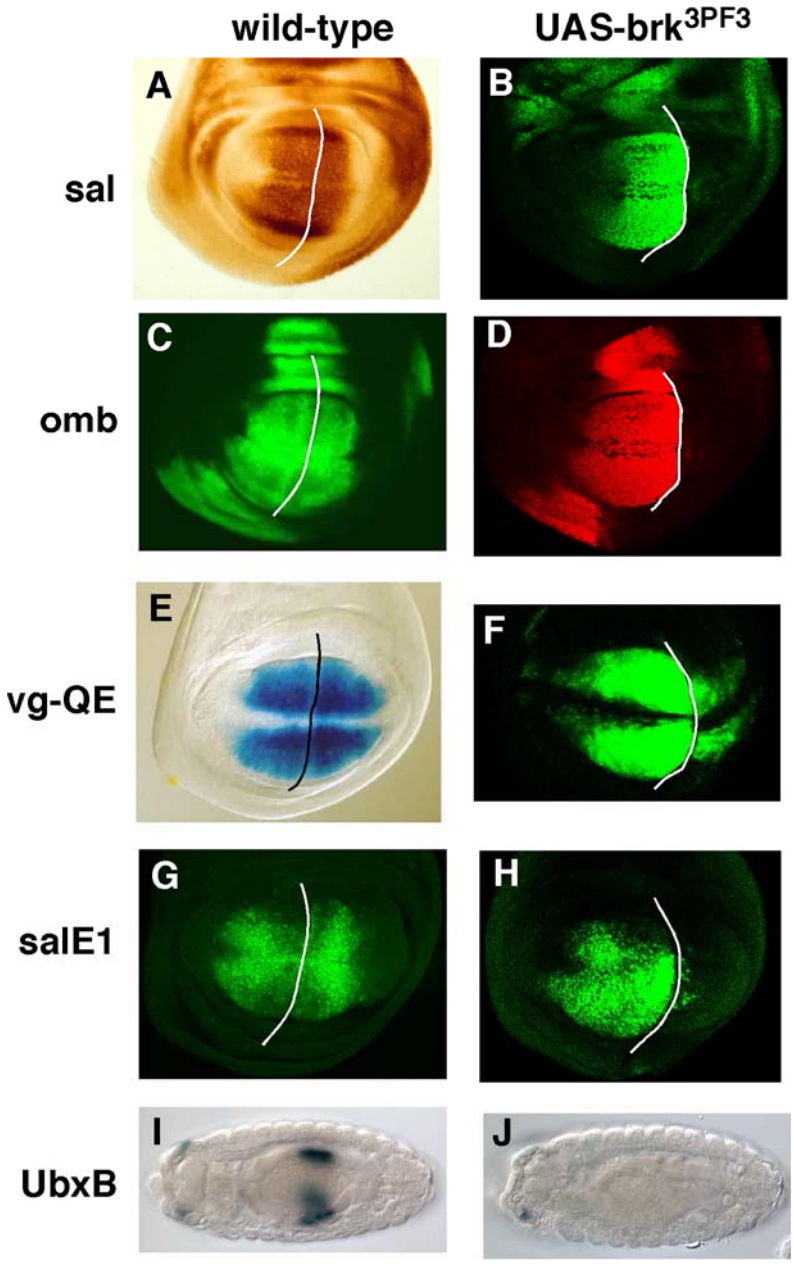

Figure 8. Analysis of Brk targets in the wing imaginal disc and embryo.

(A,C,E,G,I) Wild-type expression pattern of Brk targets analyzed in the wing imaginal disc and embryo. (B,D,F,H) Misexpression of wild-type Brk protein (Brk^{3PF3}) in the posterior of wing imaginal discs represses expression of each of the targets tested in the wing (anterior-posterior interface is marked by the line; posterior is to the right). (J) Misexpression of Brk^{3PF3} in the mesoderm of embryos represses expression of the UbxB reporter.

the analysis was completed on the expression of *sal* and *omb* in developing wing discs, with secondary analysis on the expression of vg-QE in the wing and UbxB in the embryo. In addition, the salE1 reporter was used to analyze the repressor activity of *brk* mutants and *brk* transgenes. The following provides details on how each of the targets will be assessed throughout the studies as well as evidence that each is a Brk target.

Brk functions to repress the expression of *sal*, *omb* and vg-QE in the wing and these genes were originally classified as Dpp targets. In the absence of Dpp and Brk, these genes are still expressed, indicating indirect regulation by Dpp, through repression by Brk. In *brk* mutants, the expression domains of *sal* and *omb* expand laterally, and ectopic expression of *brk* in central regions of the wing disc results in loss of both *sal* and *omb* expression (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999; Muller et al., 2003). Moreover, other studies have demonstrated that Brk binds to cis-regulatory elements of *omb* (Sivasankaran et al., 2000) and *vg* (Kirkpatrick et al., 2001).

A) *spalt* (*sal*) the expression of *sal* will be detected using an anti-Sal antibody (Kuhnlein et al., 1994) to stain wing imaginal discs.

B) *optomotor-blind* (*omb*) the expression of *omb* will be detected using *omb*-LacZ enhancer trap line and an anti-β-gal antibody to stain wing imaginal discs.

C) vestigial quadrant enhancer (vg-QE, an enhancer recapitulating a portion of *vestigial* gene expression) the expression will be detected using a vg-QE (LacZ) line and an anti-β-gal antibody to stain wing imaginal discs.

Brk was shown to repress *Ubx* in the embryonic midgut (Saller and Bienz, 2001), and to bind to *Ubx* response elements (Kirkpatrick et al., 2001; Saller and Bienz, 2001).

D) UbxB an *Ultrabithorax* (*Ubx*) reporter construct, expressed in the embryonic midgut mesoderm (Thuringer et al., 1993), will be detected in embryos using X-gal staining

E) salE1 an enhancer identified in our lab, and is the smallest region that drives GFP expression in a similar pattern as endogenous *sal*. Additional studies, including Barrio and de Celis, (2004) show that activator sequences do not overlap with Brk binding sites (Fig. 9A).

In an attempt to further understand the repressor activity of both endogenous *brk* mutants and UAS-brk transgenes, we have characterized a portion or the *sal* enhancer element, salE1. salE1 is a 471 bp fragment located 10 kb upstream of the *salm* gene, in which the *sal* locus contains two partially redundant genes, *salm* and *salr* (Fig 9A) (Barrio et al., 1996). salE1 is a subfragment of sal1.8S/E enhancer (Kuhnlein et al., 1997) and was designed based on previous studies (Guss et al., 2001) (Fig. 9A). The salE1 reporter drives expression in a slightly wider expression pattern compared to endogenous *sal* (Fig. 9C), but it is repressed in lateral regions of the wing pouch, presumably by the repressor activity of Brk.

It was shown that Brk is required for repression of salE1 expression in the lateral regions of the wing pouch in the following ways. First salE1 reporter expression is upregulated in lateral regions (where it is not normally expressed) in *brk* null (brk^{M68}) mutant clones (Fig. 10). Second, mutation of the three putative Brk binding sites in salE1 from GGCGYY (Kirkpatrick et al., 2001; Rushlow et al., 2001; Sivasankaran et al., 2000; Zhang et al., 2001b) (Fig. 9A) to GTCGYY, to generate salE1MB123, results in expansion of the expression domain to lateral regions of the wing pouch (Fig. 9E). Therefore, these binding sites must be required to repress salE1 in lateral regions. Deletion of the salE1 region encompassing the three Brk binding sites producing salGCNB (Fig 9A), a fragment similar to that analyzed previously (Guss et al., 2001), resulted in an expansion of expression into lateral regions of the wing pouch, but reporter

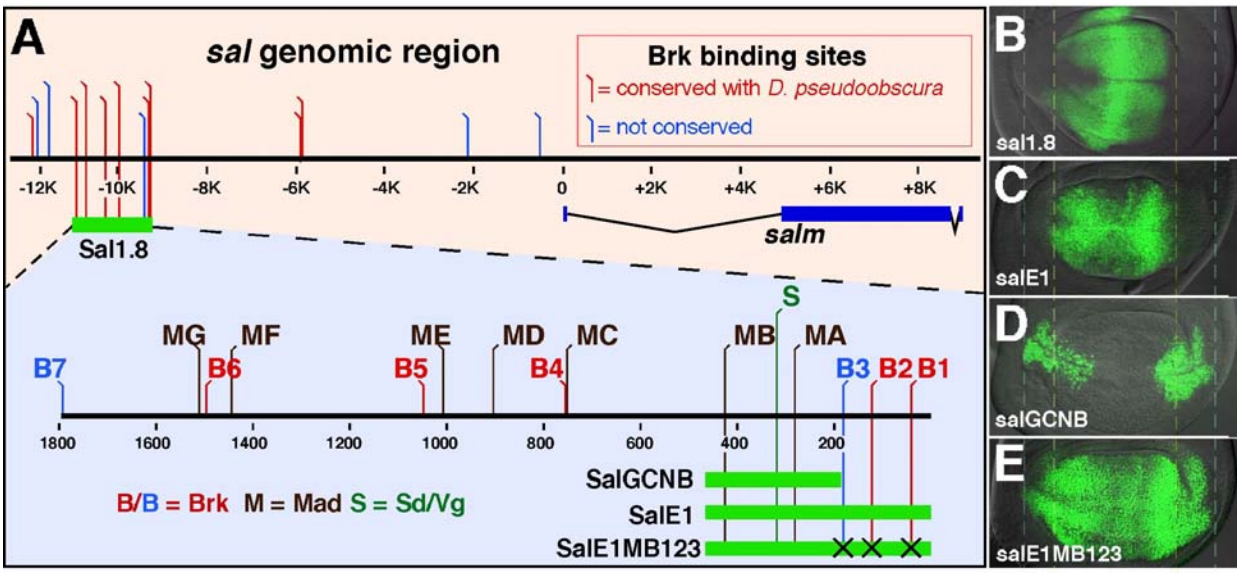

Figure 9. cis-regulatory elements at the *salm* locus.

(A) The upstream region of the *salm* gene and constructs tested for enhancer activity (driving lacZ or GFP). All putative Brk binding sites (GGCGYY) are marked along with conservation in *D. pseudoobscura*. Below, the sal1.8 fragment is magnified and seven putative Mad binding sites (MA-MG) are labeled along with seven putative Brk binding sites (B1-B7) and Scalloped/Vestigial site (S). (B) The sal1.8 fragment drives expression in the wing pouch, but in a wider pattern than *sal* itself. (C) salE1 fragment contains three Mad binding sites and three Brk binding sites and is the smallest fragment that drives reporter expression in a similar pattern as endogenous *sal*, but like sal1.8, expression is slightly wider (lateral limit marked by yellow dashed line). (D) The salGCNB fragment lacks all Brk binding sites and drives reporter expression in the lateral regions of the wing pouch (lateral limit marked by the blue dashed line), although expression is missing from the central region. (E) salE1MB123 has the three Brk binding sites mutated, and drives expression in the lateral-most region of the wing pouch, to the same width as salGCNB.

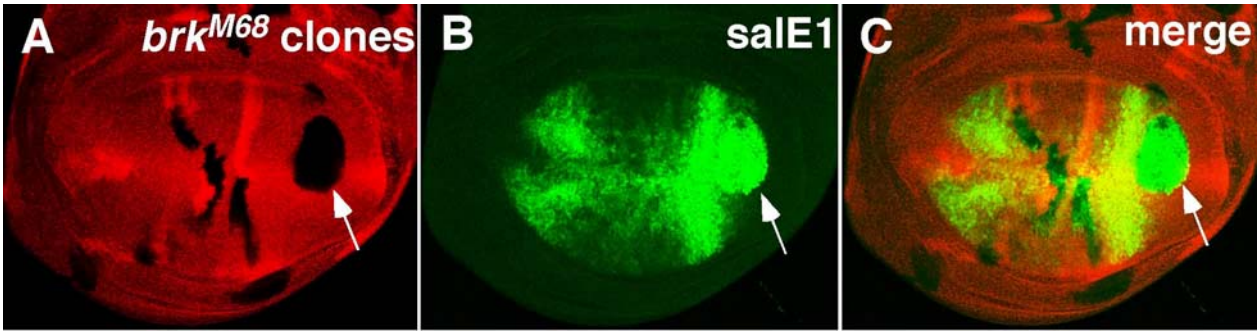

Figure 10. Expansion of salE1 reporter in *brk* mutant clones.

(A) Third instar wing discs containing *brk* null mutant clones, marked by the loss of ubiquitous β-gal in red. (B) salE1 expression is ectopically expressed in *brk* clones in the lateral wing pouch (arrow). (C) Merge of panels A and B.

expression was lost in the central region (Fig.9D). SalGCNB possesses a binding site for the activator Scalloped/Vestigial as well as two sites for activators Mad/Medea (Fig. 9A) (Barrio and de Celis, 2004). These data indicate that the Brk binding sites located in salE1 are separated from these activator sites as well as other potential activators, and are consistent with the results obtained in another recent analysis of *sal* enhancer regions (Barrio and de Celis, 2004).

2.4.2 SalE1 does not require *omb* to be activated

A recent study revealed that endogenous *sal* expression is dependent on Omb (del Alamo Rodriguez et al., 2004), so we wanted to test whether salE1 also requires *omb* for its expression. Clonal analysis of *brk omb* double mutant clones revealed ectopic salE1 expression in clones suggesting that salE1 does not require *omb* to be activated. Consequently, salE1 is not behaving exactly like the endogenous *sal* gene, since *sal* does require *omb* for its expression (GC, unpublished results).

2.4.3 Characterization of point mutations in the endogenous *brk* gene

Previously identified Brk mutations (Lammel et al., 2000; Lammel and Saumweber, 2000) were sequenced and lesions are as follows: brk^{E427} introduces a stop codon at amino acid 115, producing a truncation following the DNA binding domain (Fig 11A and 16B); brk^{F138} produces a longer version of the protein truncated at amino acid 333 (Fig 11A and 16B) before the CtBP Interaction Motif and brk^{F124} contains an amino acid substitution (R82W) in the DNA binding domain recognition helix (Fig 11A and 16B). Activity of *brk* endogenous mutants was assessed by analyzing expression of known Brk targets in the wing, *sal*, *omb* and Vg-QE, in marked homozygous mutant clones. Previous work revealed that in null *brk* mutant clones

(*brk*^{M68}), Brk targets were misexpressed in lateral regions of the wing disc (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999). Homozygous mutant clones of *brk*^{E427} and *brk*^{F124} had no perceptible activity and were not able to repress *sal*, *omb* or Vg-QE expression (Fig 11A,B and 12A,B).

Interestingly, Brk protein truncated just before the CiM and GiM, *brk*^{F138}, had significant activity compared to *brk* null mutant clones. Brk^{F138} was able to repress *omb*, but not *sal* completely or Vg-QE in mutant clones (Fig. 11A,B and 12C,D), as there was ectopic *sal* expression adjacent to the normal *sal* expression domain, but it does not expand beyond the *omb* expression domain. However, *sal* expression is dependent upon Omb (del Alamo Rodriguez et al., 2004), and so the absence of ectopic *sa*l expression in more lateral regions could be due to the lack of ectopic *omb* expression in *brk*^{F138} clones, rather than direct repression of *sal* by Brk^{F138} protein. Consequently, Brk^{F138} can repress *omb* completely and *sal* either partially or possibly not at all in the absence of the CiM and GiM. These results are consistent with the finding that *omb* is not ectopically expressed in *CtBP, gro* double mutant clones (Hasson et al., 2001). However, it was suggested that Vg-QE expression is also dependent on upon Omb (del Alamo Rodriguez et al., 2004), but Vg-QE expression is expanded into lateral *brk*^{F138} clones where omb is not expressed (Fig 12D). Possible explanations for these discrepancies are that Vg-QE expression may not always be dependent on Omb, or there may be *omb* expression in the clones, but the low levels are not detectable with the *omb* enhancer trap used.

2.4.4 CtBP or Gro is required for repression of *sal*

Brk^{F138} protein product represses *omb* without recruiting either CtBP or Gro, but either one or both co-repressors could be required to repress other target genes in the wing such as *sal*

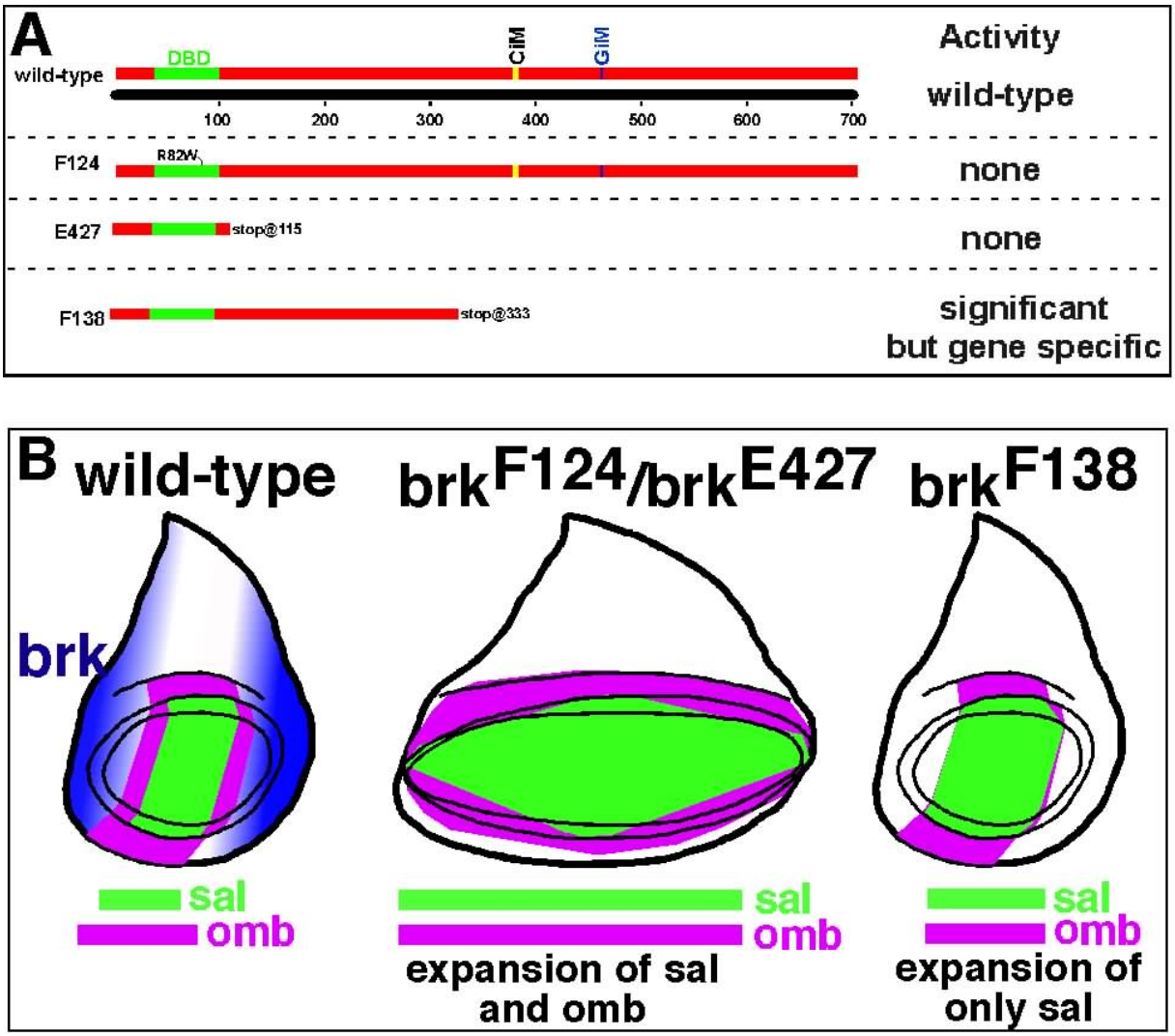

Figure 11. Schematic Representation of EMS point mutants in the endogenous *brk* gene and expression patterns of target genes.

(A) Schematic of Brk proteins containing mutations in endogenous *brk* gene and corresponding activity level for each of the proteins compared to wild-type Brk protein. $brk^{E427,}$ a protein truncated immediately after the DBD, has no activity, while a truncation producing a longer protein, but still does not include the CiM or GiM, brk^{F138}, has significant activity. An amino acid substitution within the DBD, brk^{F124}, also has no activity. (B) Schematic of wing imaginal discs showing expansion of *sal* and *omb* expression domains into lateral regions of the disc for brk^{F124} and brk^{E427}. The *sal* domain extends to the end of the *omb* domain, but does not extend beyond the *omb* expression domain in brk^{F138} mutant clones.

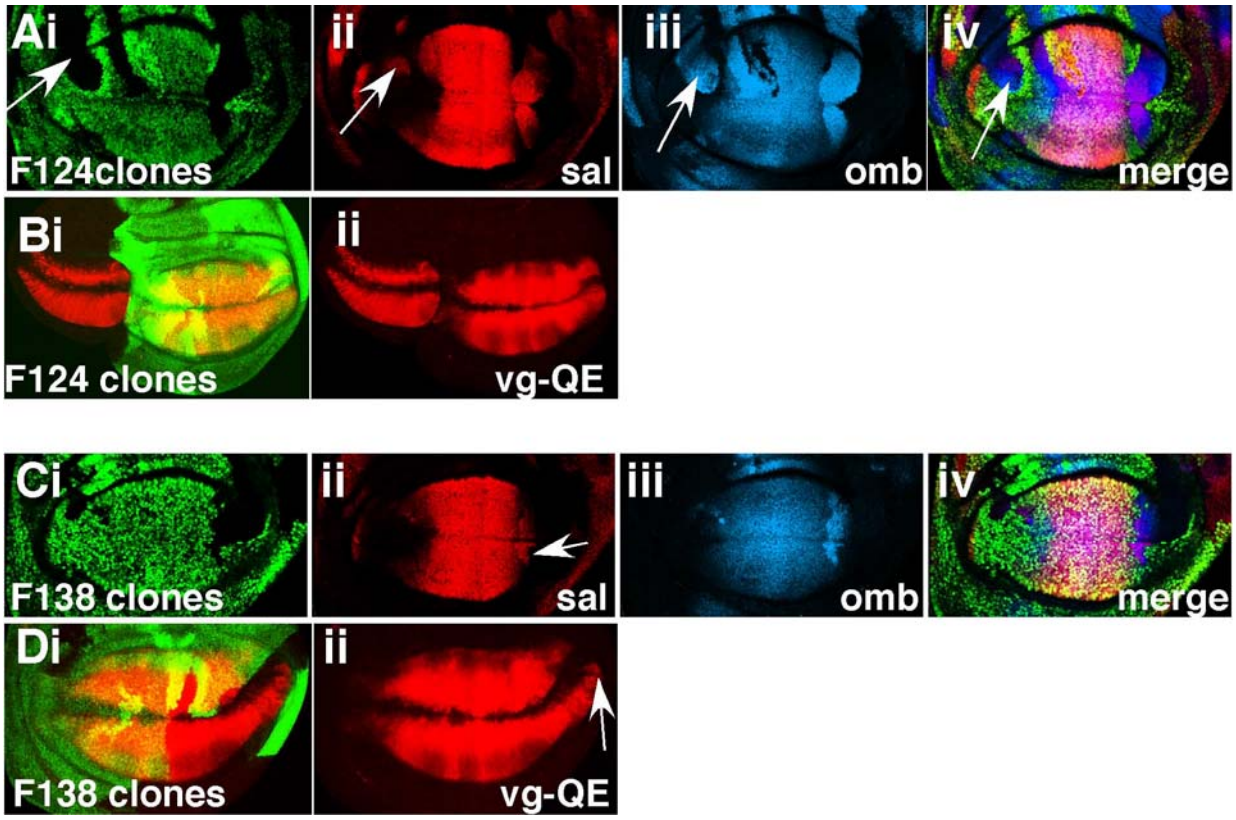

Figure 12. Phenotype of *brk* point mutants, brk^{F124} and brk^{F138}.

Third instar wing discs containing mutant clones, marked by the loss of a ubiquitous GFP transgene, and stained for *omb* (*lac-Z*, anti-βgal), Sal (antibody) and vg-QE (lacZ, anti-βgal) expression. The anterior compartment is to the left and the posterior is to the right. (A) brk^{F124} clones show ectopic expression of Sal and *omb* within the wing pouch, even in lateral postions. (B) brk^{F124} clones also show expansion of the vg-QE domain. (Cii) *sal* is ectopically expressed only in br^{kF138} clones next to the endogenous domain and does not extend to lateral regions. (Ciii) *omb* is not ectopically expressed (the upregulation of expressionin the clone is because *omb-lacZ* is on the X, as is *brk*, so the clone contains two copies of the enhancer trap). (D) vg-QE expression is expanded laterally in some brk^{F138} clones.

and Vg-QE. These findings are consistent with previous studies demonstrating that neither CtBP nor Gro are required to repress *omb* and that Gro is required to repress Vg-QE (Hasson et al., 2001). These studies have been expanded and revealed that in contrast to *omb*, Gro is required for complete repression of *sal*, and CtBP can partially compensate for the loss of Gro. Therefore, these findings contradict previous studies that neither CtBP nor Gro are required for repression of *sal* (Hasson et al., 2001). In the wing, *CtBP*/*gro* double mutant clones show the same phenotype for *sal* expression as brk^{F138} clones, i.e. ectopic expression, but only within the *omb* domain (Fig. 13A). However, in *CtBP* single mutant clones, *sal* expression is normal (Fig. 13B), showing the ectopic expression of *sal* present in *CtBP*/*gro* double mutant clones can be rescued by Gro alone. In contrast, *sal* expression in *gro* single mutant clones is somewhat expanded (Fig. 13C), but not as severely as in the *CtBP*/*gro* double mutant clones. Therefore, CtBP can supply some repressor activity to narrow the lateral limit of *sal* expression in the wing disc, but it is not as effective as Gro.

2.4.5 Brk^{F138} cannot repress salE1 reporter expression

It was previously demonstrated that brk^{F138} was able to repress expression of *omb* but not *sal* (Fig. 12C). Based on this finding, it was logical to assume that Brk^{F138} would also not be able to repress the salE1 reporter. As expected, clonal analysis revealed that Brk^{F138} was not able to repress salE1 leading to misexpression of the reporter in lateral brk^{F138} mutant cells (Fig. 14Ai-ii). Interestingly, in $brk^{F138/+}$ heterozygous cells, salE1 was expanded compared to homozygous wild type (Fig. 14Aiii), suggesting that Brk^{F138} was interfering with the ability of wild type Brk to repress salE1. Based on these data, it appears as if Brk^{F138} may be exhibiting a

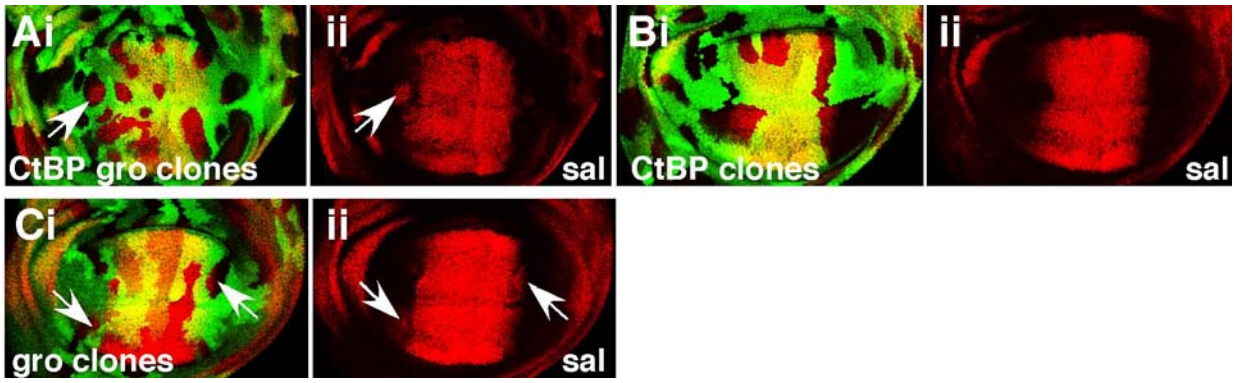

Figure 13. Phenotype of *CtBP* and *gro* mutants.

Third instar wing discs containing mutant clones, marked by the loss of a ubiquitous GFP transgene, andstained for Sal (antibody) expression. (Aii) *CtBP gro* double mutant clones are similar to brk^{F138} clones (Fig.12C), only showing ectopic Sal expression (C, arrow) immediately adjacent to the endogenous domain (when located in the *omb* domain, not shown). In contrast, Sal is not ectopically expressed in any *CtBP* single mutant clones (B), whereas there is an occasional, minor deregulation of Sal in *gro* clones (C, arrow).

dominant negative activity, but analysis of misexpression of other truncated forms of Brk protein (presented later) contradict this finding.

2.4.6 Requirement for CtBP and Gro for repression of salE1

To determine the role of co-repressors CtBP and Gro in repression of salE1, clonal analysis using *CtBP* and *gro* single and double mutants was completed. In *CtBP* single mutant clones, expression of salE1 looks normal (Fig. 14, Bi-iii), whereas in *gro* single mutant clones there was possibly some de-repression of salE1 in the anterior compartment, but the salE1 expression in the posterior looks normal (Fig14, Ci-iii). In contrast, in *CtBP*/*gro* double mutant clones, salE1 was strongly misexpressed in the lateral wing pouch (Fig 14, Di-iii). Based on these findings, repression of salE1 by CtBP and Gro is similar to endogenous *sal*, as the ectopic expression of salE1 observed in *CtBP/gro* mutant clones can be rescues by Gro alone.

2.4.7 Characterization of UAS-brk^{A438} mutants

Of the 5 mutants isolated from the UAS-brk^{A438} screen, four of the lesions produced amino acid substitutions in the DNA binding domain, D44 (L57F), S4 (A72V), C(H80L) and F2 (R81C) (Fig. 15D-G and Fig 16C). The mutations isolated were located at different regions of the DNA binding domain, and had varying effects on the activity of the resulting protein, assessed by ubiquitous expression using Gal4 driver C765, although no mutation completely abolished activity. In contrast, UAS-brk$^{A438\text{-}53}$ contained a stop codon immediately following the DNA binding domain (residue 102) and had no detectible activity *in vivo* (Fig. 15H and 16C). However, if Brk represses simply by binding-site competition, UAS-brk$^{A438\text{-}53}$ should be able to repress *in vivo*, assuming it is localized to the nucleus and is stable, because it contains the entire

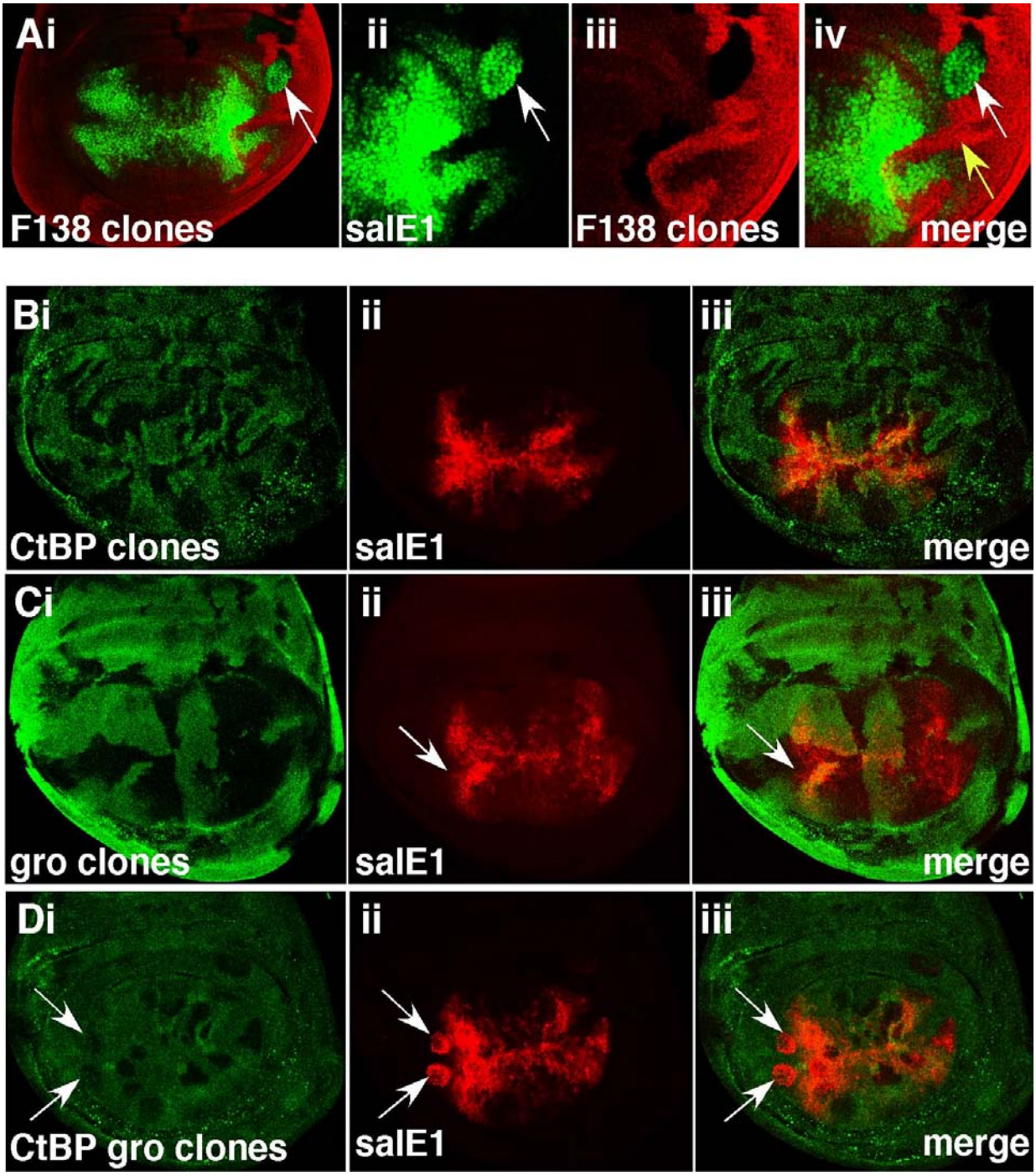

Figure 14. Expression of salE1 reporter in brk^{F138} clones and *CtBP* and *gro* mutant clones.

Figure 14. Expression of salE1 reporter in *brk*^{F138} clones and *CtBP* and *gro* mutant clones.

Third instar wing discs containing mutant clones are marked by the loss of ubiquitous β-gal expression (red for *F138* and green for *CtBP* and *gro* single and double mutant clones).(Ai-iv) salE1 (green) is ectopically expressed in *brk*^{F138} clones. In fact, expression is expanded in *brk*$^{F138/+}$ heterozygous tissue (most of the wing, weak red) compared to homozygous wild-type tissue (twin spot, dark red) (iv, yellow arrow; salE1, white arrow). (Bi-iii) salE1 expression is not affected in *CtBP* mutant clones. (Ci-iii) *gro* mutant clones show some de-repression of salE1 expression in the anterior compartment (ii). (Di-iii) In *CtBP gro* double mutant clones (i) there is misexpression of salE1 in lateral regions of the wing pouch (ii, arrows).

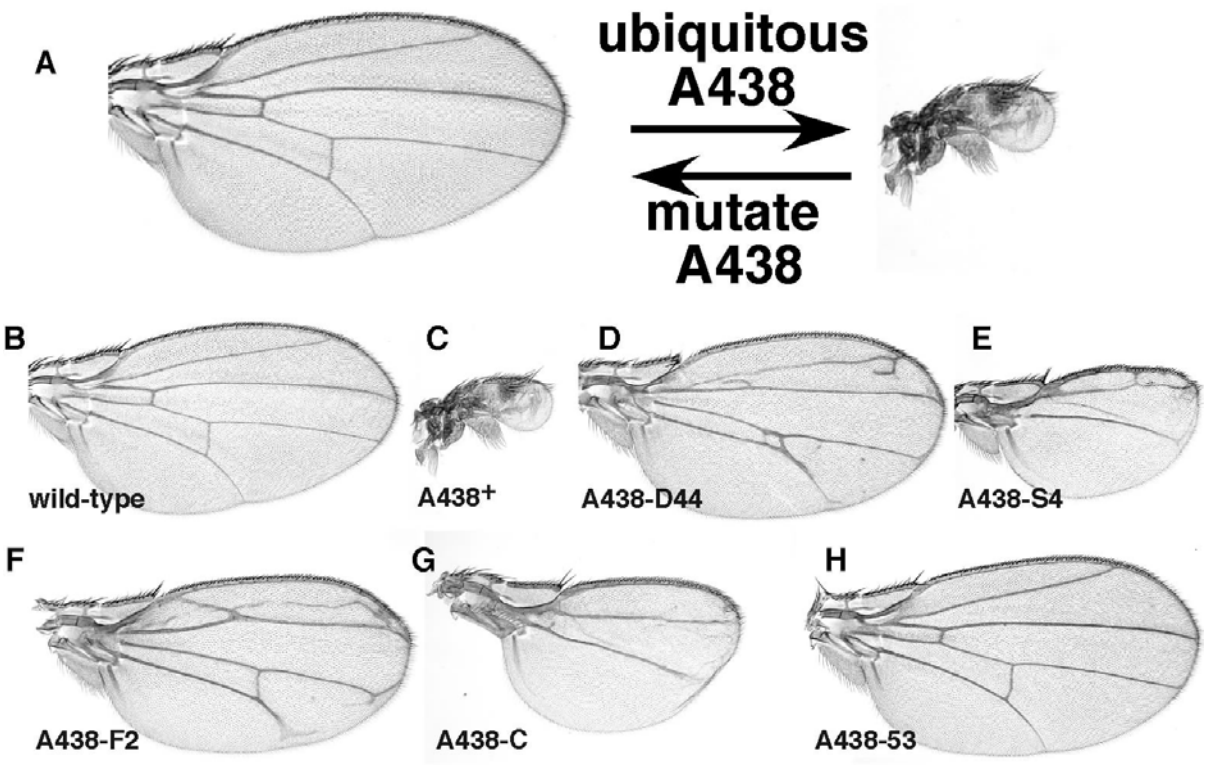

Figure 15. Mutations in UAS-brk^{A438}, which originally contained a wild-type brk transgene.

(A) Ubiquitous expression of *brk*^{A438} transgene produces a smaller wing, and mutation of the transgene results in reversion to a larger wing blade. (B) Wild-type adult wing. (C) Misexpression of wild-type A438 results in a dramatically reduced wing size. (D-H) Misexpression of mutated forms of A438, results in larger wings; from completely wild-type in the case of A438-53, indicating a complete loss of activity, to small wings in the case of A438-S4 and A438-C, indicating significant, but less than wild-type activity of Brk protein. Wings produced with A438-D44 and A438-F2 are almost wild type in size, but have venation defects indicating some activity still remains.

DNA binding domain. *In vitro* studies have demonstrated Brk residues 44-99 are sufficient to bind DNA and compete with activators (e.g. Mad) (Saller and Bienz, 2001).

Taken together, analysis of endogenous *brk* mutants and mutations in the UAS-*brk*^{A438} transgene have provided a preliminary understanding of the regions required for Brk to function as a repressor and begin to answer some of the questions previously posed. However, to completely understand which regions of Brk protein are required for its repressor activity, an additional approach was utilized, in which mutated/modified forms of Brk protein were generated and misexpressed in the developing *Drosophila* wing and embryo and the results were compared to misexpression of wild type Brk.

2.4.8 Misexpression Studies

To gain a further understanding of how Brk functions to repress Dpp target genes, a series of transgenic flies have been generated that carry mutated or modified forms of the Brk protein (Fig. 16D). Brk is a 704 amino acid protein containing an N-terminal DNA binding domain (residues 44-99), a poly-glutamine region (residues 102-120), a histine rich region (residues 151-172) and a poly-alanine tract (resides 173-189) (Fig. 16Ai). Brk also contains interaction motifs for two transcriptional co-repressors, CtBP (CiM) and Gro (GiM). Previous analysis revealed the Brk DNA binding domain is required for its function (Fig.12A,B (section 2.5) and Fig. 15D-G (section 2.9)), and so each of the *brk* transgenes contain the DNA binding domain alone or in combination with other portions of the protein.

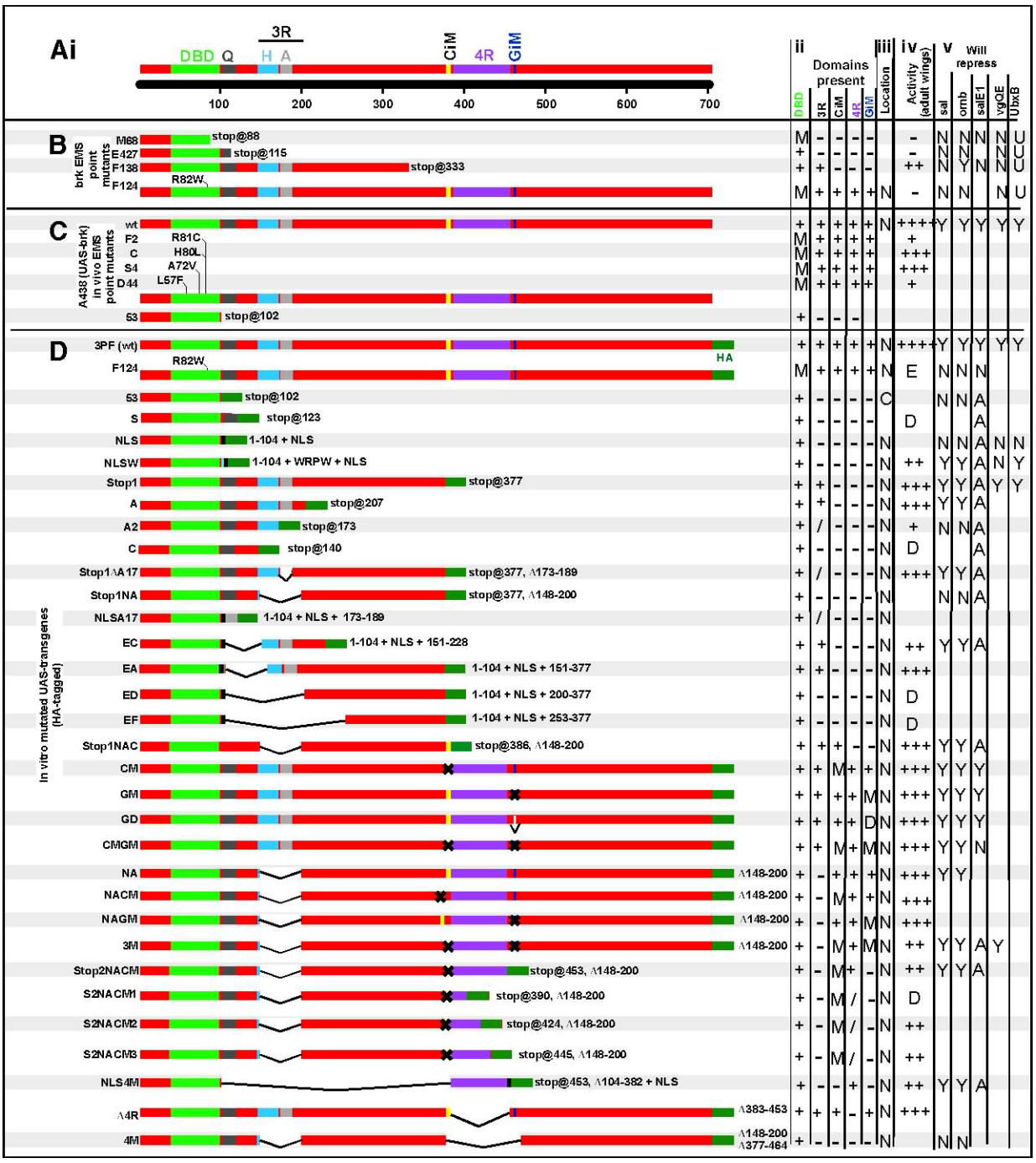

Figure 16. Mutated/modified Brk proteins and activity.

Figure 16. Mutated/modified Brk proteins and activity.

(A, Part i) Domains/motifs in the Brk protein. DBD, DNA binding domain; Q, poly-glutamine; H, histidine rich; A, poly-alanine; 3R, 4R independent repression domains. CiM and GiM, interaction motifs for the co-repressors CtBP and Groucho, respectively. (A, parts ii-v) Properties and effects of modified/mutated Brk proteins shown in B-D. (A, part ii) Domains present: present (+), deleted (-), mutated (M), or partially missing (/). (A, part iii) Nuclear (N) or cytoplasmic (C) localization (blank spaces, here and in other columns indicates that they were not tested). (A, part iv) Activity level assessed by the effect on the phenotype of adult wings ('-', no repressor activity; '++++', maximal activity; 'D', dominant negative; 'E', neomorphic). (A, part v) The ability of each protein to repress the endogenous *sal* gene, an *omb-LacZ* line and three reporters, salE1, vg-QE and UbxB. Y, repressed; N, not repressed; A. activated; U, we were unable to detect reliable differences in UbxB expression between wild-type and mutant embryos of any genotype. (B) EMS point mutants in the endogenous *brk* gene. A protein truncated within the DBD, brk^{M68}, or immediately after it, brk^{E427}, have no activity, whereas a truncation producing a longer protein, but which still does not include the CiM or GiM, brk^{F138}, has significant activity and can repress *omb*, but not the other targets. An amino acid substitution within the DBD, brk^{F124}, also abolishes activity. (C) Point mutants in a UAS-brk transgene, A438. Four result in amino acid substitutions in the DBD and reduce activity. The fifth mutation, A438-53, results in a truncation immediately after the DBD and has no activity. (D) *In vitro* mutated/modified UAS-brk transgenes. There are two basic requirements for these transgenes to repress gene expression: the DBD and a repression domain/motif. In addition to the CiM and GiM, there are two other independent repressor domains, 3R and 4R. The DBD plus any one of the four repression domains/motifs is sufficient to repress some Brk targets, although there is some variability in the ability of individual repressor domains to repress different targets. The response of salE1 reporter is unusual in that many proteins activate rather than repress its expression.

2.4.8.1 Generation and testing of Wild Type and modified/mutated forms of UAS-brk transgenes

Different portions of Brk coding regions were modified, mutated or fused to other domains, including Nuclear Localization Sequences (NLS) and repression motifs, and cloned into the pUAST plasmid (Brand and Perrimon, 1993) containing the yeast Upstream Activating Sequence. In addition, each of the constructs contains an HA epitope tag sequence to visualize localization of the proteins in wing imaginal discs. The function of the mutated/modified Brk proteins was assessed by misexpression in the developing wing and observing the effects in two different ways, first by analyzing the phenotypes of adult wings and second by looking at expression of known Brk targets in the wing. In addition, the expression of a target (*Ubx*) in the embryo was also analyzed. The modified/mutated Brk transgenes were misexpressed in the wing using two different Gal4 drivers, en-Gal4 which drives expression in the posterior of the wing (Fig. 17C) and C765 which drives ubiquitous expression in the wing (Fig. 17B), but is weaker than en-Gal4. In comparison, endogenous Brk is expressed in a lateral to medial gradient in the wing disc (Fig. 17A).

The activity of the modified/mutated Brk proteins was compared to the wild type protein by analysis of Brk target genes *sal*, *omb*, Vg-QE and salE1 in wing discs and UbxB, an *Ultrabithorax* reporter in embryos (this enhancer is known to contain overlapping Brk and Mad binding sites) (Kirkpatrick et al., 2001; Saller and Bienz, 2001). To analyze the expression of known Brk target genes in the wing (*omb*, *sal* and Vg-QE, salE1), *brk* transgenes were misexpressed in the posterior of wing discs using the en-Gal4 driver and the expression of target genes in the posterior were compared to normal expression in the anterior of the disc. Animals

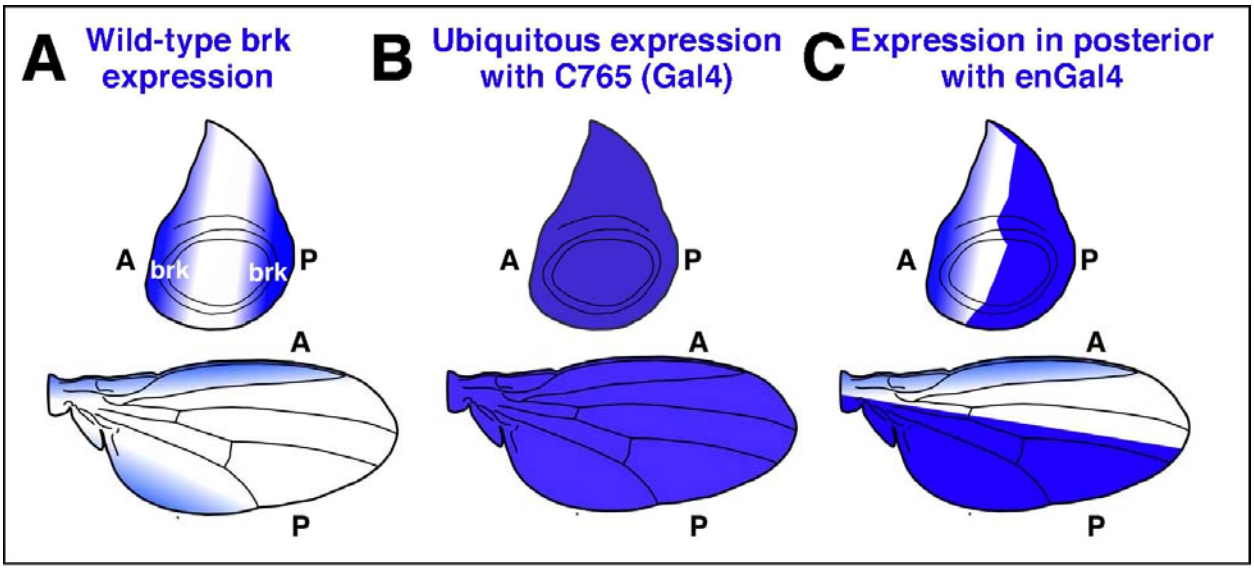

Figure 17. Misexpression of UAS-brk transgenes using two different Gal4 drivers.

(A) Expression of wild type *brk* in wing imaginal disc and how expression would project onto the adult wing. The activity of UAS-*brk* transgenes was assessed in adult wings using two Gal4 drivers, C765 and enGal4. Wing imaginal disc and adult wing showing ubiquitous expression using the C765 driver (B), and expression exclusively in the posterior compartment using enGal4 (C).

were raised at different temperatures to vary the amount of protein produced, as Gal4 is cold sensitive, so more protein is produced at higher temperatures. The modified/mutated forms of Brk protein generated a wide range of repressor activity based on the corresponding wing adult phenotype (Fig. 18). The assignment of activity levels observed in adult wings was based on the following criteria:

(++++) Wild-type level (no modified/mutated protein achieved this level). No adults' eclosed with en-Gal4 even when reared at 17°C. With C765 at 20°C, there was an almost complete loss of wing blade.

(+++) Some adults were obtained with en-Gal4 at 20-25°C, with substantial loss of posterior wing tissue and veins. With C765 at 25°C, there was almost a complete loss of the wing blade.

(++) Adult flies were obtained with en-Gal4 at 25-30°C, their wings had loss of tissue or fusion of veins IV and V and loss of the posterior cross vein. With C765 at 30°C, the wings were slightly smaller and had vein defects including extra cross veins.

(+) Adult flies obtained with en-Gal4 at 25-30°C with loss of the posterior cross vein. With C765 at 30°C there was little or no effect on the wings.

(D) Dominant negative: Adult flies obtained with en-Gal4 and C765 at 25-30°C and have excess posterior wing tissue and veins, sometimes producing a 'blistered' phenotype in the posterior.

(E) Neomorphic: Adult flies obtained with en-Gal4 and C765 at 25-30°C, wings have ectopic veins, particularly near vein V. The Brk protein in these animals may have a different function compared to wild-type protein.

(-) No activity. No abnormal phenotype under 25°C; at 30°C there was often some disruptions to wing venation such as extra small veins around the posterior cross vein and vein V. This was distinct from the other phenotypes above and may be caused by an extremely weak dominant negative activity.

At least three lines of each construct were tested apart from Brk^{F124} (one line). Although there was some variability in the level of activity from line to line, in general, most lines from any one construct fell into the same category of activity level. To be assigned to one of the above categories, at least two lines from a construct had to have a similar level of activity, with most constructs having at least three lines with similar levels of activity.

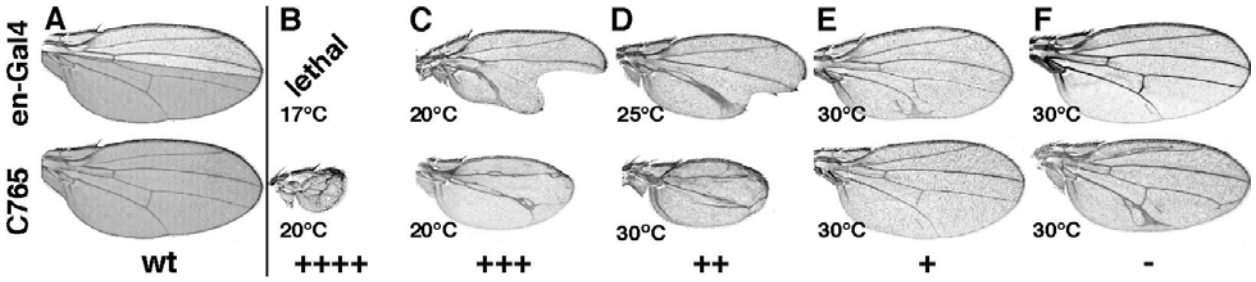

Figure 18. Phenotype of adult wings following misexpression of modified/mutated forms of *brk*.

UAS-brk transgenes were driven by en-Gal4 in the posterior compartment and C765 ubiquitously (C765 is weaker than en-Gal4). (A) Wild-type wings shaded to indicate the expression domains of the Gal4 drivers. (B-F) The activity level of different Brk proteins was classified into five categories from '++++' (full, wild-type level) to '-' (no repressor activity); examples of each category are shown, for comparison, the temperature must be taken into account (Gal4 is cold sensitive). (B) Full activity of 3PF3 (wild-type). With en-Gal4, no animals survived to adult; with C765 the wing size is drastically reduced at 20°C. (C-F) Mutated/modified Brk proteins have weaker activity than wild-type. (C) Stop1, 20°C (top); CM, 20°C (bottom). (D) 3M, 25°C (top); 3M, 30°C (bottom). (E) A2, 30°C. (F) NLS, 30°C (top); F124, 30°C (bottom). At high temperatures, ectopic veins are produced with F124, which we attribute to gain of function, as it has no effect on Brk target gene expression.

2.4.8.2 Activity of Wild Type Brk

As stated above, the activity of modified/mutated forms of Brk was compared to wild type Brk protein, Brk^{3PF3}. UAS-brk^{3PF3}; en-Gal4 flies do not survive to adults, even at low temperatures (17°C), whereas ubiquitous expression of the full-length *brk* transgene using C765 allows the animals to emerge at 20°C, but they have an almost complete loss of the wing blade (Fig 19B). However, UAS-brk^{3PF3}; en-Gal4 animals survive to the late larval or early pupal stages at 20°C, allowing analysis of *brk* target gene expression at this temperature. Wing imaginal discs stained for *sal*, *omb* and Vg-QE revealed that *sal* and *omb* are completely repressed in the posterior and Vg-QE was almost completely repressed (Fig. 19D,E). In comparison, analysis of larvae reared at 17°C revealed that *sal* is completely repressed (Fig. 19Cii), but some *omb* expression can be detected (Fig 19Ci), demonstrating that wild type Brk represses *sal* better than *omb*.

Analysis of *brk* null mutant clones revealed ectopic salE1 expression in lateral regions of the wing pouch (Fig. 10, section 2.4), and we tested to see if wild type Brk was able to repress salE1 reporter expression. UAS-brk^{3PF3} was misexpressed in the posterior of the wing disc using en-Gal4 and showed that salE1 expression was repressed in the posterior (Fig. 19F), providing further evidence for the role of Brk in repressing the salE1 reporter in lateral regions of the disc. Furthermore, misexpression of UAS-brk^{3PF3} in the posterior of salE1MB123 (salE1 with 3 Brk binding sites mutated) discs did not repress reporter gene expression in the posterior, but rather had no effect (Fig. 19G). Therefore, as expected Brk binding sites in salE1 are critical for Brk to repress salE1 reporter expression.

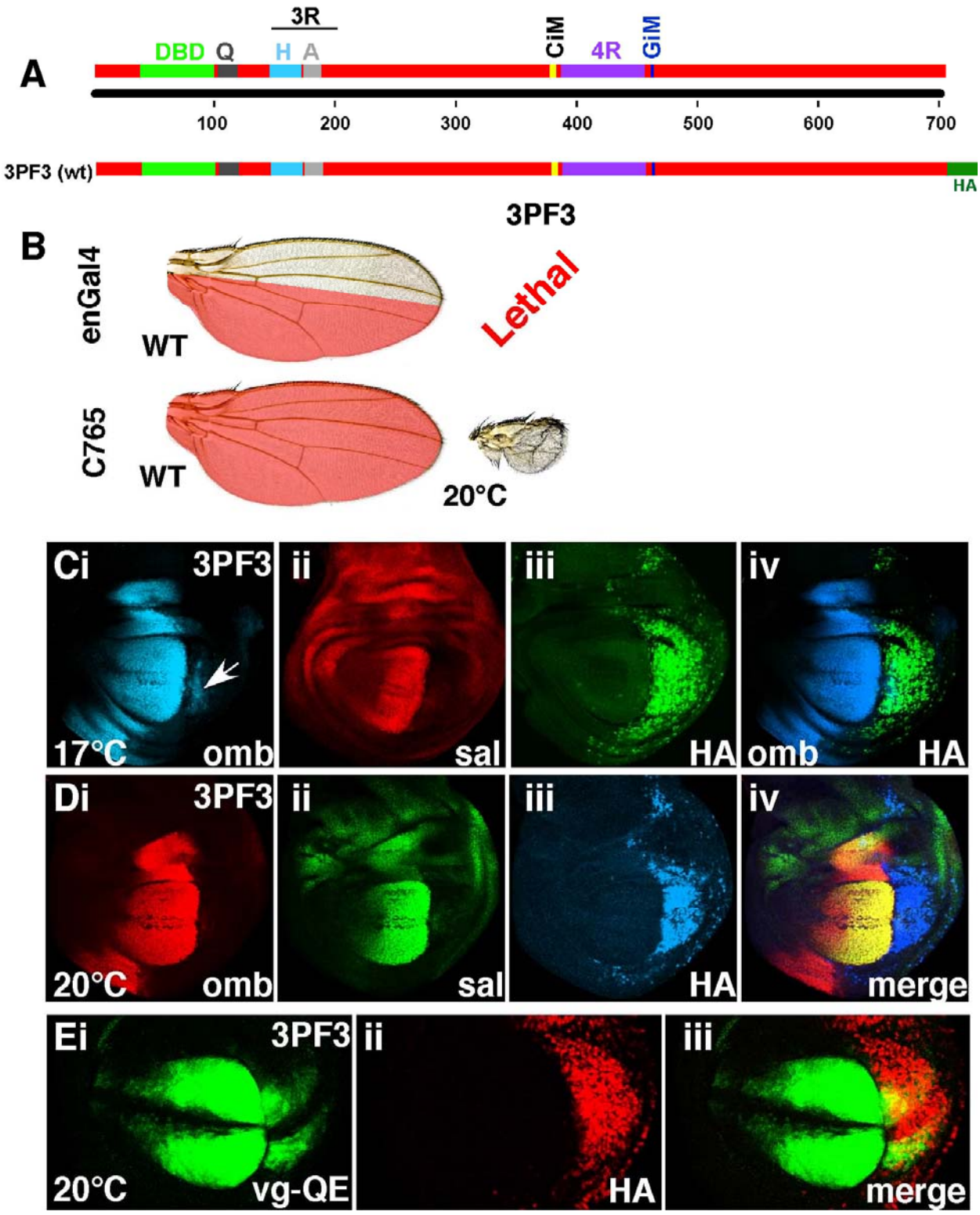

Figure 19. Misexpression of wild-type Brk (3PF3) in the developing wing and embryo.

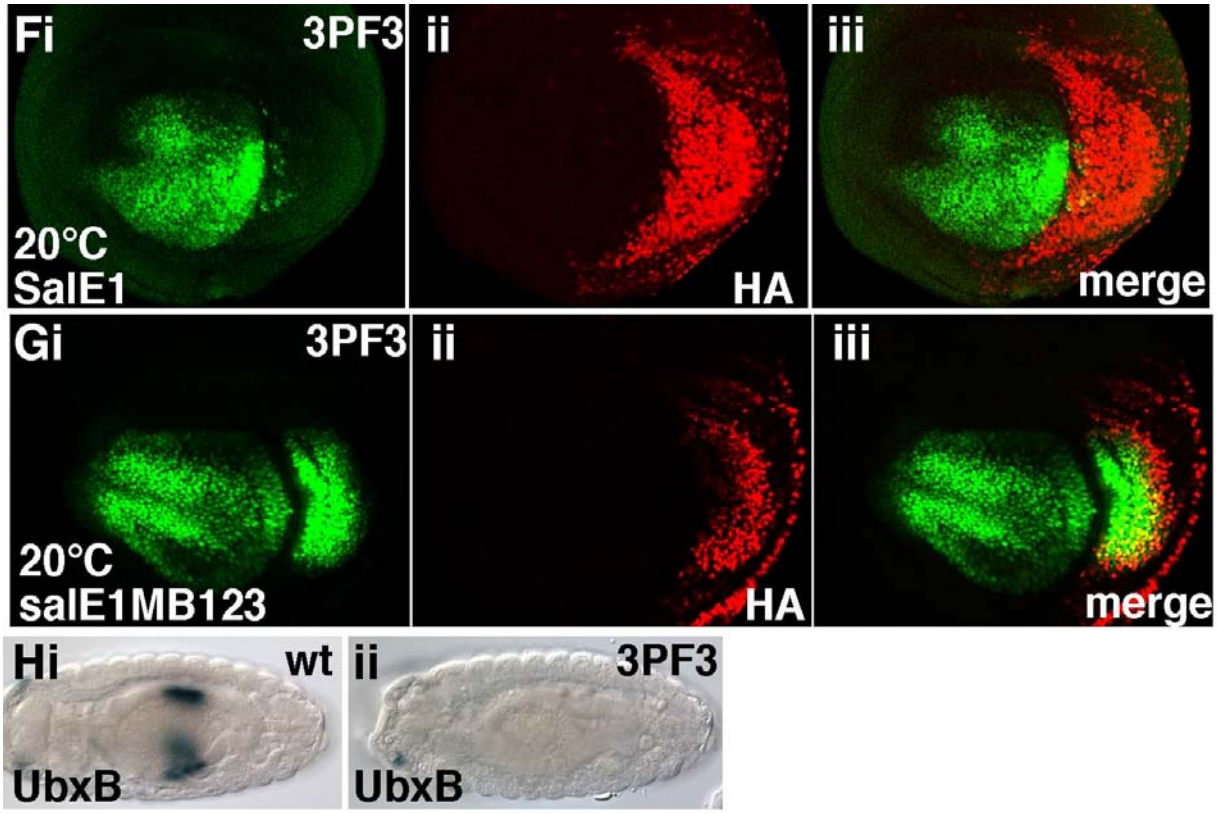

Figure 19. Misexpression of wild-type Brk (3PF3) in the developing wing and embryo.

(A) Schematic of Brk and HA-tagged wild-type Brk (3PF3) protein containing DBD, interaction motifs CiM and GiM, and independent repressor domains, 3R and 4R. In Figures 19-35, the construct is shown below the Brk protein (B) Adult wings. Color overlays of wild-type wing showing the regions where Brk^{3PF3}, and other modified proteins throughout the study were misexpressed; enGal4 is expressed in the posterior, while C765 is ubiquitous, but weaker than enGal4. Brk3PF3, a wild-type form of Brk protein, induces early pupal lethality with enGal4, and an almost complete loss of the wing blade with C765. (C-F) Repression of *sal*, *omb*, vg-QE and salE1 following misexpression of 3PF3 in the posterior of wing discs using enGal4 (posterior is to the right in this and other figures and is compared to anterior expression on the left). Expression of Sal is visualized with anti-Sal antibody; *omb* and vg-QE are visualized using lacZ (anti-βgal antibody). salE1 and salE1MB123 are GFP reporter constructs. Transgene expression is indicated by HA panel (anti-HA antibody). This methodology will be used for Figures 19-35. (Ci-iv) At 17°C, *sal* is completely repressed (red), but some *omb* (blue) expression is present. (Di-iv) At higher temperatures (20°C) (results in higher levels of transgene expression), both *sal* (green) and *omb* (red) are completely repressed. (Ei-iii) 3PF3 substantially reduces the level and width of vg-QE expression in the posterior. (Fi-iii) 3PF3 almost completely represses salE1 reporter expression in the posterior (20°C). But mutation of the three Brk binding sites in salE1 results in the inability of Brk to repress salE1 expression (Gi-iii). (H) UbxB drives reporter gene (*lacZ*, visualized using X-gal) expression in the visceral mesoderm of embryos. (Hii) Misexpression of 3PF3 throughout the mesoderm completely represses UbxB reporter gene expression.

Previous studies demonstrated ectopic wild type Brk completely repressed expression of an *Ultrabithorax* reporter, UbxB, in the visceral mesoderm of the embryo (Saller and Bienz, 2001). We repeated the experiments completed in the Bienz Lab to confirm that our wild type Brk transgene also repressed UbxB reporter expression in the visceral mesoderm in the embryo. Misexpression of UAS-brk^{3PF3} in the mesoderm with the 24B Gal4 driver results in complete repression of UbxB reporter gene expression compared to control embryos with no transgene (Fig 19H). Taken together, these studies revealed that full-length, wild type Brk is capable of repressing all five Brk targets in the wing and embryo.

Misexpression of wild-type Brk in wing discs (UAS-brk^{B459}) (25°C) results in a dramatic alteration of the wing disc morphology from a relatively flat morphology, to a deep invagination at the interface between cells ectopically expressing *brk* in the posterior and normal cells in the anterior (Moser and Campbell, 2005). Recent studies demonstrated that loss of Dpp signaling in cells in the wing pouch results in extrusion from the epithelium (Gibson and Perrimon, 2005; Shen and Dahmann, 2005). Cells in the invagination move basally relative to the apical/basal polarity of the epithelium (Moser and Campbell, 2005). As a result of the alteration in disc shape, analysis of gene expression patterns required both the basal and apical XY confocal sections. Analysis revealed that at lower temperatures (20°C), *sal* was completely repressed and *omb* was detected in the posterior of discs (Moser and Campbell, 2005). These results are similar to those obtained using UAS-brk^{3PF3}, showing more Brk is required to repress *omb* than *sal.* However, analysis of gene expression in this study predominantly focused on apical to medial confocal sections.

2.4.8.3 Analysis of modified/mutated forms of Brk protein

2.4.8.3.1 Requirement for a functional DNA binding domain

As indicated previously, clonal analysis of endogenous mutant *brk*^{F124}, containing an amino acid substitution in the recognition helix (R82W) of the DNA binding domain, revealed a protein with little or no activity, as *brk*^{F124} mutant clones were reminiscent of *brk* null alleles with misexpression of *sal, omb* and vg-QE in lateral regions of the wing pouch (Fig. 12A,B). In addition, four mutations isolated from the UAS-brk^{A438} EMS screen, corresponding to lesions in the DNA binding domain, reduced activity of the protein, but these mutations did not abolish activity (Fig 16C). An HA tagged version of the *brk*^{F124} mutant (Fig 16D, 20A), UAS-brk^{F124}, containing the same amino acid substitution in the DNA binding domain, was produced and it also did not possess repressor activity as shown by normal *sal* and *omb* expression (Fig. 20C).

Misexpression of UAS-brk^{F124} in the posterior of wing discs revealed that Brk protein with a non-functional DNA binding domain was not capable of repressing salE1 (Fig 16D), as salE1 expression is identical in the anterior and posterior compartments. Since Brk^{F124} could not repress salE1, it argues that this protein may be compromised in its ability to bind DNA. However, when Brk^{F124} was misexpressed at high levels using C765 (30°C), ectopic veins were present in adult wings (Fig 20B), possibly due to aberrant activity of the protein, indicating the protein has some activity but it is not equivalent to wild type protein. This protein may have a novel activity possibly due to titrating out factors such as Gro or CtBP, e.g. reduction of Gro may result in ectopic wing veins. Overall, mutations in the DNA binding domain of *brk* either reduces or completely abolishes the repressor activity of the protein, indicating Brk requires a functional DNA binding domain for repressor activity.

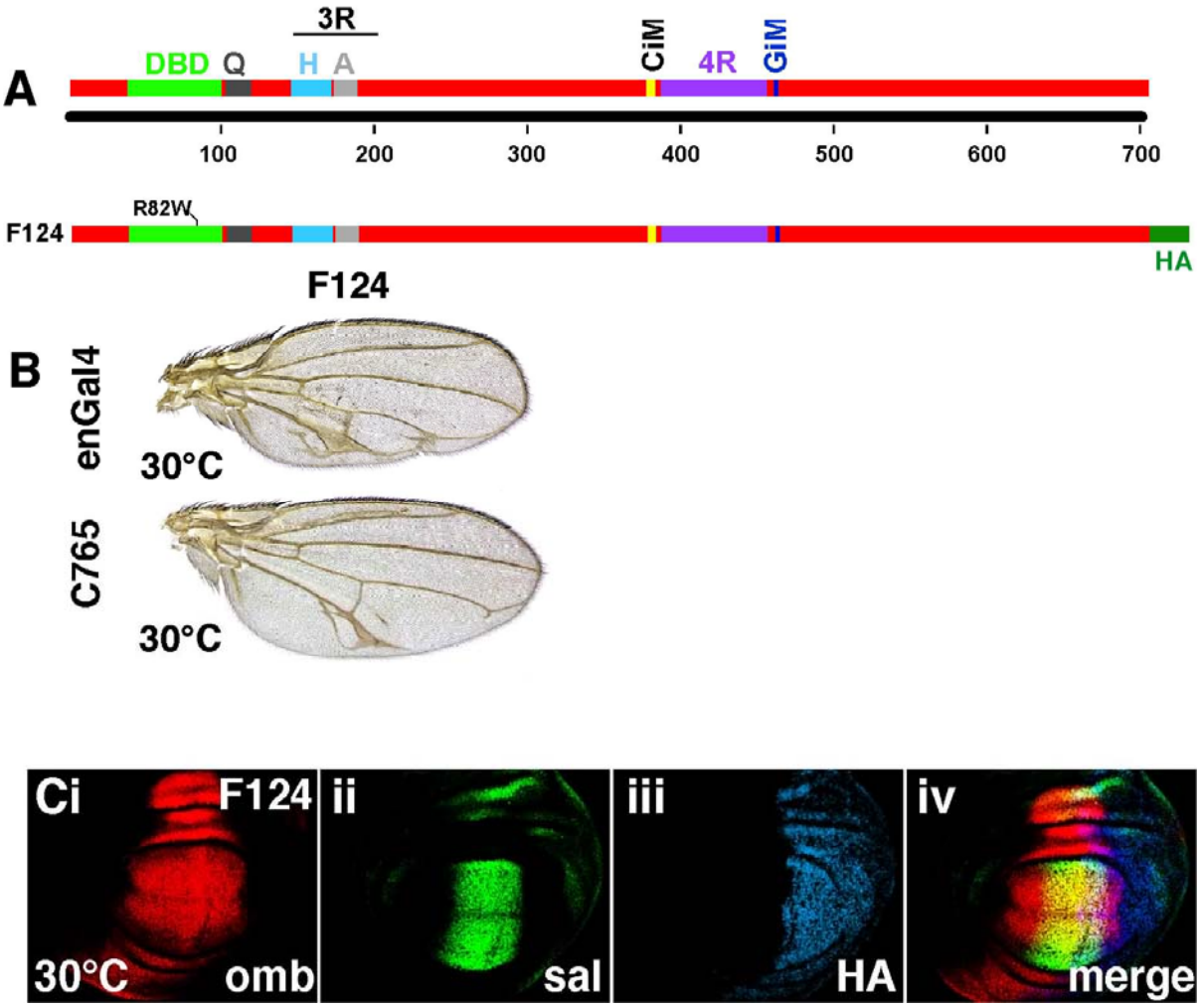

Figure 20. Misexpression of UAS-brk^{F124} in the developing wing.

(A) Schematic of HA-tagged Brk^{F124} protein containing an amino acid substitution in the DBD, but with functional interaction motifs CiM and GiM, and independent repressor domains, 3R and 4R. (B) Adult wing phenotype. Misexpression of Brk^{F124} at high levels (30°C) with enGal4 and C765 produced ectopic veins in adult wings, indicating the protein has some activity, but it is not equivalent to wild type protein. (Ci-iv) Misexpression of Brk^{F124} in the posterior of wing discs did not repress either *sal* (green) or *omb* (red) expression.

2.4.8.3.2 Brk proteins containing only a DNA binding domain or DBD plus 'Q' region have no repressor activity

If Brk can repress simply by binding-site competition, then a protein possessing only the DNA binding domain and which is localized to the nucleus should be able to repress target genes. Previous *in vitro* studies have demonstrated that Brk residues 44-99 are sufficient to bind DNA and compete with activators such as Mad (Saller and Bienz, 2001). Endogenous mutant brk^{E427}, truncated at residue 115, and UAS-$brk^{A438\text{-}53}$, truncated at residue 102, produce proteins primarily containing the DNA binding domain (Fig. 16B,C). However, neither of these proteins possessed repressor activity *in vivo* (Fig. 16B,C). One possible reason for the inactivity of these proteins could be the lack of a Nuclear Localization Sequence (NLS), thereby making the proteins predominately cytoplasmic. Characterization of the localization of these proteins was not possible using available antibodies, so an equivalent HA tagged version of UAS-$brk^{A438\text{-}53}$, Brk^{53}, was produced (Fig. 16D, 21A). Misexpression of UAS-brk^{53} produced a wild type wing indicating the protein does not possess activity (Fig. 21B). Brk^{53} was not able to repress expression of *sal* or *omb* in the posterior of wing discs (Fig. 21D). Subsequent analysis of the localization of this protein in wing discs using an anti-HA antibody revealed that Brk^{53} protein was not restricted to the nucleus (Fig. 21E), which could explain its lack of activity. Moreover, extension of the protein to include the poly-glutamine region 'Q region' to generate Brk^{S} (Fig. 16D, 21A), truncated at residue 123, also produced a protein with no repressor activity (Fig. 16D). In fact, Brk^{S} seemed to be functioning as a dominant negative form of the protein shown by the excess wing tissue producing a "blistered" wing (Fig. 21B). The truncated form of the protein may be interfering with the ability of endogenous Brk to repress target genes, resulting in excess wing vein and tissue formation. Overexpression of Brk^{S} with a deletion *brk*-Gal4 line

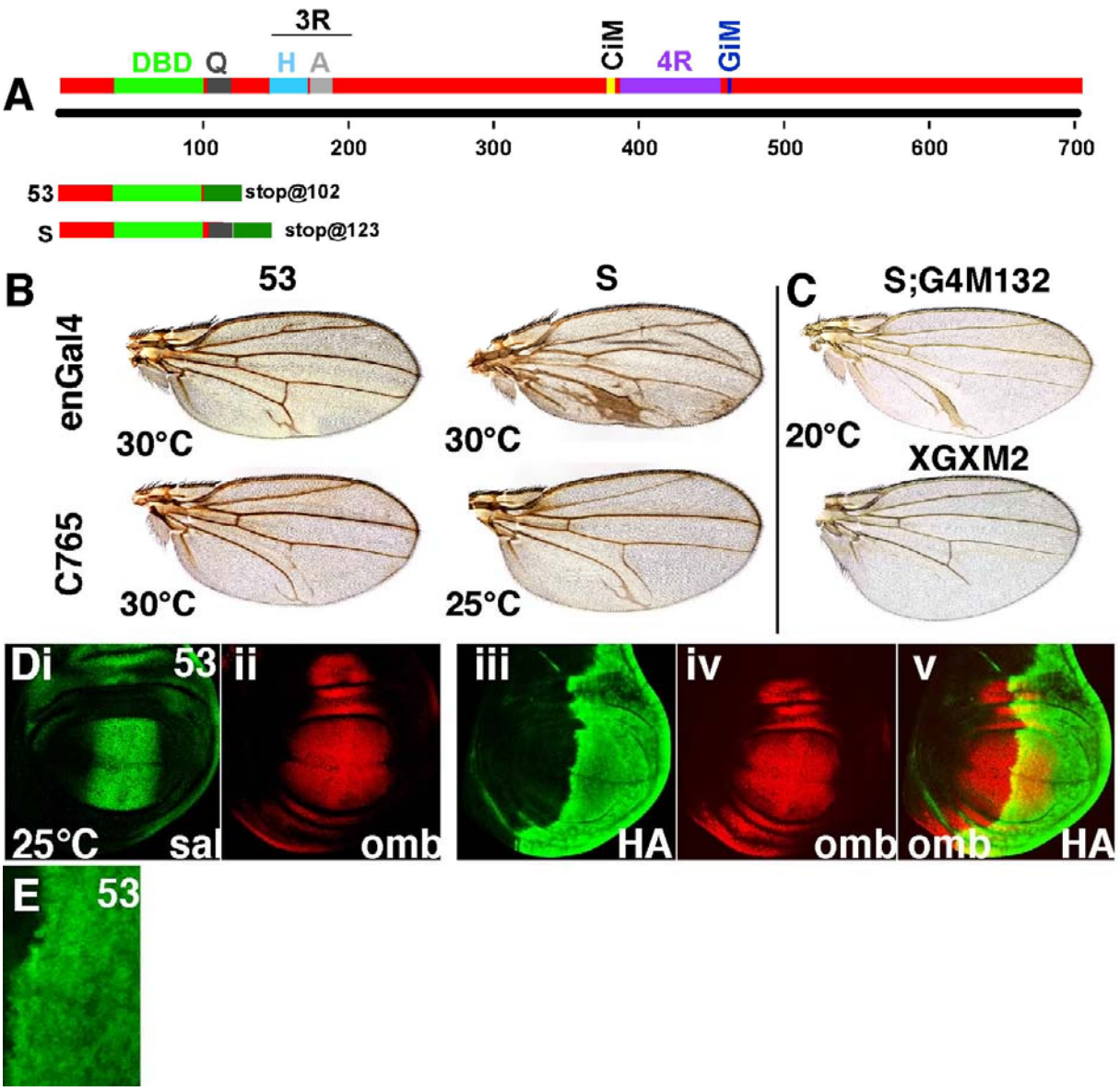

Figure 21. Misexpression of truncated forms of Brk (53 and S) in the developing wing.

(A) Schematic of HA-tagged Brk^{53} and Brk^{S} proteins, truncated immediately following the DBD, and after the poly-glutamine region, respectively. Both of these proteins lack interaction motifs for CiM and GiM, as well as independent repressor domains 3R and 4R. (B) Misexpression of Brk^{53} or Brk^{S} produced predominantly wild type adult wings with enGal4 and C765. Brk^{S} may be functioning as a dominant negative, shown by the excess wing tissue in the posterior portion of the wing. (C) Overexpression of Brk^{S} with brk-Gal4 deletion line (G4M132), results in excess tissue and veins in the posterior and an expansion of the posterior similar to a weak loss of function *brk* mutant (XGXM2). (Di-v) Brk^{53} cannot repress expression of *sal* (green) or *omb* (red) in the posterior of wing discs, but analysis revealed that Brk^{53} was not restricted to the nucleus. (i, ii) expression of *sal* and *omb*, respectively. (iii, iv,v) expression of HA-53 and *omb*. (E) Higher magnification image focused on the large peripodial membrane cells showing expression of 53 is not restricted to the nucleus.

(G4M132) produced adult wings with a similar phenotype to a weak loss of function *brk* mutant (XGXM2) (Fig. 21C). Brk^{53} and Brk^{S} were not able to repress salE1 reporter expression when misexpressed in the posterior of wing discs, but surprisingly, they were able to activate expression indicating that some Brk^{53} does get into the nucleus (Fig. 16D). These data suggest that salE1 is not acting the same as the endogenous *sal* gene.

2.4.8.3.3 Addition of a Nuclear Localization Sequence does not change the repressor activity of truncated Brk

Since Brk^{53} did not possess activity and was predominantly cytoplasmic, an SV40 T Antigen Nuclear Localization Sequence (Kalderon et al., 1984) was added to Brk^{53}, making Brk^{NLS} protein (Fig. 16D, 22A). In contrast to Brk^{53}, Brk^{NLS} was localized in the nucleus (Fig. 22Dv, 16D) where it could potentially bind DNA and repress target genes. However, Brk^{NLS} also did not have detectible repressor activity, as shown by the presence of wild type wings in adults following misexpression using both en-Gal4 and C765 (Fig 22B). Furthermore, Brk^{NLS} was not able to repress either *sal* or *omb* when misexpressed in wing discs (Fig 22Di-iv). Misexpression of UAS-brk^{NLS} in the posterior of wing discs did not repress salE1 reporter gene expression. Similar to Brk^{53}, Brk^{NLS} also activated salE1 reporter expression, providing strong evidence that Brk^{NLS} can bind DNA *in vivo* (Fig. 22E). Surprisingly, these findings suggest that the N-terminal region of Brk protein may possess a cryptic activation domain. In contrast, misexpression of UAS-brk^{NLS}, in the posterior of wing discs did not have any effect on salE1MB123 expression, either activating or repressing the reporter (Fig. 22F).

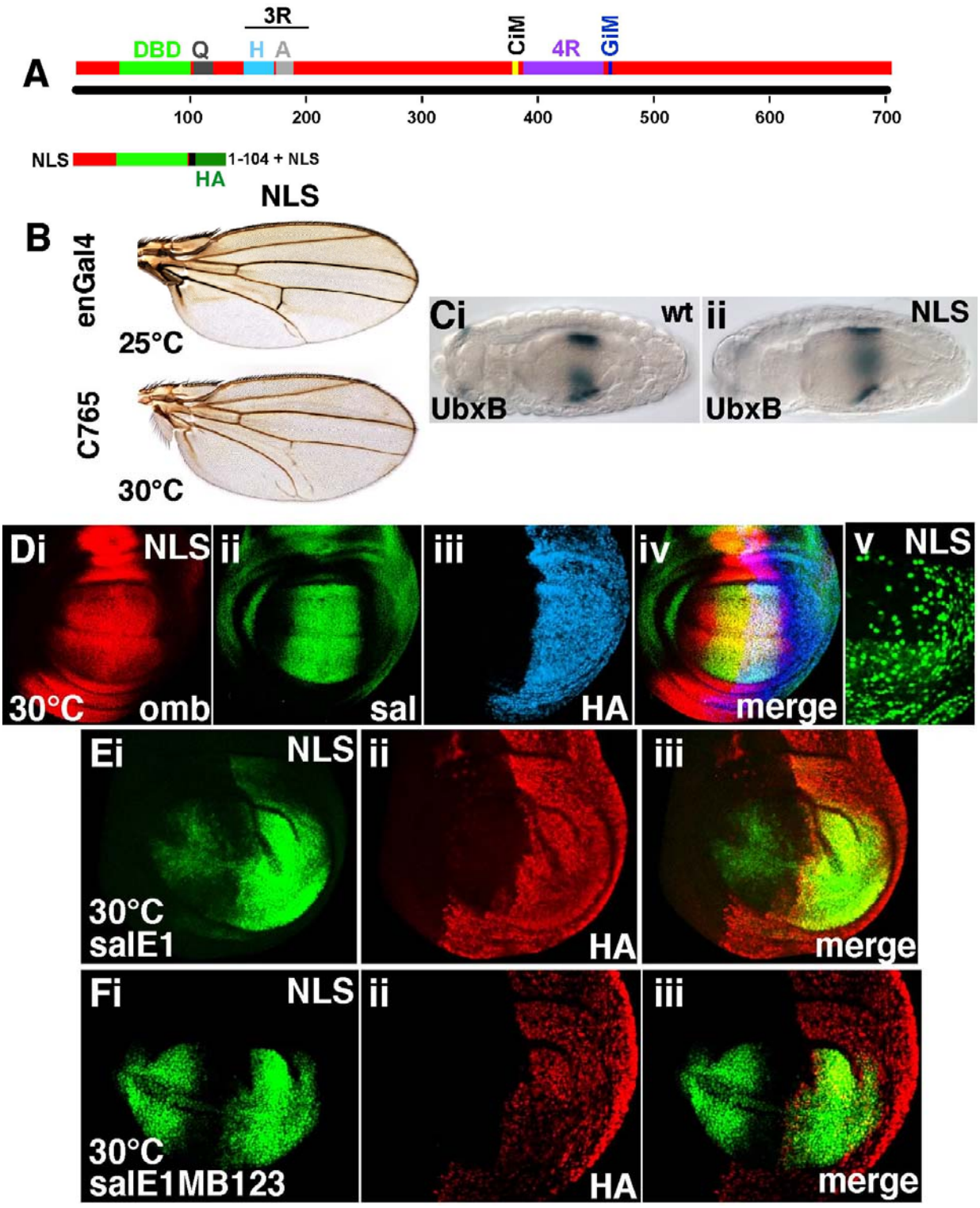

Figure 22. Misexpression of UAS-brk^{NLS} in the developing wing and embryo.

Figure 22. Misexpression of UAS-brk^{NLS} in the developing wing and embryo.

(A) Schematic of HA-tagged Brk^{NLS}, a protein truncated immediately after the DNA binding domain that contains a nuclear localization sequence. (B) Phenotype of adult wings. Brk^{NLS} does not have any detectable repressor activity, shown by wild-type wings following misexpression with either Gal4 driver. (C) Misexpression of NLS in the mesoderm has no effect on UbxB reporter gene expression in the embryo. (D-F) Analysis of *sal*, *omb*, salE1 expression in wing discs. (Di-iv) At 30°C, NLS has no effect on the expression of *sal* (green) or *omb* (red) when misexpressed in the posterior of wing discs. (Dv) Higher magnification image focused on the large peripodial membrane cells showing expression of NLS is exclusively nuclear (HA staining). (Ei-iii) Misexpression of NLS in the posterior of wing discs results in activation of salE1 reporter expression. (Fi-iii) NLS does not activate salE1MB123 (salE1 with three Brk binding sites mutated) reporter expression (compare posterior to anterior expression).

2.4.8.3.3.1 **Brk^{NLS} can bind DNA *in vitro***

To confirm whether or not Brk^{NLS} is capable of binding DNA *in vitro*, DNA binding assays were completed using the UbxB enhancer signal responsive sequence as the probe. Brk residues 44-99 were previously shown to bind the UbxB enhancer, (Saller and Bienz, 2001). The UbxB enhancer is a 260 bp fragment that can drive reporter gene expression in a pattern similar to endogenous Ubx (Thuringer et al., 1993), but its expression is slightly wider. In addition, the signal responsive sequences contained within the UbxB midgut enhancer contains three Brk binding sites as well as three Mad binding sites which are overlapping (Fig. 23A) (Saller and Bienz, 2001).

In vitro translated Brk^{NLS} was incubated in the presence of radiolabeled UbxB probe (36 bp in length and centered around the overlapping Mad/Brk sites shown in Fig. 23A), and run on a 4% PAGE Gel (Ausubel et al., 1993). Analysis revealed a shift of the labeled UbxB probe to two slower migrating bands, the lower migrating band possibly corresponds to one Brk^{NLS} bound and the higher migrating band corresponds to two Brk^{NLS} molecules bound (Fig. 23B, Lanes 3,4,5,6,11,12). In addition, incubation of the Brk^{NLS}-UbxB complex with anti-HA antibody produced a supershift of the complex (Fig. 23B, Lane 9), confirming that Brk^{NLS} is indeed binding to the UbxB probe and that the antibody recognizes the HA tagged *in vitro* translated protein. Furthermore, experiments adding either 10X or 100X of unlabeled UbxB self competitor probe resulted in a predominant loss of the higher migrating species with 10X (Fig. 23B, Lane 12) and complete loss of the higher migrating species and partial loss of the lower migrating species in the presence of 100X unlabeled probe (Fig. 23B, Lane 13) compared to the absence of competitor probe control (Fig. 23B, Lane 11). In addition, there is an increase in the

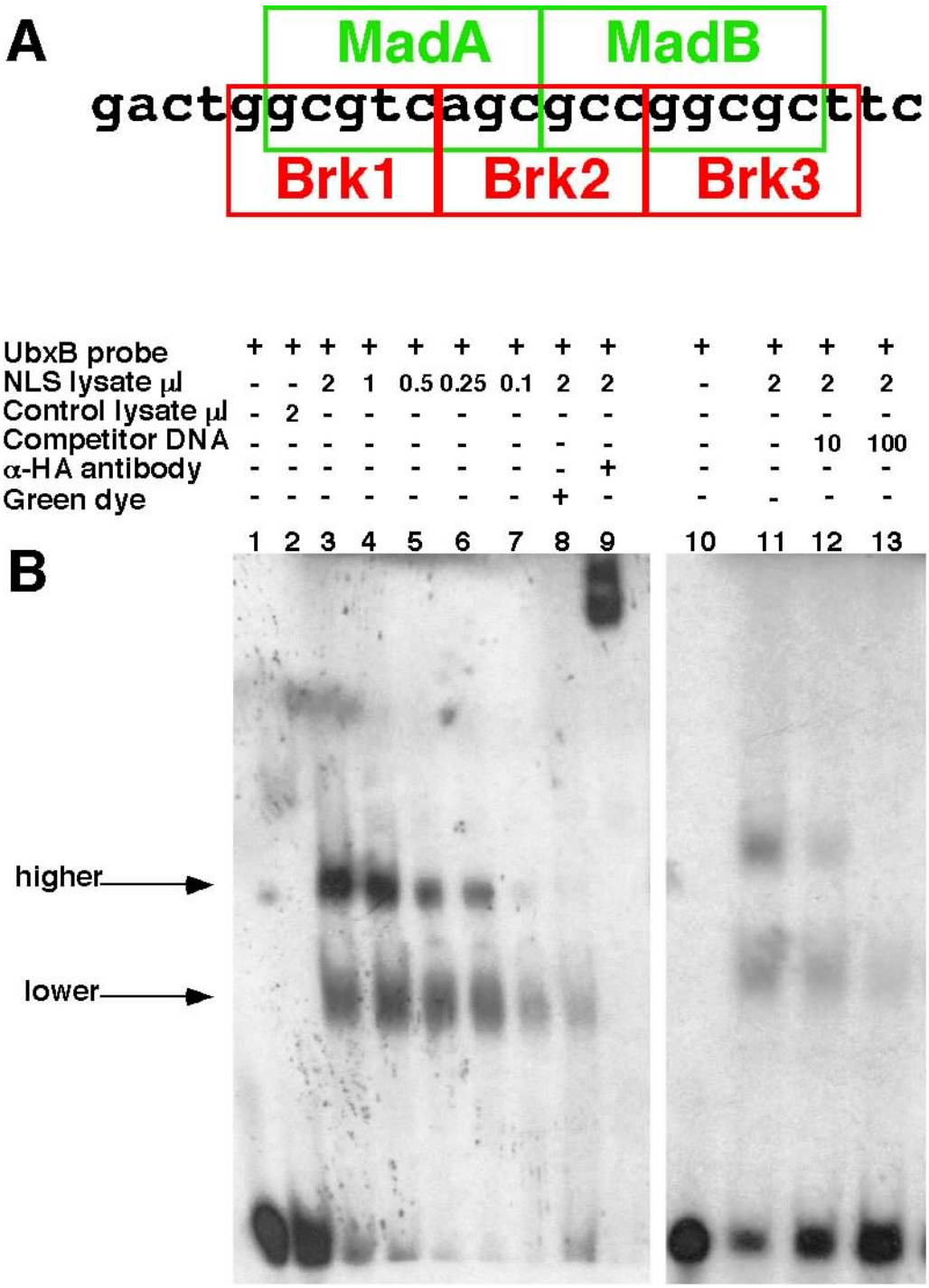

Figure 23. Gel Mobility shift assay using HA-Brk^{NLS} rabbit reticulocyte lysate and UbxB probe demonstrating Brk^{NLS} binds to DNA *in vitro*.

Figure 23. Gel Mobility shift assay using HA-Brk^{NLS} rabbit reticulocyte lysate and UbxB probe demonstrating Brk^{NLS} binds to DNA *in vitro*.

(A) Relevant portion of UbxB DNA probe showing overlapping Brk (Red boxes) and Mad (activator) binding sites (Green boxes)(adapted from Saller & Bienz, 2001). (B) Gel shift assays demonstrating Brk^{NLS} binds to DNA *in vitro*. (Lanes 1,10) 32P labeled UbxB probe, no lysate. (Lane 2) Control lysate with no Brk^{NLS} expression plasmid added. (Lane 3) Addition of NLS lysate to UbxB probe results in a slower migration of the UbxB probe. We hypothesize that the higher migrating band corresponds to binding of two Brk^{NLS} molecules and the lower band to one molecule. (Lanes 4,5,6,7) Addition of decreasing amounts of NLS lysate. (Lane 8) Same as lane 3, but with addition of green dye. The same amount of UbxB probe was added to each lane. (Lane 9) Addition of anti-HA antibody to a similar reaction as in lane 3, results in a supershift of the protein-DNA complex. (Lanes 11,12,13) NLS lysate plus 0X, 10X and 100X unlabeled competitor UbxB probe. Increasing amounts of competitor DNA first results in a loss of the higher migrating band suggesting that the second Brk may be more weakly bound.

amount of radiolabeled probe not being shifted in the presence of the 10X and 100X unlabeled probe compared to the control, demonstrating the unlabeled probe is in fact competing for binding to Brk^{NLS}. Taken together, these data suggest that Brk^{NLS} is capable of binding DNA *in vitro* and indicates that this protein is probably capable of binding DNA *in vivo*, thereby arguing against simple binding-site competition as the mechanism Brk uses for repression of target genes.

2.4.8.3.3.2 Brk does not use competition as a mechanism for repression of target genes

Brk^{NLS} contains the region previously shown to bind DNA *in vitro* (residues 44-99) (Saller and Bienz, 2001), binds DNA *in vitro* (Fig. 23) and is localized in the nucleus (Fig. 22Dv); therefore, Brk^{NLS} should be able to bind DNA *in vivo* and argues against Brk using binding-site competition as a mechanism for repression. However, the *in vitro* studies suggesting Brk uses binding-site competition as a mechanism for competition were completed using genes expressed in the embryo (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001), and it is possible that Brk does not use this mechanism in the wing. Subsequently, we tested one of the embryonic targets, UbxB, a *Ubx* reporter construct expressed in the embryonic midgut mesoderm (Thuringer et al., 1993) and found that Brk^{NLS} was not able to repress this target either (Fig. 22C). Consequently, these data argue against Brk competing with activators, even with enhancers where Brk and activator (Mad) binding sites overlap.

2.4.8.3.4 Addition of a minimal repression domain/motif to Brk DBD restores repressor activity

Although Brk^{NLS} was shown to bind DNA *in vitro*, possible reasons for the lack of Brk^{NLS} activity are that it cannot bind DNA *in vivo* or it cannot fold properly. To test these possibilities, another construct was generated by inserting 4 amino acids corresponding to a minimal Gro Interaction Motif, WRPW, between the Nuclear Localization Sequence and the HA tag of Brk^{NLS} to produce Brk^{NLSW} (Fig 24A, 16D). The WRPW motif has been shown to function as a repression motif via recruitment of the co-represssor Groucho (Aronson et al., 1997; Fisher et al., 1996). Addition of the WRPW repression motif confers considerable activity to Brk^{NLSW}, although not as strong as that of wild type Brk. UAS-brk^{NLSW}; C765 flies have a significant reduction in the size of the wing blade (Fig. 24B) and there is loss of posterior wing tissue in en-Gal4 animals (Fig. 24B). Furthermore, analysis of UAS-brk^{NLSW}; en-Gal4 wing discs revealed that *sal* is completely repressed and *omb* partially repressed (Fig. 24D). The ability of Brk^{NLSW} to repress *sal* more effectively than *omb* is similar to the repression profile observed with wild type Brk protein. In contrast, Brk^{NLSW} cannot repress Vg-QE expression (Fig. 24E), but can strongly repress UbxB expression in the embryonic midgut mesoderm (Fig. 24C). Moreover, Brk^{NLSW}, a protein possessing only the GiM was also not able to repress salE1 but rather activated this reporter, similar to other truncated proteins (Fig. 24F).

2.4.8.3.5 Brk protein possesses an additional repression domain/motif, 3R, between the DNA binding domain and the CiM

As shown previously, Brk^{NLS} does not possess repressor activity in this assay, but Brk^{F138} has considerable activity (Section 2.5, Fig. 12C,D), suggesting that the region lying between the DNA binding domain and the CtBP Interaction Motif (CiM) must contain an additional

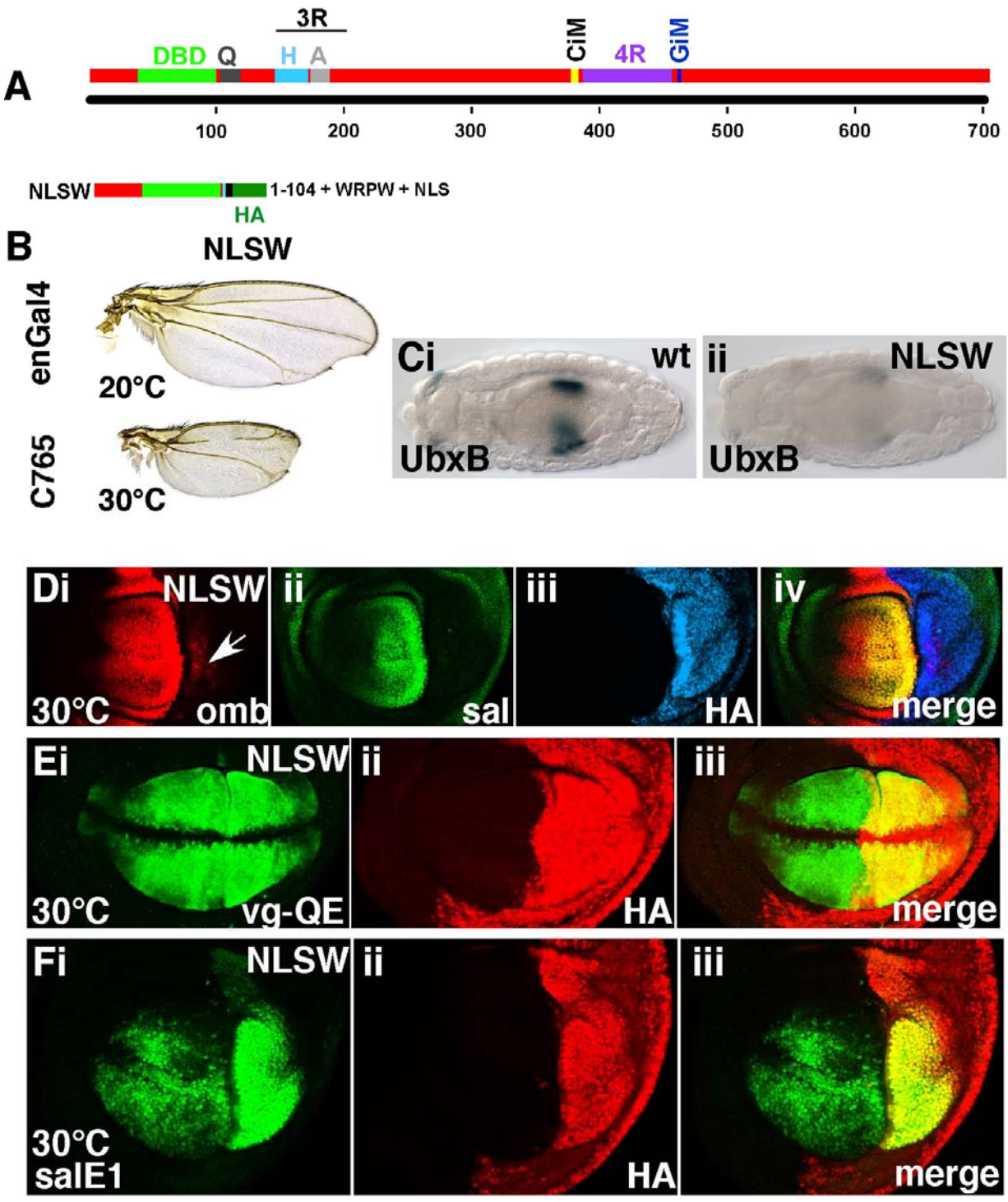

Figure 24. Misexpression of UAS-brk^{NLSW} in the developing wing and embryo.

Figure 24. Misexpression of UAS-brk^{NLSW} in the developing wing and embryo.

(A) Schematic of HA-tagged Brk^{NLSW}, a protein truncated immediately after the DNA binding domain that contains a nuclear localization sequence and a Groucho interaction motif (GiM), WRPW. (B) Adult wing phenotype. NLSW, a protein identical to NLS, but has the GiM added, has significant activity resulting in loss of wing tissue and veins. (C) NLSW represses UbxB reporter gene expression in embryos. (D-F) Analysis of *sal*, *omb*, vg-QE and salE1 expression. (Di-iv) Misexpression of NLSW in the posterior of wings discs using en-Gal4 results in complete repression of *sal* (green) and almost complete repression of *omb* (red, arrow). (Ei-iii) NLSW has no effect on vg-QE expression when misexpressed in the posterior. (Fi-iii) NLSW activates salE1 reporter gene expression in the posterior of wing discs, similar to NLS.

repression domain. To test this possibility, Brk^{Stop1} a protein similar to Brk^{F138}, being truncated immediately before the CiM and lacking the GiM (residue 377) (Fig. 25A, 16D), was produced to verify that any activity from Brk^{F138} was not due to read through of the stop codon. At 20°C, UAS-brk^{Stop1}; C765 animals have significantly reduced wing size and misexpression in the posterior compartment with en-Gal4 reduces the amount of posterior wing tissue (Fig. 25B). Furthermore, misexpression of Brk^{Stop1} in the posterior of wing discs at 25°C results in complete repression of target genes *omb*, *sal* and Vg-QE (Fig. 25E,F). When larvae were reared at 20°C to reduce the expression of the transgene, *omb* was still almost completely repressed with some weak expression present in the posterior (Fig. 25Di). However, at 20°C, *sal* was strongly expressed in the posterior adjacent to the A/P boundary, but did not extend laterally compared to its endogenous expression domain (Fig. 25Dii). These findings show that Brk^{Stop1} represses *omb* more effectively than *sal* and therefore is the opposite of wild type Brk repression of these target genes. In addition to repression of *omb* and *sal*, Brk^{Stop1} also represses UbxB and vg-QE reporters (Fig. 25C,F). In fact, Brk^{Stop1} can repress UbxB more efficiently than Brk^{NLSW} (Fig. 24D) and vg-QE better than wild type. Misexpression of Brk^{Stop1} in the posterior of wing discs activated expression of salE1 throughout the posterior wing pouch as well as in the hinge and notum (body wall) (Fig. 25G).

2.4.8.3.5.1 Identification of the 3R repression domain

To identify the specific region between the DNA binding domain and the CiM required for repressor activity, a series of progressively smaller Brk truncations were generated (Fig. 26A, 16D). Truncations of the region between the DNA binding domain and the CiM revealed Brk^{A} as the shortest protein with repressor activity. Brk^{A} is truncated at residue 206 (Fig 26A) and has

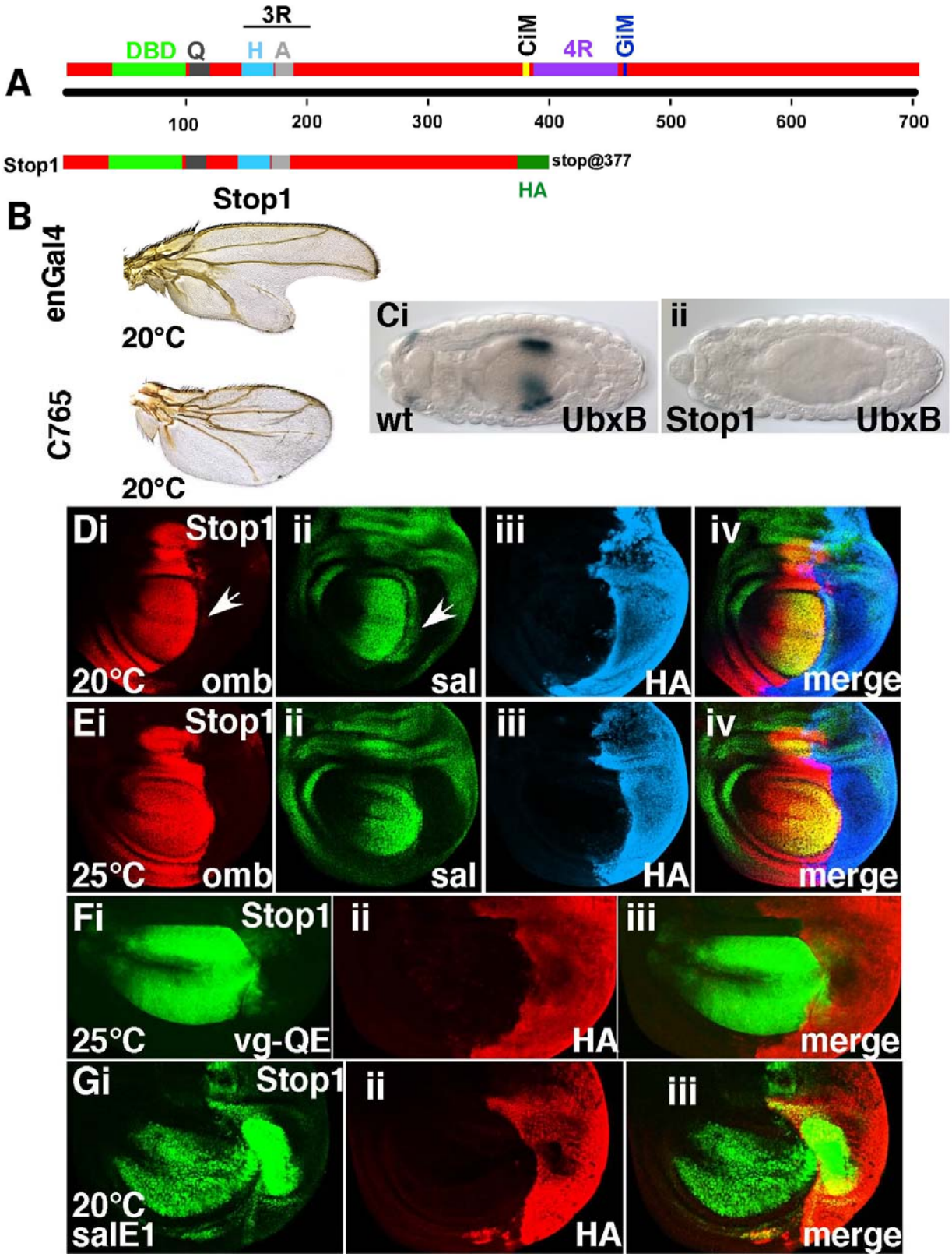

Figure 25. Misexpression of UAS-brk^{Stop1} in the developing wing and embryo.

Figure 25. Misexpression of UAS-brk^{Stop1} in the developing wing and embryo.

(A) Schematic of HA-tagged Brk^{Stop1}, a protein truncated before the CiM and GiM. (B) Adult wing phenotype. Stop1 has significant activity, resulting in loss of wing tissue and veins with both Gal4 drivers. (C) Misexpression of Stop1 in the embryonic mesoderm completely represses UbxB reporter expression. (D-G) Analysis of *sal*, *omb*, vg-QE and salE1 expression in wing discs. (Di-iv) Misexpression of Stop1 in the posterior of wing discs at 20°C results in an almost complete loss of *omb* (red) (there is some residual expression, arrow in i), whereas *sal* is still expressed at high levels in the posterior (green-arrow in ii). (Ei-iv) At 25°C, Stop1 represses *sal* and *omb* completely. (Fi-iii) Stop1 almost completely represses vg-QE expression in the posterior at 25°C. (Gi-iii) Misexpression of Stop1 in the posterior of wing discs results in activation of salE1 reporter rather than repression.

repressor activity similar to Brk^{Stop1} (Fig 16D). At 25°C, UAS-brk^{A} ; C765 adults have smaller wings compared to wild type and there is a significant loss of posterior wing tissue using en-Gal4 (Fig. 26B). Brk^{A} contains three distinct sequences: a poly-glutamine stretch, a histidine rich region and a poly-alanine stretch (Fig. 26A). All of these sequences may be important for this region to act as a repressor domain, but no single region alone is capable of repressor activity. Similar to Brk^{Stop1}, misexpression of UAS-brk^{A} in the posterior of wing discs activated expression of salE1 throughout the posterior wing pouch as well as in the hinge and notum (Fig. 16D)

Truncation of Brk at residue 173 (Brk^{A2}), eliminating the poly-alanine region and some unique sequence at the C-terminus, produces a protein with minimal repressor activity (Fig. 16D) as shown by the nearly wild type wings following misexpression with both C765 and en-Gal4 drivers at 30°C (Fig 26B). The wings have a few excess veins surrounding vein V and some excess tissue with en-Gal4. In addition, *sal* and *omb* expression are not repressed by Brk^{A2} (Fig 16D). UAS-brk^{C} produces a protein truncated at residue 140, to eliminate the histidine rich region, and renders a nearly inactive form of the protein with excess veins and tissue, when misexpressed in the wing using C765 and en-Gal4 (Fig 26B). In fact, Brk^{C} seems to be acting as a dominant negative demonstrated by the excess wing tissue and blistery appearance present in the posterior of the wing. Interestingly, both Brk^{A2} and Brk^{C} were able to activate salE1 reporter gene expression, not repress its expression (Fig. 16D).

2.4.8.3.5.2 Dissection of the region between the DNA binding domain and the CiM

To further dissect the region between the DNA binding domain and the CiM required for repressor activity, a series of additional constructs were generated containing portions of this

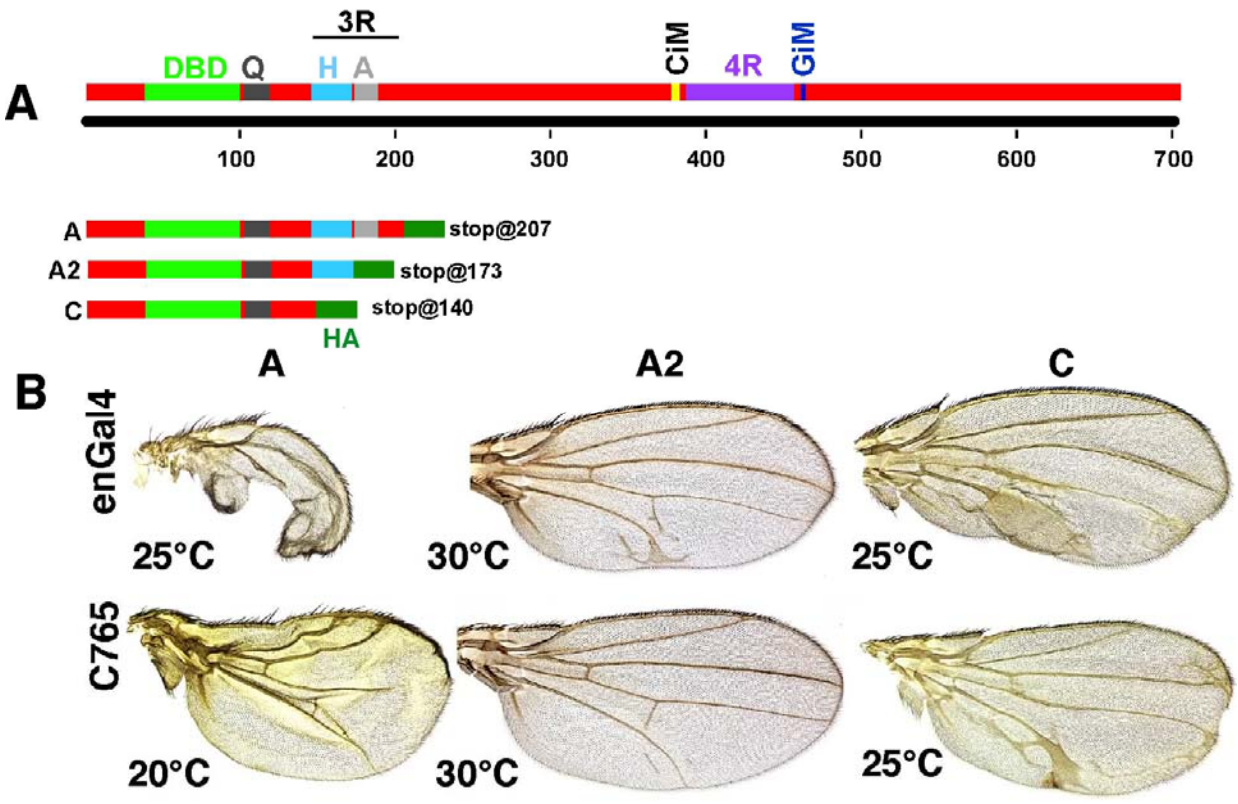

Figure 26. Misexpression of UAS-brk^{A}, UAS-brk^{A2} and UAS-brk^{C} in the developing wing.

(A) Schematic of HA-tagged proteins, Brk^{A}, Brk^{A2} and Brk^{C} containing progressively smaller truncations that includes the 3R repressor domain, includes a portion of 3R or does not include 3R, respectively. (B) Phenotype of adult wings following misexpression with enGal4 and C765. Brk^{A} has significant repressor activity resulting in a reduction of wing tissue and/or loss of wing veins. In contrast, Brk^{A2} has minimal repressor activity, whereas Brk^{C} expression results in excess wing tissue formation, and thus appears to be acting as a dominant negative.

region attached to the DBD + NLS. UAS-brk^{EA} contains the DBD/NLS plus residues 152 to 377 (Fig. 27A, 16D) and produced a protein with intermediate to high levels of activity demonstrated by misexpression at 20°C with en-Gal4 and C765. UAS-brk$^{EA;}$ en-Gal4 adult wings are notched at the distal posterior portion of the wing (Fig 27B), and at 25°C, many of the animals died as pharate adults. In addition, UAS-brk^{EA}; C765 animals have smaller wings when reared at 25°C and fusion of veins II and III and IV and V when reared at 20°C (Fig. 27B). Another protein Brk^{EC}, contains the DBD/NLS plus residues 151 to 228 (Fig. 27A, 16D) with no poly-glutamine region and this protein had considerable activity, suggesting the poly-glutamine region is not required for repressor activity. At 25°C, UAS-brk^{EC}; en-Gal4 produced wings with missing posterior tissue (Fig. 27B). However, misexpression with C765 produced predominantly wild type wings at 25°C (Fig. 27B). Misexpression of UAS-brk^{EC} (30°C) in the posterior of wing discs revealed that both *omb* and *sal* are repressed in the posterior compartment, with low levels of *omb* and *sal* detected near the A/P border (Fig. 27C). In common with Brk^{Stop1}, Brk^{EC} activated salE1 reporter gene expression rather than repressing its expression (Fig. 16D). In contrast, two additional transgenes UAS-brk^{ED} and UAS-brk^{EF} containing the DBD/NLS plus residues 200 to 377 and 253 to 377, respectively produced proteins with no significant repressor activity. In fact, misexpression of UAS-brk^{ED} and UAS-brk^{EF} using the en-Gal driver (25°C) produced adult wings with excess posterior tissue (blistery) indicative of a dominant negative effect (Fig. 27B) rather than any true repressor activity.

Several transcriptional repressors have poly-alanine tracts, some of which reside in regions known to act as repression domains (Gerwin et al., 1994; Han and Manley, 1993a; Han and Manley, 1993b; Licht et al., 1994). To determine the importance of the poly-alanine tract, the 17 alanine residues (173-189) from Brk^{Stop1}were deleted, producing Brk$^{Stop1\Delta A17}$ (Fig. 28A,

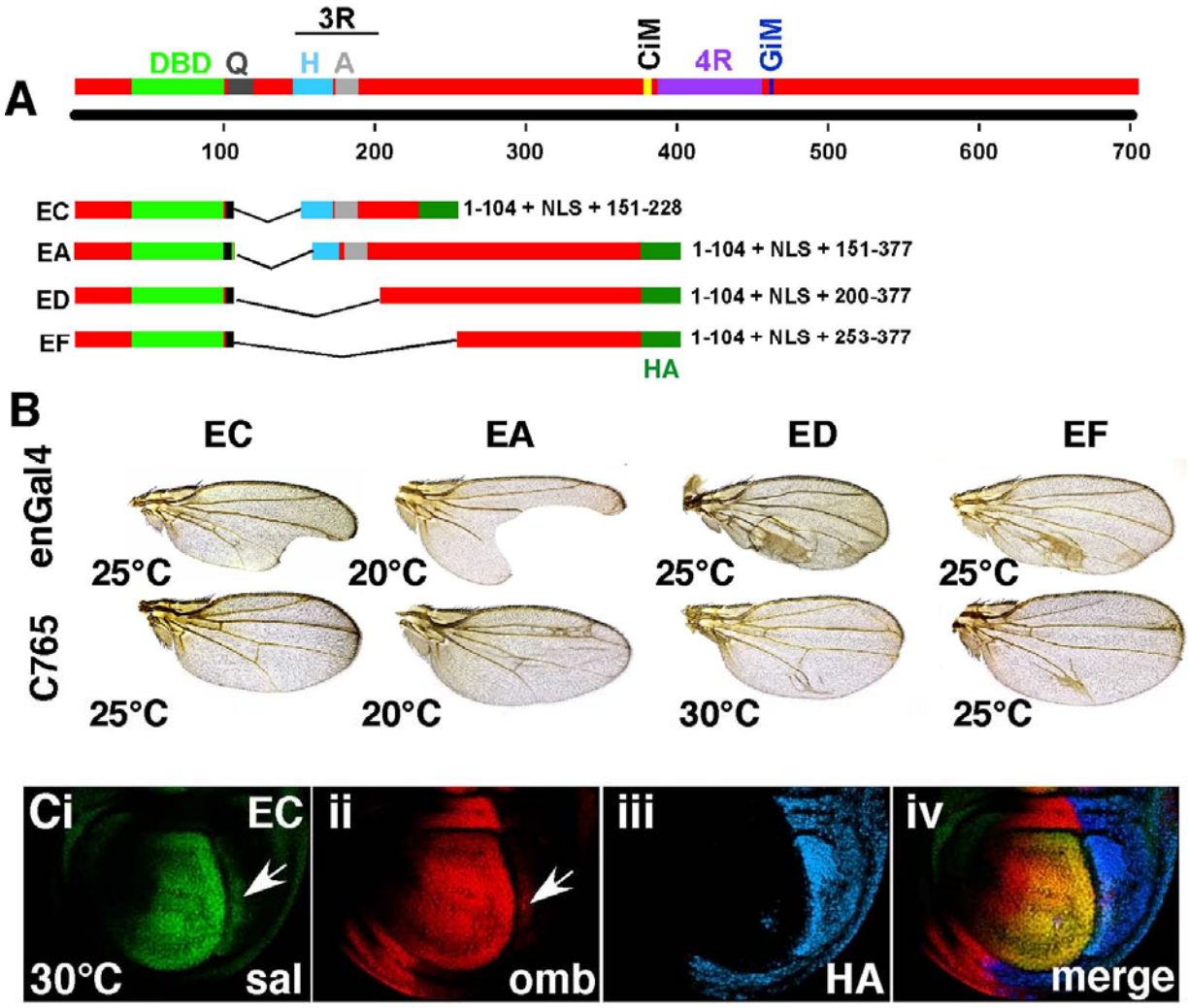

Figure 27. Misexpression of UAS-brk^{EC}, UAS-brk^{EA}, UAS-brk^{ED}, UAS-brk^{EF} in the developing wing.

(A) Schematic of HA-tagged proteins, Brk^{EC}, Brk^{EA}, Brk^{ED}, and Brk^{EF} containing the DBD, NLS plus different portions of the region between the DBD and CiM. (B) Phenotype of adult wings following misexpression with enGal4 and C765. Brk^{EC} has moderate repressor activity and results in a reduction of wing tissue and/or loss of wing veins with enGal4. Brk^{EA}, contains almost the entire region between the DBD and CiM has significant activity, and results in loss of wing tissue and fusion of veins with en-Gal4 and C765, respectively. Brk^{ED} and Brk^{EF} do not possess repressor activity, but rather possess a dominant negative activity. (Ci-iii) Brk^{EC} partially represses both *sal* (green, arrow) and *omb* (red, arrow) when misexpressed in the posterior of wing discs with en-Gal4.

16D) that still had considerable repressor activity (Fig. 16D). In fact, $Brk^{Stop1\Delta A17}$ repressed both *sal* and *omb* in the posterior of wing discs (Fig. 16D). On the other hand, deletion of the poly-alanine stretch along with additional sequence on either side (residues 148-200) to make $Brk^{Stop1NA}$ results in the loss of repressor activity (Fig. 16D, 28B). Analysis of UAS-$brk^{Stop1NA}$; en-Gal4 wing discs revealed that neither *sal* nor *omb* were repressed in the posterior (Fig 28C) suggesting that the deleted region confers repressor activity. Misexpression of both $Brk^{Stop1\Delta A17}$ and $Brk^{Stop1NA}$ in the posterior of wing discs resulted in activation of salE1 reporter gene expression rather than repression (Fig. 16D).

Based on the above analysis, the region of Brk protein corresponding to residues 151-206 is the minimal region sufficient to confer repressor activity and has been named 3R (Winter and Campbell, 2004) for the third repression domain in addition to the CiM and GiM (Fig. 16Ai). The 3R region consists of a histidine rich region, a stretch of poly-alanine residues and additional unique sequence at the C-terminal end. Further analysis of this region is necessary to determine essential sequences in this region, as it appears the poly-alanine stretch may not be absolutely required for repressor activity as demonstrated with $Brk^{Stop1\Delta A17}$ (Fig. 28B). Additional support that the poly-alanine region in 3R is not acting as an autonomous repressor region comes from analysis of another construct UAS-brk^{NLSA17} (fusion of 17 alanines to Brk^{NLS})(Fig. 28A, 16D). Brk^{NLSA17} also did not possess repressor activity as shown by predominantly wild type wings upon misexpression with either en-Gal4 or C765 (Fig. 28B).

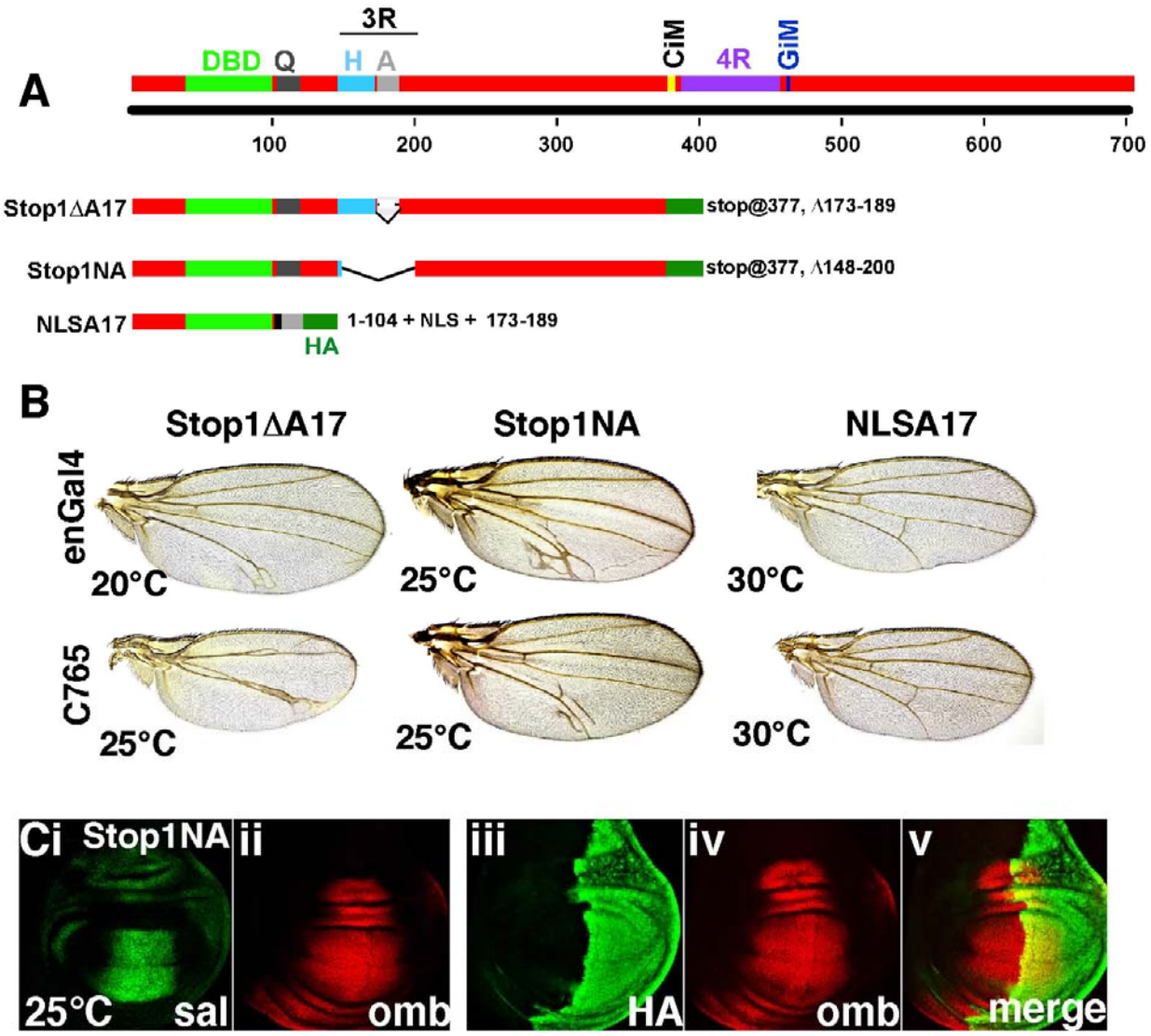

Figure 28. Misexpression of UAS-brk$^{Stop1\Delta A17}$, UAS-brk^{Stop1NA} and UAS-brk^{NLSA17} in the developing wing.

(A) Schematic of HA-tagged Brk proteins. Brk$^{Stop1\Delta A17}$, Brk^{Stop1NA} and Brk^{NLSA17} containing a deletion of poly-alanine region, deletion of 3R region and addition of poly-alanine region to NLS, respectively. (B) Phenotype of adult wings following misexpression with enGal4 and C765. Stop1ΔA17 has significant activity resulting in loss of wing tissue and veins, whereas Stop1NA and NLSA17 do not have detectable repressor activity. (Ci-v) Analysis of *sal* and *omb* expression. Misexpression of Stop1NA in the posterior of wing discs does not repress *sal* or *omb* expression.

2.4.8.3.6 Brk containing only the CiM has significant activity

$Brk^{Stop1NA}$, a protein truncated immediately before the CiM and deletion of the 3R region, did not possess repressor activity. However, addition of 10 amino acids to include the CiM to $Brk^{Stop1NA}$, produced $Brk^{Stop1NAC}$ (Fig. 29A, 16D) that had considerable activity following misexpression with en-Gal4 and C765 at 25°C (Fig 29B), with posterior wing tissue missing and additional cross veins, respectively. Moreover, $Brk^{Stop1NAC}$ almost completely repressed *sal*, whereas *omb* is somewhat repressed in wing discs (Fig. 29C). Therefore, $Brk^{Stop1NAC}$ behaves in a similar manner to wild type Brk, as it was more effective at repressing *sal* compared to *omb*. In contrast, $Brk^{Stop1NAC}$ was not capable of repressing salE1. In fact, this truncated protein like other truncated forms of Brk actually activated salE1 reporter gene expression (Fig. 16D).

2.4.8.3.7 Inactivation of a single repression domain/motif decreases Brk activity

To determine if the loss of a single repression domain/motif of Brk reduced its activity in comparison to wild type, a series of constructs mutating or deleting repression domains were generated. Mutation of the CiM from residues PMDLSLG to AMAAALA produced Brk^{CM} (Fig. 30A, 16D) that possessed significant repressor activity demonstrated by misexpression in the wing. UAS-brk^{CM}; en-Gal4 animals have almost a complete loss of posterior wing tissue at 25°C and loss of wing veins IV and V at 20°C (Fig. 30B). At 25°C UAS-brk^{CM}; C765 flies have very small wings and at 20°C wing are somewhat smaller with fusion of wing veins II and III as well as IV and V (Fig. 30B).

Mutation of the GiM from FKPY to FAAA or deletion of the GiM to produce Brk^{GM} and Brk^{GD}, respectively also rendered proteins with significant repressor activity, having similar adult wing phenotypes as those described for Brk^{CM}, but slightly more severe (Fig. 30B). In

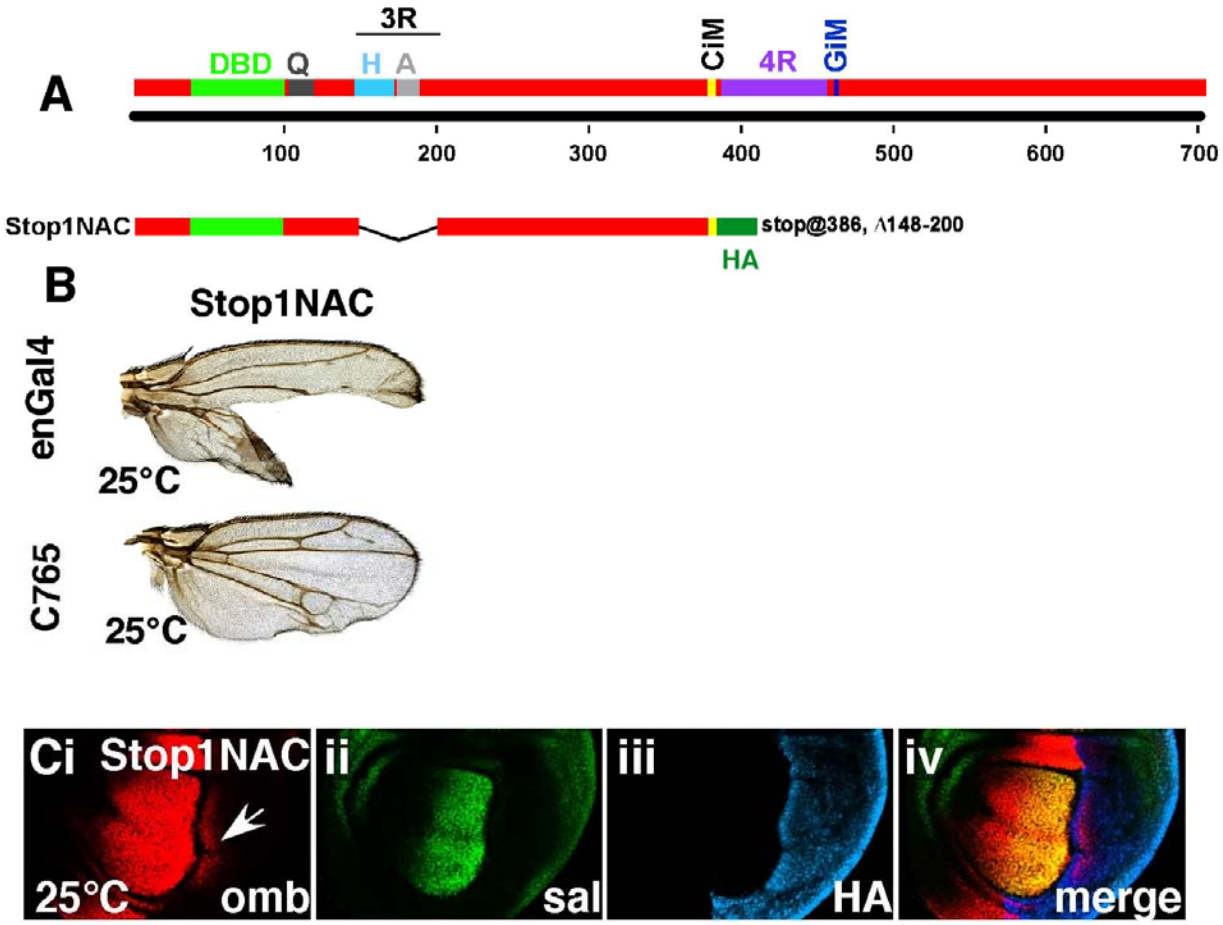

Figure 29. Misexpression of UAS-brk^{Stop1NAC} in the developing wing.

(A) Schematic of HA-tagged Brk^{Stop1NAC}, a protein truncated immediately after the CiM, but has the 3R repression domain region deleted. (B) Phenotype of adult wings. Brk^{Stop1NAC} has significant repressor activity resulting in a loss of wing tissue and veins when misexpressed withenGal4 and C765. (Ci-iv) At 25°C, Stop1NAC, which possesses only a CiM, completely represses *sal* (green) and almost completely represses *omb* (red, arrow).

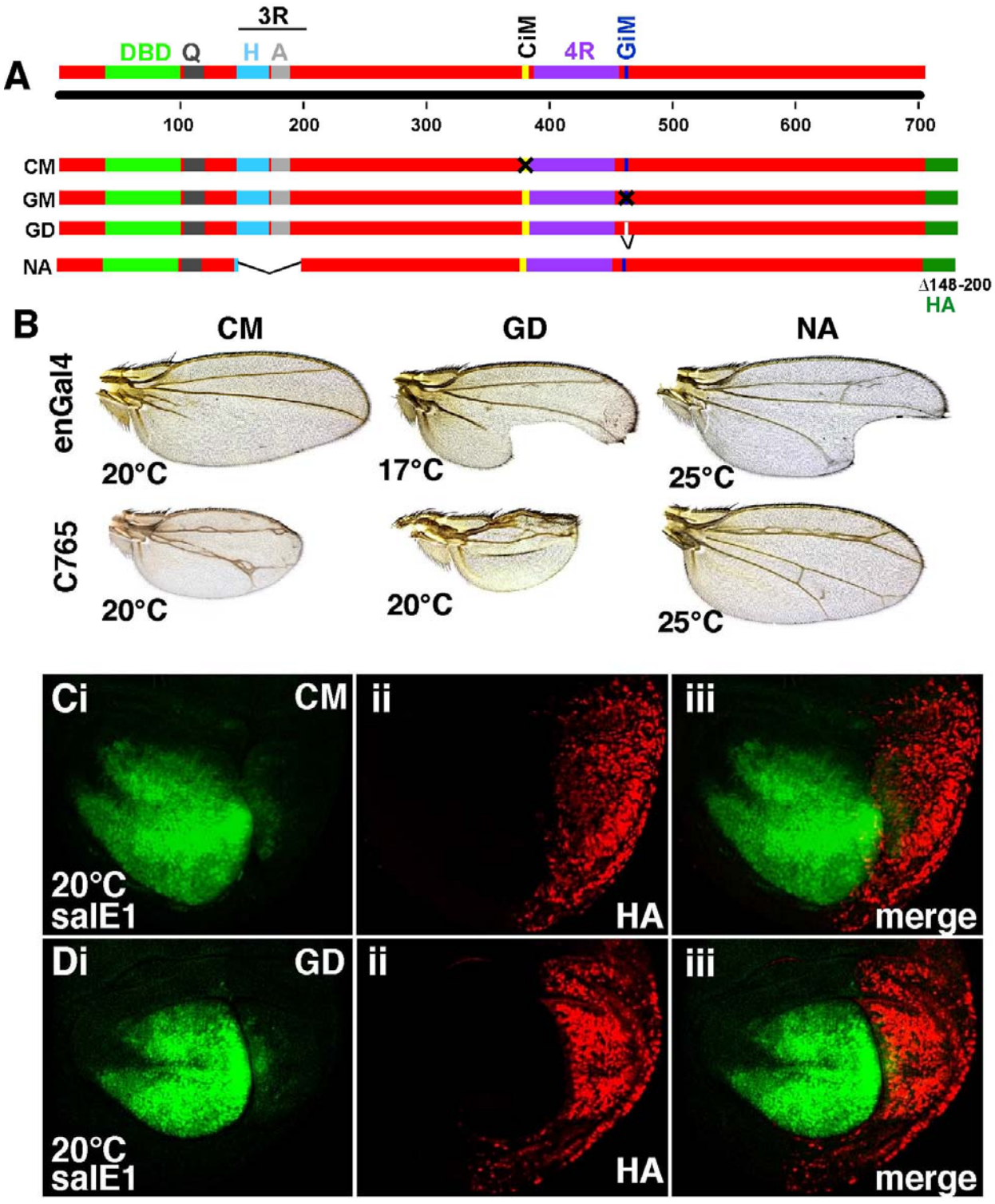

Figure 30. Misexpression of UAS-brk^{CM}, UAS-brk^{GD} and UAS-brk^{NA} in the developing wing.

Figure 30. Misexpression of UAS-brk^{CM}, UAS-brk^{GD} and UAS-brk^{NA} in the developing wing.

(A) Schematic of HA-tagged proteins Brk^{CM}, Brk^{GD}, and Brk^{NA} which contain a mutated CiM, deleted GiM and deleted 3R, respectively. (B) Phenotype of adult wings. CM and GD, containing mutation of the CiM or deletion of the GiM, produce proteins with significant repressor activity resulting in loss of wing tissue and veins when misexpressed using enGal4 or C765, even at lower temperatures (17°C or 20°C). Brk^{NA} also has significant activity resulting in a reduction of wing tissue and fusion of wing veins. (C-D) Analysis of salE1 reporter expression. Misexpression of CM and GD in the posterior of wing discs results in almost complete repression of salE1 reporter expression at 20°C.

addition, deletion of the 3R region (residues 148-200) forming Brk^{NA} also produced a protein with comparable activity to the other proteins having mutations/deletions to a single repression motif (Fig 30B). However, in contrast to the activity of wild type Brk^{3PF3} (Fig. 19B), flies did survive to adults following misexpression of each of these single mutant transgenes with en-Gal4 at 20°C, demonstrating each protein was less active than wild type Brk protein. Additional analysis revealed each of the above mentioned proteins could repress *sal* and *omb*, but expression levels of each target gene were too similar to each other and to that seen in the presence of wild type protein to make any distinctions (data not shown). Analysis of Brk^{CM}, a full-length protein with the CiM mutated, revealed that it could repress salE1 reporter expression in the posterior of wing discs (Fig.30C). In addition, Brk^{GD}, a full-length Brk protein with the GiM deleted, was also capable of repressing salE1 (Fig 30D). Mutations similar to those generated in CM and GM result in the loss of Gro and CtBP binding in other studies (Hasson et al., 2001; Zhang and Levine, 1999)

2.4.8.3.8 Inactivation of multiple repression domains/motifs also decreases the activity of Brk

Subsequent analysis of the effect of mutation and/or deletion of multiple repression domains/motifs revealed that each protein had reduced activity compared to wild type Brk, but had similar activity levels as the single mutations/deletions. The following constructs were generated: UAS-brk^{CMGM}, with mutations of the CiM and GiM listed previously; UAS-brk^{NACM}, containing a deletion of the 3R region (residues 148-200) and mutation of the CiM; and UAS-brk^{NAGM}, with a deletion of the 3R region and mutation of the GiM (Fig. 31A, 16D). When each of these transgenes was misexpressed using en-Gal4, adults survived only with Brk^{CMGM} at 25°C

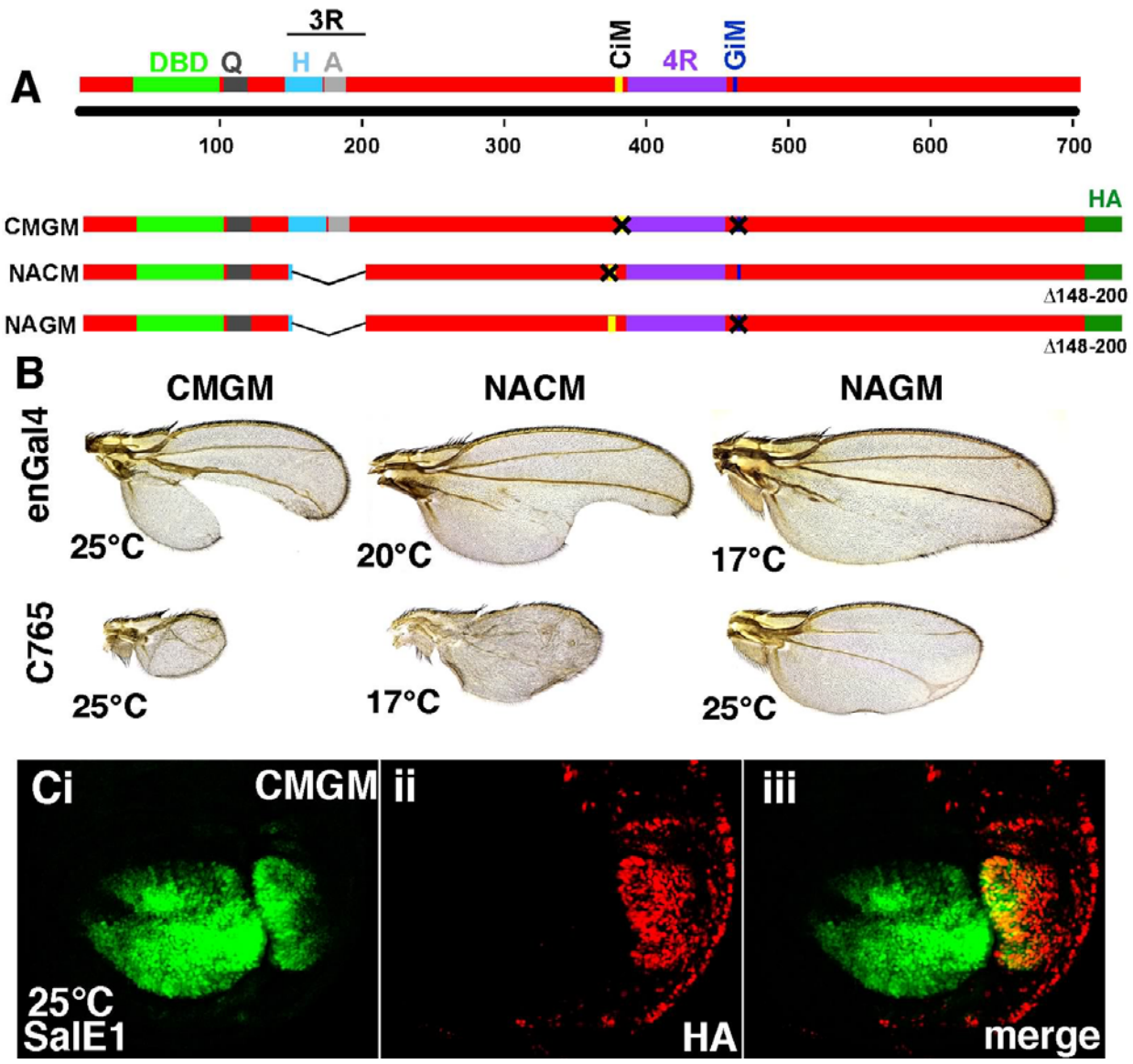

Figure 31. Misexpression of UAS-brk^{CMGM}, UAS-brk^{NACM} and UAS-brk^{NAGM} in the developing wing.

(A) Schematic of HA-tagged proteins Brk^{CMGM}, Brk^{NACM}, and Brk^{NAGM} containing mutated CiM and GiM, deleted 3R and mutated CiM, and deleted 3R and mutated GiM, respectively. (B) Phenotype of adult wings. Misexpression of these modified Brk proteins using enGal4 and C765 results in significant loss of wing tissue and veins demonstrating that each of the proteins still has significant repressor activity. (Ci-iii) Analysis of salE1 reporter expression. Misexpression of CMGM in the posterior of wing discs results in activation of salE1 reporter expression rather than repression.

and the flies contained wings with significant, almost complete loss of posterior wing tissue (Fig. 31B). At 20°C, adults survived following misexpression of all three transgenes, but once again the posterior portion of the wing tissue was missing (Fig. 31B). On the other hand, misexpression of transgenes using C765 produced animals with small wings at 25°C for all three double mutations/deletions (Fig. 31B). These results confirm that Brk protein containing multiple mutations/deletions of repression domains/motifs still have significant repressor activity, indistinguishable from proteins with a single mutation or deletion. Interestingly, mutation of both the CiM and GiM significantly reduced the ability of Brk^{CMGM} to repress salE1, suggesting that either an intact CiM or GiM are required for repression of this reporter (Fig. 31C).

2.4.8.3.9 Brk must possess a fourth repression domain/motif

The experimental data discussed thus far demonstrate that Brk requires a functional DNA binding domain, plus at least one repression domain/motif to confer repressor activity. Furthermore, if 3R, the CiM and GiM are the only repression domains/motifs present in the Brk protein, then mutation or deletion of all three simultaneously should render the protein inactive. To test this hypothesis, UAS-brk^{3M} was generated that contains previously mentioned mutations in the CiM and GiM as well as a deletion of the 3R region (residues 148-200) (Fig 32A, 16D). Misexpression of UAS-brk^{3M} using both en-Gal4 and C765 produced adults at 25°C containing missing posterior wing tissue and smaller wing size (Fig. 32B), respectively, not the anticipated wild type wing observed with an inactive form of the protein. In addition, Brk^{3M} repressed *sal*, *omb* and Vg-QE in wing discs (Fig. 32C,D). Consequently, since Brk^{3M} still possessed significant repressor activity, there must be at least one additional repression domain/motif in

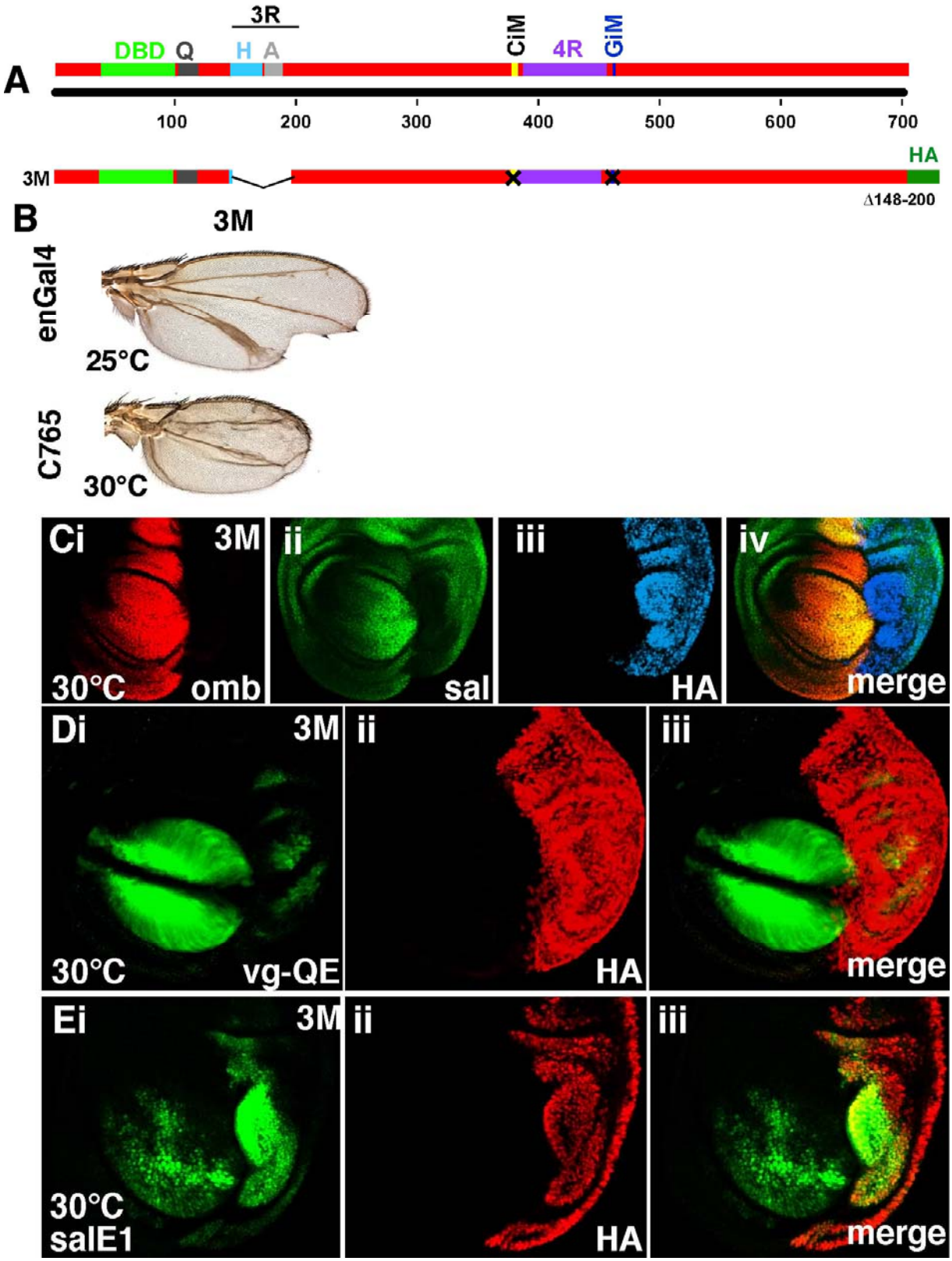

Figure 32. Misexpression of UAS-brk^{3M} in the developing wing.

Figure 32. Misexpression of UAS-brk^{3M} in the developing wing.

(A) Schematic of HA-tagged protein, Brk^{3M} containing mutations in the CiM and GiM, and deleteion of the 3R region. (B) Phenotype of adult wings. Although the 3R region is deleted and the CiM and GiM are mutated, 3M still had significant activity resulting in a loss of wing tissue and veins. (C-E) Analysis of sal, omb, vg-QE and salE1 expression. (Ci-iv) Misexpression of 3M in the posterior of wing discs with enGal4 (30°C) results in complete repression of *sal* (green) and *omb* (red). (Di-iii) Brk^{3M} can almost completely repress vg-QE expression in the posterior of wing discs. (Ei-iii) In contrast, 3M cannot repress salE1 reporter expression; but rather it activates salE1.

Brk. Further analysis of the ability of Brk^{3M} to repress salE1 revealed a predominantly full-length form of Brk protein that activated salE1 reporter rather than repressed its expression (Fig. 32E). These data suggest either the CiM or GiM may be required to repress salE1, since Brk^{CMGM} was also not able to repress this reporter.

2.4.8.3.9.1 Identification of putative fourth repression domain/motif

In an attempt to identify the fourth repression domain/motif, successive truncations of Brk^{3M} revealed UAS-$brk^{Stop2NACM}$ (Fig. 33A, 16D) as the shortest protein with repressor activity even though it was truncated before the GiM, the CiM was mutated and 3R region was deleted. UAS-$brk^{Stop2NACM}$; en-Gal4 (25°C) animals have wings with posterior tissue missing (Fig 33B), whereas UAS-$brk^{Stop2NACM}$; C765 (25°C) flies have extra cross veins or fusions between veins II and III, IV and V; or complete loss of veins III and V. (Fig. 33B). Analysis of gene expression in wing discs stained for *sal* and *omb* revealed complete repression of both *sal* and *omb* (Fig. 33C). In contrast, $Brk^{Stop2NACM}$ was not able to repress salE1, but similar to other truncated Brk proteins activated this reporter (Fig. 16D). $Brk^{Stop2NACM}$ is truncated immediately before the GiM and is identical to $Brk^{Stop1NA}$ with the exception of the region between the CiM and the GIM. Therefore, additional constructs were generated to confirm if this region between the CiM and GiM was in fact the fourth repression domain. Brk^{NLS4R} has the region between the CiM and the GiM (residues 383-453) fused to the DNA binding domain plus an NLS (Fig. 33A, 16D). This protein has an intermediate level of activity shown by adult wings having the distal posterior wing tissue missing (en-Gal4) and fusion of wing veins II and III (C765) at 25°C. Misexpression of UAS-brk^{NLS4R} in the posterior of wing discs revealed that *sal* was more repressed than *omb* (Fig. 33D). However, Brk^{NLS4R} was not able to repress salE1 but rather

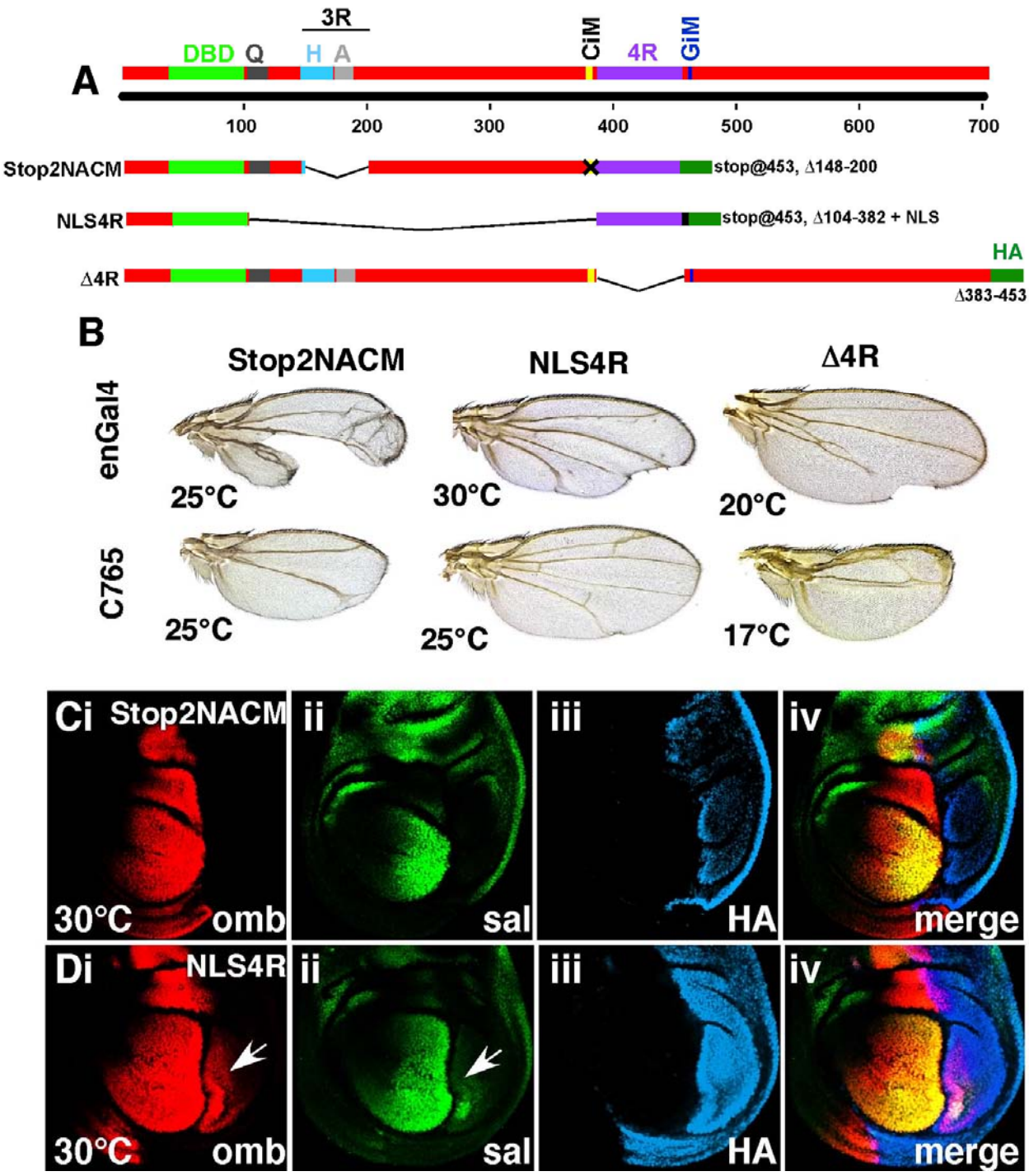

Figure 33. Misexpression of UAS-brk^{Stop2NACM}, UAS-brk^{NLS4R} and UAS-brk$^{\Delta 4R}$ in the developing wing.

Figure 33. Misexpression of UAS-brk^{Stop2NACM}, UAS-brk^{NLS4R} and UAS-brk$^{\Delta 4R}$ in the developing wing.

(A) Schematic of HA-tagged proteins, Brk^{Stop2NACM}, Brk^{NLS4R} and Brk$^{\Delta 4R}$. Stop2NACM is truncated immediately before the GiM and has a mutated CiM and deleted 3R region. NLS4R contains N-terminal 104 aa, NLS plus the region between the CiM and GiM, and Δ4R has a deletion of the region between the CiM and GiM. (B) Phenotype of adult wings. At 25°C, Stop2NACM has significant activity resulting in a loss of wing tissue and veins when misexpressed with both enGal4 and C765. NLS4R also has considerable activity resulting in a loss of wing tissue with enGal4. Brk$^{\Delta 4R}$, like other single mutations/deletions, has significant activity shown by a reduction of wing tissue and loss of veins. (C-D) Analysis of *sal* and *omb* expression. (Ci-iv) Misexpression of Stop2NACM in the posterior of wing discs (iii) at 30°C results in a complete loss of *sal* (green) and *omb* (red) expression. (Di-iv) NLS4R can partially repress expression of *sal* (green) and *omb* (red) in the posterior.

activated reporter expression (Fig. 16D). Previously mentioned experiments revealed that mutation or deletion of a single repression domain/motif reduced the activity of the protein compared to that of wild type Brk. When the region between the CiM and GiM is deleted ($Brk^{\Delta 4R}$), the protein still possesses significant activity as demonstrated by misexpression using en-Gal4 and C765, with the posterior wing tissue missing and smaller wing size, respectively (Fig. 33B).

In an attempt to identify the residues between the CiM and GiM responsible for the repressor activity, a series of truncations of $Brk^{Stop2NACM}$ were generated producing the following transgenes: UAS-$brk^{S2NACM1}$ (truncated at residue 390); UAS-$brk^{S2NACM2}$ (truncated at residue 424) and UAS-$brk^{S2NACM3}$ (truncated at residue 445) (Fig. 34A, 16D). Each of the transgenes was misexpressed in the wing using en-Gal4 resulting in wings with posterior tissue missing as well as a blistery appearance near vein V for $Brk^{S2NACM1}$; a loss of posterior tissue at the distal tip for $Brk^{S2NACM2}$ and a severe loss of posterior tissue and fusion of veins IV and V for $Brk^{S2NACM3}$ at 25°C (Fig 34B). In addition, ubiquitous expression of the transgene (C765) in the wing produced a slightly smaller wing and partial loss of vein II for $Brk^{S2NACM1}$; extra veins near vein V for $Brk^{S2NACM2}$ and extra veins near vein V as well as a very slight excess portion of vein II for $Brk^{S2NACM3}$ (Fig 34B).

If this region is the fourth repression domain, then the protein should no longer possess repressor activity if the 3R region as well as the region including the CiM through the GiM were deleted (residues 377-464). Brk^{4M} (Fig. 35A, 16D) contains both of these deletions and renders an inactive form of the Brk protein based on predominantly wild type appearance of adult wings (Fig. 35B). There are, however, some ectopic wing veins in the proximity of vein V (en-Gal4) that could be due to a dominant negative effect of this protein. Initial attempts to determine the

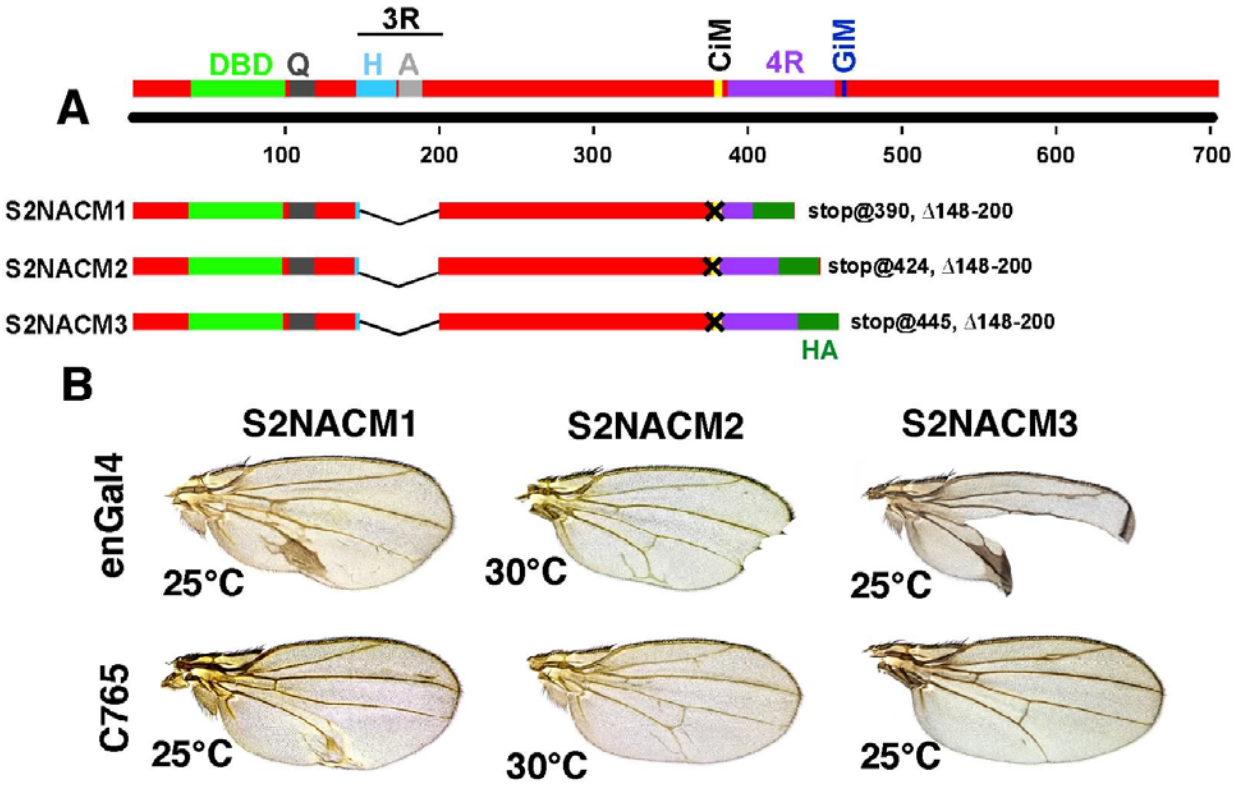

Figure 34. Misexpression of UAS-brk^{S2NACM1}, UAS-brk^{S2NACM2} and UAS-brk^{S2NACM3} in the developing wing.

(A) Schematic of HA-tagged proteins, Brk^{S2NACM1}, Brk^{S2NACM2} and Brk^{S2NACM3} containing progressively larger truncations within the putative 4R region. (B) Phenotype of adult wings. Misexpression of the truncated Brk proteins in the posterior (enGal4) or ubiquitously (C765) results in varying phenotypes; S2NACM1 has a dominant negative activity, S2NACM2 has moderate activity with loss of distal most posterior tissue with enGal4, and S2NACM3 has significant activity, with almost complete loss of posterior tissue with enGal4.

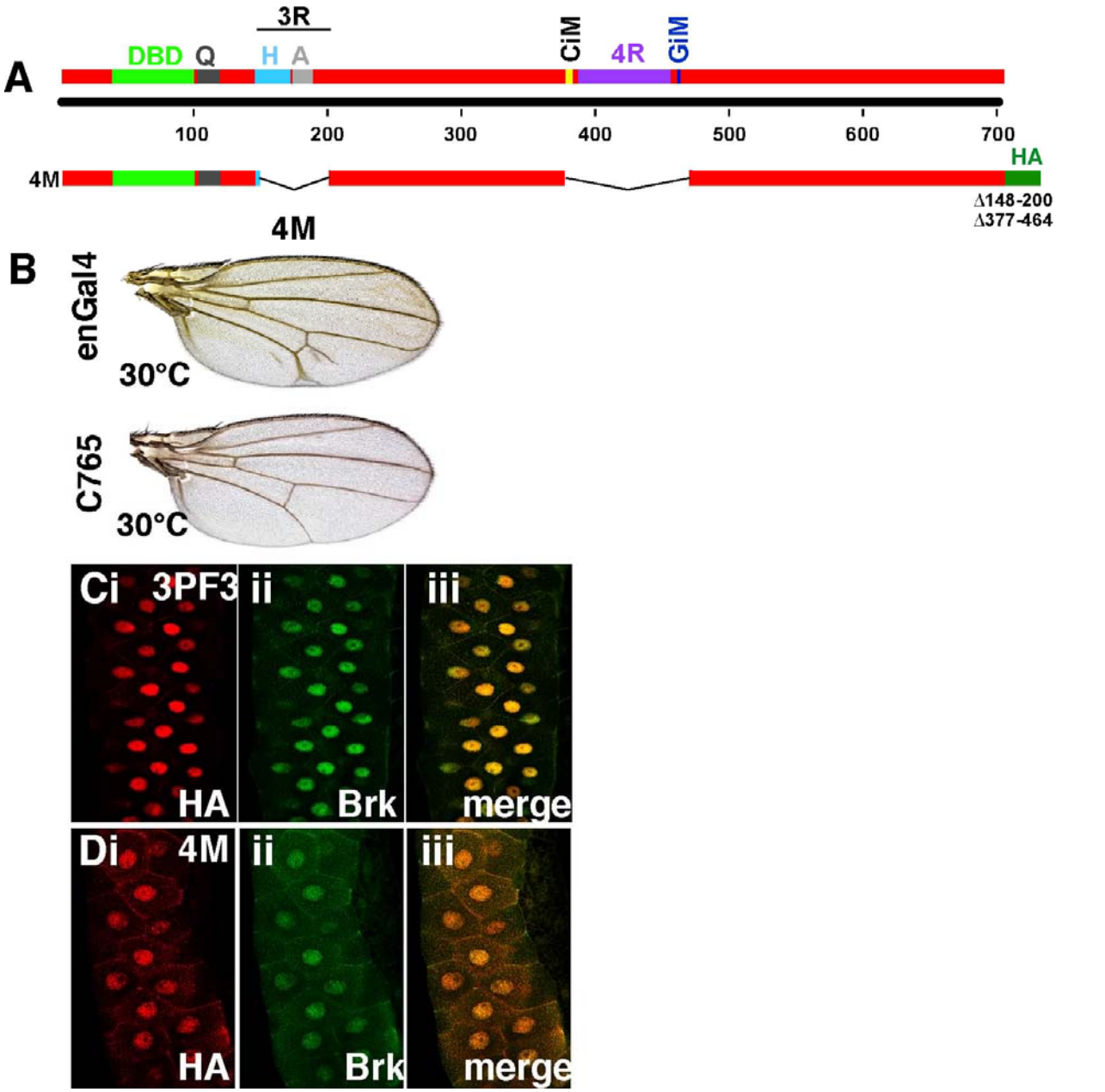

Figure 35. Misexpression of UAS-brk^{4M} in the developing wing and overexpression in larval salivary glands.

(A) Schematic of HA-tagged protein, Brk^{4M} with a deletion of the 3R region and deletion of the region including the CiM through the GiM. (B) Adult wing phenotype. Misexpression of 4M with enGal4 and C765 results in predominantly wild-type wings, suggesting the 4R region is the 4th repressor domain of Brk. (C-D) Overexpression of wild-type Brk (3PF3) and 4M in salivary glands using a *brk*-Gal4 driver (3SB). (Ci, Di) Expression of HA tagged proteins showing nuclear and membrane localization. (Cii, Dii) Expression of endogenous Brk protein (Brk antibody).

gene expression of *sal* and *omb* revealed that both *sal* and *omb* were not repressed by Brk^{4M} (data not shown). However, subsequent triple staining experiments for *sal*, *omb* and the HA tagged protein did not detect the HA epitope in wing discs. Further analysis of Brk^{4M} in larval salivary glands, where endogenous Brk is highly expressed, using a brk-Gal4 line to drive UAS-brk^{4M} revealed expression of the HA epitope on Brk^{4M} (Fig. 35Di) compared to staining using anti-BrkR3 antibody which showed high gene expression of *sal* and *omb* revealed that both *sal* and *omb* were not repressed by Brk^{4M} (data not shown). However, subsequent triple staining experiments for *sal, omb* and the HA tagged protein did not detect the HA epitope in wing discs. Further analysis of Brk^{4M} in larval salivary glands, where endogenous Brk is highly expressed, using a brk-Gal4 line to drive UAS-brk^{4M} levels of endogenous Brk expression (Fig. 35Dii). In contrast to Brk^{4M}, wild type Brk^{3PF3}, or any other modified Brk protein, showed high levels of expression using either the anti-HA antibody or the anti-BrkR3 antibody (Fig. 35Ci-ii). These findings suggest that Brk^{4M} is being expressed, but at low levels (multiple lines tested), and the HA antibody is not detecting this protein in wing discs. The above data suggest that the region between the CiM and GiM serves as the fourth repression domain of Brk and has been named 4R.

2.5 Discussion

Dpp acts as a morphogen in the *Drosophila* wing and it is distributed in a medial to lateral gradient that in turn produces the lateral to medial Brk expression gradient, complementary to Dpp itself. Dpp target genes *sal* and *omb* are repressed by Brk, and the lateral limits of their expression domain are determined by their sensitivity to Brk (Campbell and Tomlinson, 1999; Entchev et al., 2000; Jazwinska et al., 1999a; Minami et al., 1999; Muller et

al., 2003; Teleman and Cohen, 2000). Therefore, *omb*, which is less sensitive to Brk, is expressed in a wider domain compared to *sal*. One of the key questions that the above study tries to address is: Why are some genes, such as *sal*, repressed by lower levels of Brk protein than others such as *omb*? The mechanism Brk uses to repress gene expression has been investigated to determine if it is the same for different Dpp target genes. If the mechanism is the same for each gene, then the sensitivity observed between genes is simply quantitative and Brk would operate the same, but just more effectively at the more sensitive target genes and this could be achieved, for example, by *sal* having more binding sites or sites with higher affinity. On the other hand, if the mechanism is not the same for each gene, then the difference in the genes sensitivity may result from the ability of Brk to use different mechanisms to repress target genes. Here we demonstrate Brk can in fact utilize different mechanisms to repress *sal* and *omb*.

2.5.1 Brk does not appear to repress by simple competition

One of the most basic modes of transcriptional repression involves competition between a repressor and an activator for binding to DNA, when the repressor and activator have the same or overlapping binding sites in an enhancer common in prokaryotes. It is reasonable to assume that a transcription factor, localized in the nucleus, should only require a DNA binding domain to bind DNA and compete for overlapping binding sites in an enhancer. Possibly, the affinity of each protein could play a role in its ability to bind and "out compete" the other protein. It has been shown that Brk contains an N-terminal sequence specific DNA binding domain (Fig. 16A) (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller et al., 2002; Sivasankaran et al., 2000; Zhang et al., 2001b) and mutations in the DNA binding domain completely abolish repressor

activity (Fig. 12A,B; 16B,C,D; 20) or reduce the activity demonstrating the DNA binding domain is absolutely required for Brk function.

Previous studies suggested Brk functions by simple competition with activators (Mad) for overlapping binding sites *in vitro* (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001). The data presented herein argue against Brk functioning by simple competition. Brk^{NLS}, a nuclear protein containing only the DNA binding domain cannot repress any of the Brk target genes tested *in vivo* in the wing (Fig. 22), as well as the UbxB reporter in embryos that possesses overlapping Brk and Mad binding sites which Brk and Mad can compete for *in vitro* (Kirkpatrick et al., 2001; Saller and Bienz, 2001). Brk^{NLS} can bind the UbxB enhancer *in vitro* (Fig. 23B), but one concern may be that Brk^{NLS} cannot bind DNA *in vivo*. However, Brk^{NLS} actually activated salE1 (Fig. 22E), which was repressed by wild type Brk, providing strong evidence that this protein can bind DNA *in vivo*. salE1 is activated in cells that are not expressing endogenous Brk, indicating Brk^{NLS} is not acting as a dominant negative. Furthermore, misexpression of UAS-brk^{NLS} in salE1MB123 wing discs did not activate expression (Fig. 22F), demonstrating the three Brk sites are required for repression or activation of the reporter, and providing even more evidence that brk^{NLS} can bind DNA *in vivo*. Therefore, it seems Brk cannot repress by competition, but requires additional repression domains/motifs. In addition, a variation of Brk^{NLS}, Brk^{NLSW} which is the same as Brk^{NLS} except for the addition of four amino acids WRPW (Fig. 16D, 24A), shown to recruit the co-repressor Gro (Aronson et al., 1997; Fisher et al., 1996), can repress the Dpp targets, *sal* and *omb* (Fig. 24D). These data suggest that Brk^{NLS} should also be able to bind DNA and repress target genes *in vivo* by competition, but this was not the case, so it argues against simple binding site competition as a

mechanism for Brk repression. Other studies just assume that overlapping binding sites alone are sufficient for simple competition.

The N-terminal 104 amino acids are sufficient to allow Brk to bind to its normal targets, but the protein is unable to repress genes without an active repression domain such as that provided by the GiM and recruited by the WRPW motif. An active repression domain may be required for the protein to access DNA in chromatin. Therefore, Brk requires the DNA binding domain plus an active repression domain for its activity, and based on these data it appears that Brk^{NLS} does fold properly.

Although many repressors are thought to use competition as a mechanism based on *in vitro* binding studies, there is little direct support in eukaryotes that this occurs *in vivo*. The present study provides *in vivo* evidence that a Brk protein consisting of a functional DNA binding domain and which is localized in the nucleus does not repress target genes *in vivo*, even those shown to contain overlapping binding sites for repressor and activator *in vitro* (UbxB) (Fig. 23A). One of the only *in vivo* studies providing evidence for competition showed that *Drosophila* embryonic repressor Krüppel (Kr) could repress a synthetic enhancer possessing overlapping binding sites with activators Dorsal and Bicoid (Nibu et al., 2003). Kr uses CtBP as a co-repressor for repression, but repression in the Nibu study (2003) was CtBP independent. However, additional studies of Kr are necessary to determine if additional regions outside the DNA binding domain and CtBP interaction motif function as repressor domains (Licht et al., 1990), similar to that of the 3R domain in Brk. A region of Kr spanning amino acids 62-92 has been identified as a putative repressor domain (Nibu et al., 2003). Overall, in prokaryotes, binding site competition *in vivo* has been demonstrated (Ptashne and Gann, 2001), but strong support is lacking in eukaryotic organisms for this phenomenon *in vivo*. *In vivo*, multiple

proteins form complexes that can activate or repress transcription, and so there is not just competition with a single transcription factor as *in vitro*. Therefore, my studies question whether or not binding-site competition is really a mechanism of repression in eukaryotic organisms.

2.5.2 Brk protein possesses at least three repression domains/motifs

My *in vivo* studies argue strongly against Brk repressing target genes by simple competition for binding sites. Therefore, Brk must contain repression domains/motifs that confer Brk with its repressor activity. Previous studies have identified regions of Brk protein serving as interaction motifs for co-repressors CtBP and Groucho (referred to as the CiM and GiM) (Hasson et al., 2001; Saller and Bienz, 2001; Zhang et al., 2001b). Interestingly, neither CtBP nor Gro is required for repression of a Brk target gene *omb* (Hasson et al., 2001). Such a finding is consistent with data showing that brk^{F138}, encoding a protein truncated before the CiM and GiM, is capable of repressing *omb* (Fig. 12Ciii). Further support that the CiM and GiM are not required for repression of *omb* comes from analysis of truncated Brk proteins Brk^{Stop1}, Brk^{EC} and Brk^{A} which all repress *omb* even though they lack a CiM or GiM (Fig. 25D,E, 16D, 27C). However, other truncated Brk proteins that do not contain the 3R region of Brk or the other three repression domains (Brk^{A2}, $Brk^{Stop1NA}$) (Winter and Campbell, 2004) cannot repress *omb* expression (Fig. 16D, 28C). Furthermore, it is not known whether 3R can function as an autonomous repression domain outside of Brk or the positioning requirement for Brk sites and activator/promoter sites for the functioning of 3R. Additional studies are necessary to answer these questions.

2.5.3 Differential activity of Brk repression domains/motifs

Brk contains three (probably four) repression domains/motifs, and this poses the question of why multiple repression domains are needed? Analysis revealed that the three Brk repression domains/motifs are not equivalent, as some domains are more effective at repressing some target genes, but not others. In fact, wild type Brk protein, as well as those possessing only the CiM ($Brk^{Stop1NAC}$) and GiM (Brk^{NLSW}), can repress both *sal* and *omb*, but they can repress *sal* more effectively than *omb* (Fig. 29C, 24D). However, salE1 is repressed by wild type Brk (Brk^{3PF3}), but salE1 cannot be repressed by truncated forms of Brk containing only the CiM ($Brk^{Stop1NAC}$) or the GiM (Brk^{NLSW}) (Fig. 16D, 24F), showing this *sal* enhancer does not behave like the endogenous *sal* gene. Each of the above mentioned truncated forms of Brk were able to repress endogenous *sal*. The co-repressor CtBP can only function over short distances (Arnosti et al., 1996; Gray and Levine, 1996; Gray et al., 1994), suggesting Brk and activator binding sites in salE1 may be located too far apart to be repressed by $Brk^{Stop1NAC}$. In contrast, Gro is known as a long-range repressor (Barolo and Levine, 1997; Cai et al., 1996; Dubnicoff et al., 1997; Paroush et al., 1994), therefore, Brk^{NLSW} should be able to repress salE1. However, Brk^{NLSW} was not able to repress salE1 either. The inability of Brk^{NLSW} to repress salE1 may be due to the artificial nature of this protein, where the GiM is situated very close to the DNA binding domain thereby causing a steric hindrance that interferes with long-range repression. Many transcriptional repressors (e.g. Hairy family members) containing a GiM have the interaction motif located at a distance from the DNA binding domain (Fisher et al., 1996; Rushlow et al., 1989).

Studies of *CtBP* and *gro* single mutant clones or double mutant clones revealed that Gro is required for repression of *sal*, and that CtBP can provide some activity for repression of *sal* in *gro* mutant clones, but the repression is limited (Fig. 13C). However, neither CtBP nor Gro are

required for repression of *omb* (Hasson et al., 2001). Further analysis of *CtBP* and *gro* single and double mutant clones showed that in the absence of *gro*, there was some de-repression of salE1 reporter gene expression in the anterior, but the not in the posterior (Fig. 14C). salE1 expression was not affected in *CtBP* single mutant clones, but *CtBP gro* double mutant clones showed strong misexpression of salE1 in the wing pouch (Fig. 14D). These findings suggest that Gro is required to repress the salE1 reporter due to the positioning of Brk sites and activator sites that are over 150 bp apart. However, CtBP can partially repress in the absence of Gro.

The third repression domain of Brk, 3R (Winter and Campbell, 2004), is sufficient for repression of *omb* (Fig. 12Ciii, 25D,E), as well as the UbxB enhancer in embryos (Fig. 25Cii), and to repress *sal* also. However, this may be indirect (see below). In fact, proteins containing only the DNA binding domain plus the 3R domain can repress *omb* better than *sal,* which is the opposite of wild type Brk or those proteins containing only the CiM or GiM (Fig. 29C, 24D). Even though 3R may appear to repress *sal* (Fig. 25D,E), the repression may be indirect because previous studies show that *sal* requires Omb to be expressed (del Alamo Rodriguez et al., 2004) and if *omb* is directly repressed by 3R, then *sal* expression would be eliminated as well. For example, although *sal* is repressed by Brk^{Stop1} at 25°C, it may be indirect because *sal* requires Omb and *omb* is completely repressed at this temperature (Fig. 25Eii) (del Alamo Rodriguez et al., 2004). In contrast, most other proteins such as wild-type Brk and Brk^{NLSW}, do appear to repress *sal* directly because for these proteins, *sal* expression is lost at lower Brk transgene protein concentration than *omb,* i.e. loss of *omb* cannot account for loss of *sal*. Surprisingly, at 20°C (Brk^{Stop1}) *omb* expression cannot be detected in some cells where *sal* is being expressed. Previous studies (del Alamo Rodriguez et al., 2004) as well as our own studies demonstrate that *sal* expression is completely dependent upon Omb, so the most plausible explanation for these

findings is that *omb* is actually expressed in these cells, but the levels may be too low for detection with the *omb*-LacZ line used for the studies.

Analysis of the ability of 3R to repress Vg-QE produced confusing, contradictory results. In brk^{F138} mutant clones, there was expansion of Vg-QE expression suggesting that the 3R repression domain cannot repress this enhancer. In contrast, Brk^{Stop1} (a similar truncated protein) could effectively repress Vg-QE upon misexpression using the UAS/Gal4 system (Fig. 25F). One possible explanation for this discrepancy could be the high levels of expression achieved with the UAS/Gal4 system may have enabled repression of this target, compared to more physiological levels of brk^{F138} protein produced in the mutant clones which did not allow 3R to repress Vg-QE. A previous study demonstrated ectopic expression of UAS-*engrailed* in the posterior of wing discs reduced expression of En protein (Tabata et al., 1995), similar to the reduction of Vg-QE expression when UAS-brk^{Stop1} was misexpressed in the current study.

The ability of a single repression domain to be sufficient to repress a given target gene may rely on the positioning of Brk binding sites relative to activator sites (or promoter sites) of the target. For example, the UbxB reporter contains overlapping Brk and Mad (activator) binding sites (Kirkpatrick et al., 2001; Saller and Bienz, 2001). Moreover, an important Brk binding site may overlap with an activator site in the *omb* enhancer (Sivasankaran et al., 2000). In contrast, activator and Brk binding sites are separated in the cis-regulatory elements of the *sal* gene (Barrio and de Celis, 2004) and the salE1 enhancer (Fig. 9A). Brk proteins containing only the 3R domain can repress *omb* and UbxB, but not *sal* or salE1. Clonal analysis of brk^{F138} mutant cells revealed Brk^{F138} was not able to repress salE1 reporter gene expression and there was ectopic salE1 expression in lateral clones (Fig. 14A). In addition, truncated forms of Brk protein containing the 3R region (Brk^{Stop1}, Brk^{A}, Brk^{EC}) were also not able to repress salE1 reporter

expression when misexpressed in the posterior of wing discs; rather they activated expression (Fig. 16D). These results suggest that 3R can only repress over short distances rather than long range (over 150 bp) and that activator and Brk binding sites must overlap or be in close proximity for the 3R region to repress.

2.5.4 Multiple repression domains of Brk protein

The current study has identified two additional regions of Brk protein that act as repression domains along with the previously identified CiM and GiM. Why does Brk possess at least three, possibly four independent repression domains/motifs? There are two probable answers: qualitative, where different repression domains/motifs are required to repress different target genes and quantitative, where more domains/motifs confer increased repressor activity. There are several known transcription factors containing multiple repression domains, and they have multiple domains for either qualitative and quantitative reasons or both. A prime example comes from the pair rule protein Runt in the *Drosophila* embryo that requires Gro to repress one stripe of the pair rule genes, *even skipped* (eve) and *hairy* but not to repress *engrailed* (Aronson et al., 1997). In addition, the gap protein Knirps represses different stripes of *eve*, and requires CtBP to repress stripes 4 + 6, but not to repress stripes 3 + 7. However, increasing the levels of Knirps enables it to repress stripes 4 + 6 without CtBP suggesting a quantitative rather than qualitative difference (Struffi et al., 2004). Furthermore, the repressor activity of Eve protein is increased in the presence of Gro (Kobayashi et al., 2001).

As stated above, there are differences in the ability of the three repression domains/motifs of Brk to repress different target genes. For instance, 3R is sufficient for repression of *omb* but not *sal* or SalE1. But, either CiM or GiM seem to be able to repress both *sal* and *omb* (Fig. 29C,

24D), so why does Brk need the 3R domain as well? Brk seems to be completely active in its ability to repress *omb* in the absence of CtBP and Gro, and recruitment of both CtBP and Gro does not appear to increase Brk activity in terms of increased repression of *omb*. If this was not the case, then the width of the *omb* expression domain should shift in brk^{F138} mutant cells, which do not have a CiM or GiM, or in *CtBP*/*gro* double mutant clones, but this does not happen (Hasson et al., 2001). Therefore, the 3R domain may be more efficient than either CiM or GiM in repressing *omb,* thereby providing Brk with an appropriate level of activity to set up the *omb* domain in the correct position.

Brk must recruit either CtBP or Gro to repress some target genes such as *sal* (Fig. 13) and *brk* itself (Hasson et al., 2001), or just Gro for others such as Vg-QE (Hasson et al., 2001). So, why does Brk even need to recruit CtBP? Misexpression of Brk proteins with a mutation of CiM alone or in combination with a mutation or deletion of GiM and 3R show reduced activity compared to wild type (Fig. 16D). However, there is no evidence for the specific role of CtBP for repression of any Brk target gene in the wing, as *CtBP* mutant clones do not have any effect on expression of known Brk targets in the wing (Fig. 13B) (Hasson et al., 2001). Analysis of Brk sequences from four diverse insect species reveal that CtBP and Gro interaction motifs have been conserved for millions of years (Fig. 7) suggesting recruitment of CtBP must be important for Brk function. Therefore, it is possible that CtBP is required for Brk function in other tissues outside the wing such as the embryo (Hasson et al., 2001) or for other unidentified Brk targets in the wing.

Transcriptional repression can act over either long or short distances with Gro acting via the former and CtBP the latter. Consequently, it seems odd that Brk would recruit both co-repressors based on these properties. Presumably transcription factors would recruit either CtBP

or Gro (Zhang and Levine, 1999), and it appears that recruitment of CtBP would provide the advantage of enabling a transcription factor to repress at a single enhancer without interfering with the activity of another closely positioned enhancer. On the other hand, recruitment of Gro by a transcription factor at one enhancer may in fact interfere with the activity of a more distant enhancer, but such a simple model is not always the case (Nibu et al., 2001). Experiments using chimeric repressors, containing the DNA binding domain from short-range repressors and Gro interaction sequences from the long-range repressor Hairy, revealed that long-range repression mediated by Gro (via Hairy) did not always produce the dominant inactivation of linked enhancers, suggesting that repressors need activators present to bind DNA (Nibu et al., 2001). Therefore, almost all transcription factors only recruit one co-repressor. In addition to Brk, two other transcription factors, Hairy and Hairless, have been shown to recruit both CtBP and Gro. Surprisingly, CtBP seems to function as an antagonist of Gro when recruited by Hairy as compared to its standard function as a co-repressor (Phippen et al., 2000; Zhang and Levine, 1999). For Brk, all experimental evidence suggests that CtBP contributes to the repressor activity of Brk, not an antagonistic role. In the case of Hairless, genetic evidence suggests that CtBP and Gro both confer repressor activity to the transcription factor (Barolo and Posakony, 2002), but it is not known whether CtBP is required to increase the activity of Hairless, or if it is required for some targets that Gro is not able to completely repress.

Extensive analysis of the 3R region of Brk revealed that it is sufficient for normal repression of *omb* and UbxB, but not for complete repression of *sal*, suggesting this region is only sufficient when Brk sites are in close proximity to activation sites. Further studies need to be completed to determine the binding site proximity requirements for the 4R region. Brk^{NLS4R}

can repress both *sal* and *omb* partially, but has not been tested for its ability to repress the UbxB enhancer.

As stated previously, the best way to fully understand the function of each repression domain would be to replace endogenous *brk* with transgenes having one or two repression domains that have been mutated or deleted. The current study analyzed the effects of misexpression of modified/mutated forms of Brk protein in regions where endogenous *brk* is normally not expressed. Although this was not the ideal approach to use, great strides have been made in beginning to understand what regions of the protein are important for Brk repressor activity and more importantly, how each of these regions serve to act differentially on target genes.

2.5.5 Requirement of CiM and/or GiM for repression of salE1

Full-length forms of Brk protein containing mutation or deletion of the CiM or GiM (Brk^{CM} and Brk^{GD}) were capable of repressing salE1 expression (Fig. 30C,D). On the other hand, mutation of both the CiM and GiM (Brk^{CMGM}, Brk^{3M}) produced proteins that were not able to repress salE1 expression (Fig. 31C, 32E), and Brk^{3M} actually activated salE1 expression (Fig. 32E). Therefore, either the CiM or GiM are required for repression of salE1. Based on the analysis of *CtBP gro* single and double mutant clones as well as the misexpression of mutated forms of Brk protein, it appears as if salE1 enhancer requires the recruitment of co-repressor Gro and possibly CtBP. However, $Brk^{Stop1NAC}$ was also not able to repress salE1, possibly due to the fact that CtBP cannot mediate long-range repression, and Gro recruitment is required for repression of this reporter. If this were the case, Brk^{GD} should not have repressed salE1 expression, but it did (Fig. 30D). Possible explanations for the inability of $Brk^{Stop1NAC}$ to repress

salE1 include: 1) artificial nature of the reporter construct or 2) there may be sequences in the C-terminal region that work in conjunction with Gro/CtBP to repress this reporter. Additional evidence to support the requirement for GiM (Gro) and/or CiM (CtBP) for repression of salE1 comes from Brk^{CMGM}, Brk^{3M} and *CtBP gro* double mutant clones. In each of these cases, CtBP and Gro are either not available for recruitment, or cannot bind to a mutated interaction motif, and salE1 cannot be repressed. Similar to endogenous *sal*, repression of the salE1 reporter requires recruitment of a co-repressor to assist Brk.

2.5.6 Importance of putative Brk binding sites in salE1 enhancer

Although wild type Brk (Brk^{3PF3}) repressed salE1 expression and Brk^{Stop1} and Brk^{NLS} activated salE1, mutation of the three putative Brk binding sites present in salE1 (salE1MB123) have a different effect. Mutation of the three binding sites results in expansion of expression into lateral regions of the wing pouch, and misexpression of each of the above mentioned transgenes in the posterior of the wing does not change the expression of salE1MB123 either by repressing or activating (Fig. 19Gi, 22Fi). Therefore, one or more of the Brk binding sites contained in salE1 are important for the repressor activity of wild type Brk and activation by Brk^{Stop1} and Brk^{NLS}. Additional analysis of the effect of mutation of each of these sites individually or in combination revealed that binding sites 1 and 2 are important for repression, but binding site 3 is a lower affinity site. It is interesting to note that binding site 3 is the site not conserved between *D. melanogaster* and *D. pseudoobscura* (Fig. 9A).

2.6 Conclusions

In order for Brk to possess repressor activity *in vivo*, the protein must contain a functional DNA binding domain. Moreover, Brk does not repress target genes simply by binding site competition, but rather it requires specific repression domains/motifs for its activity. In addition to the CiM and GiM, Brk possesses two other independent repression domains/motifs designated 3R and 4R, for third and fourth repression domains, respectively (Fig 36). The 3R repression domain is sufficient for normal repression of *omb* and UbxB, but not *sal*. The co-repressor, Gro is required for normal repression of *sal* and is sufficient even in the absence of CtBP. However, in the absence of Gro, CtBP can provide partial activity for repression of *sal*, but it is not completely effective. Whether a single repression domain is sufficient for Brk to repress a particular target gene may depend upon the positioning of Brk binding sites relative to activator sites. For example, the UbxB reporter has overlapping Brk and activator (Mad) sites (Kirkpatrick et al., 2001). In contrast, the cis-regulatory elements of *sal* suggest that Brk and activator binding sites are separated (Barrio and de Celis, 2004).

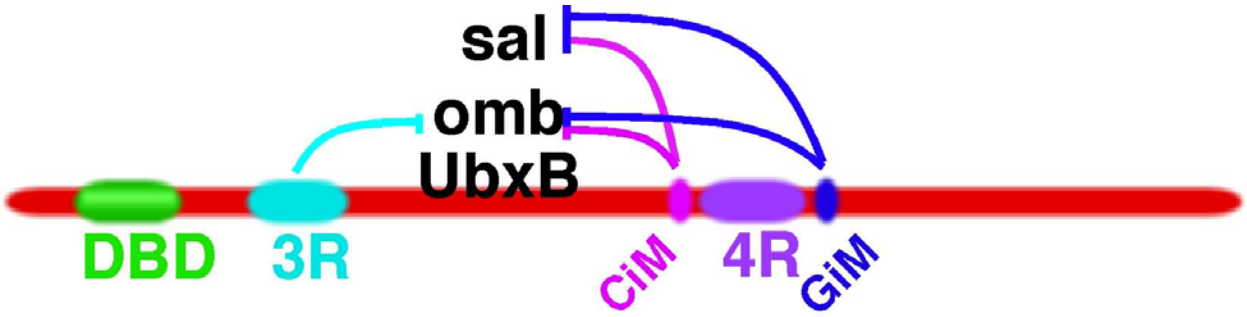

Figure 36. Repression of Dpp targets by Brinker.

Brk does not repress genes simply by competition, but requires repression domains/motifs in combination with its DNA binding domain (DBD). Brk possesses four independent repression domains/motifs: the CtPB interaction motif (CiM), Groucho interaction motif (GiM), 3R and 4R. 3R represses *omb* and UbxB, but not *sal*. The CiM and GiM can repress *sal*, *omb* and UbxB.

2.7 Future Studies

Based on the above analysis, the 3R region of Brk seems to require Brk and activator sites to be located in close proximity for repression of target genes such as UbxB and *omb*. In contrast, further analysis needs to be completed to determine the requirements for the 4R region of Brk and whether Brk and activator sites need to be in close proximity or if they can be separated.

Other experiments will attempt to answer some of the following questions. To begin with, which repression domains are truly required by endogenous Brk for its repressor activity and why are these domains required? Analysis of the functional domains of Brk relied on misexpression of the modified/mutated forms of the protein, in regions where Brk is not normally expressed, and comparison to wild type Brk. To gain a more complete understanding of the role each of the repression domains plays in Brk function, the endogenous *brk* gene will be replaced with the modified/mutated forms of the protein allowing the requirements of each domain to be assessed. The easiest way to replace endogenous *brk*, with the *brk* transgenes, would be to drive the transgenes with a *brk*-Gal4 line in a *brk* mutant background. My attempts to replace endogenous *brk* in this manner were unsuccessful, and so another approach will have to be utilized. The initial approach utilized deletion lines of the *brk*-Gal4 line, but none of the seven deletion lines isolated were able to rescue when crossed with wild-type (3PF3). The deletion lines may have important enhancer/promoter elements missing/disrupted and do not drive *brk* expression enough to resuce the phenotype. An alternate approach is to drive *brk* transgenes with a B14 genomic fragment, plus 320 bp of endogenous promoter region, plus *brk* coding region, shown to rescue a *brk* mutant (brk^{XA}) (completed by M. Moser), but not *brk* null

mutant, *brk*^{M68}. B14 is a 4.7 kb enhancer fragment, containing a 53 bp silencer region and enhancer region plus additional sequence, that drives *brk* expression in a pattern similar to endogenous *brk* (Muller et al., 2003). Flies containing modified/mutated forms of Brk will be examined to see if they can rescue *brk*^{XA} mutants (a hypomorphic allele).

Analysis of Brk^{A} and Brk^{EC} narrowed the 3R region to include residue 151-206 and Brk^{A2} truncated at residue 173, indicating that 3R could be included in residues 173 to 206, or these residues work in combination with more N-terminal residues. In contrast, the 4R domain has been narrowed down to only the region lying between the CiM and GiM (residues 383-452). To further analyze this region, smaller regions will be fused to DBD+NLS and their activity assayed *in vivo* as previously described. To overcome the potential problems of the repressor domain being located too close to the DNA binding domain, it may be necessary to fuse smaller regions to Brk^{Stop1NA} (no repressor activity), i.e. a linker region between the DNA binding domain and repression domain might be necessary. Moreover, do these regions contain short motifs for recruitment of co-repressors such as CtBP or Gro, or are they composed of larger regions that constitute autonomous repression domains or act as antagonists of activator proteins? To evaluate the ability of 3R and 4R to function as autonomous repressor domains, each of the regions will be fused to heterologous DNA binding domains (e.g. Gal4) and assayed for their ability to repress reporter genes driven by enhancer or promoter regions with inserted UAS sites. Spacing between the UAS sites and activator sites or the promoter (driving LacZ) is different for each reporter, enabling a determination of whether a particular region of a protein can act as a repression domain. Moreover, this method may be able to distinguish whether the region acts by quenching activators or directly repressing the transcriptional machinery, as well as the distance over which it will act. In addition, to assess whether the repression domains act

as antagonists of activators, UAS sites can be introduced into enhancers that contain Mad binding sites. Additional experiments will be completed to determine whether or not the 3R region can only repress targets when Brk and activator sites are in close proximity.

Since Brk^{NLSW} was not able to repress salE1, it may be a result of the lack of spacing between the DNA binding domain and the GiM creating a smaller "artificial" protein. Clonal analysis revealed that Gro was needed to repress the salE1 enhancer so it was thought that Brk^{NLSW} should be able to repress salE1, but it did not. However, would the addition of the WRPW motif to $Brk^{Stop1NA}$ enable the truncated protein to repress salE1? Addition of the GiM to $Brk^{Stop1NA}$ would allow for spacing between the DNA binding domain and the GiM. However, this approach may not work either because $Brk^{Stop1NAC}$ was not able to repress salE1.

In addition, larger enhancer regions may behave more like the endogenous *sa*l gene, therefore the ability of *brk* mutants and modified/mutated forms of the Brk protein to repress could be tested using larger enhancer regions such as sal1.8 or even sal10.2. Once the importance of different sites within the enhancer are determined, additional constructs can be generated that change the spacing between Brk sites and analyzed for the ability of wild type and modified/mutated forms of Brk to repress reporter gene expression. Furthermore, additional Brk binding sites can be added to determine if these increase the ability of wild type and modified/mutated forms of Brk to repress expression. Finally, a determination of the requirements for each Brk repression motif/domain (CiM, GiM, 3R and 4R) can be made in terms of the distance Brk and activator sites need to be for repression by each of the domains.

Overall, this study revealed that Brk does not function identically to repress each of its targets. The DNA binding domain is essential for Brk to repress target genes and the DNA binding domain alone is insufficient for repressor activity. Contrary to previous suggestions, I

demonstrated that Brk cannot repress simply by competing with activators, but requires specific repression domains (active repression) in combination with its DNA binding domain. Brk possesses four independent repression domains, but these domains are not equivalent. Thus, although *sal* and *omb* show quantitative differences in their response to Brk, this may actually be based more on qualitative differences in the mechanisms that Brk uses to repress them.

Although the current study answered the question whether or not Brk functions identically to repress each of its targets, the analysis relied on ectopic expression of modified/mutated form of the Brk protein in regions where Brk is not normally expressed. A better approach would be to replace the endogenous *brk* gene with modified/mutated forms of Brk to fully understand the role of each repression domain/motif in Brk function. However, this approach requires a *brk* rescue construct that was not available when these studies were implemented. Ectopic expression of modified/mutated *brk* transgenes using the Gal4/UAS system enabled me to identify two additional regions of Brk protein that function as independent repression domains.

As mentioned, there are numerous experiments that need completed to determine how each repression domains/motifs of Brk functions to repress target genes. However, the most important experiments would include those replacing endogenous *brk* with modified/mutated proteins forms of the protein and further analysis of the 4R region similar to that completed for the 3R region. In addition, experiments to verify whether or not the 3R and 4R regions function as autonomous repression domains are extremely important.

2.8 Materials and Methods

2.8.1 Comparison of *brinker* sequences from insect species

brinker sequences from *Anopheles gambiae* (accession number AY57899), *Drosophila pseudoobscura* (included in AADE01000981) and *Bombyx mori* (combination of AU004448 and AV402267) were obtained through BLAST searches of NCBI databases and compared to *Drosophila melanogaster.*

2.8.2 Fly strains utilized for studies

The following alleles or transgenes were used: brk^{F124}, brk^{F138}, brk^{M68}, $CtBP^{87De-10}$, gro^{E48}, Vg-QE (P3-lacZ), hs-GFP ($Avic$\GFP$^{hsT:Hsap\MYC}$), hs-flp (P{hsFLP}22), FRT18A (P{ry[+t7.2]=neoFRT}18A), FRT82B (P{neoFRT}82B, Ubi-GFP (P{Ubi-GFP(S65T)nls}3R), omb-lacZ (P{lacW}bi^{Pol-1}), C765 (Scer\Gal4^{C-765}), en-Gal4 (P{en2.4-Gal4}e16E), W^{1118}, W^{1118} P{ey-FLP.N}2; P{bossT:Arus/HRP}2, P{neoFRT}82B P{arm –LacZ.V}83B.

2.8.3 Generation of *in vitro* mutated/modified UAS-brk transgenes

Constructs containing modified/mutated forms of *brk* were generated by PCR amplification from *brk* cDNA (tmbNB14-2) and cloned into appropriate sites of pBluescript containing two copies of the HA epitope sequence to the C-terminus (pFlu3PRX). The sequence is (GS/EF/RS)MAGNIYPYDVPDYA GYPYDVPDYAG and HA sequence is underlined. Brk wild type and modified/mutated transgenes were cloned into pUAST vector (Brand and Perrimon, 1993) *as Kpn*I/*Xba*I fragments for misexpression studies.

Location of mutations, modifications or deletions are indicated in Figure 16. To generate wild type *brk*, brk^{3PF3} , a *Hind*III/*Hind*III fragment from *brk* cDNA (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a) was cloned into *Hind*III cut pBluescript. An *Eco*RI site was introduced at the end of the coding region by PCR, and a *Kpn*I/*Eco*RI fragment (nt 1-2643) was cloned into pFlu3PRX to add the HA tag at the C-terminus. An HA tagged version of endogenous *brk* mutant, brk^{F124}, (containing an R82W substitution in the DNA binding domain), was generated by cloning a *Bsi*WI/*Spe*I (nt 67-1762) cut PCR product derived from brk^{F124} genomic DNA into *Bsi*WI/*Spe*I cut brk^{3PF3}. Primer pairs used to generate each of the modified/mutated forms of Brk protein can be found in Appendix 1. C-terminal truncations of *brk* to produce Brk^{53}, Brk^{S}, Brk^{Stop1}, Brk^{A}, Brk^{A2} and Brk^{C} were generated by PCR using an external primer M13 forward (pBluescript) and internal primers flanked by *Eco*RI, *Bam*HI or *Bgl*II restriction sites. Brk^{Stop1NA} was generated by inverse PCR to remove the 3R region (deletion of residues 148-200 to a *Bgl*II site (RS)) using brk^{Stop1} as template. Brk$^{Stop1\Delta A17}$, and Brk^{Stop1NAC} were generated as indicated above, but template DNA was brk^{Stop1NA} or brk^{NA}, respectively. Brk^{Stop2NACM}, Brk^{S2NACM1}, Brk^{S2NACM2} and Brk^{S2NACM3} were generated by PCR as indicated above using brk^{3M} as template DNA that has the 3R region deleted and mutation of the CtBP interaction motif (CiM) from PMDLSLG to AMAAALA as a *Not*I site. Brk^{NLS} and Brk^{NLSW} were generated by PCR using an internal primer flanked by the sequence of the Nuclear Localization Sequence (NLS) from SV40 T Antigen (Kalderon et al., 1984), PPKKKRKV, plus WRPW for Brk^{NLSW}. Constructs with regions of Brk protein attached to the Brk^{NLS} were generated by cloning residues 151-228 for Brk^{EC}, residues 152-377 for Brk^{EA}, residues 200-377 for Brk^{ED}, residues 253-377 for Brk^{EF} and residues 383-453 for Brk^{NLS4R} as *Bgl*II cut fragments into *Bam*HI cut Brk^{NLS}. Mutations of CtBP and Groucho Interaction Motifs and deletions of

internal regions (3R and 4R) were generated by inverse PCR using two internal primers flanked by restriction sites to change the sequence as indicated and religation of the PCR product. Brk^{CM} was designed to mutate the CiM from PMDLSLG to AMAAALA and create a *Not*I site, whereas Brk^{GM} mutated the GiM from FKPY to FAAA and created a *Not*I site. Mutations similar to those generated result in a loss of CtBP and Gro binding to Brk (Hasson et al., 2001; Zhang and Levine, 1999). Cloning a *Spe*I/*XbaI* fragment from brk^{GM} into *Spe*I/*Xba*I cut brk^{CM}, to mutate the CiM and GiM, generated Brk^{CMGM}. Brk^{NA} was generated by inverse PCR to remove amino acids 148-200 and replace with a BglII site (RS). Brk^{3M} was generated by inverse PCR using brk^{CMGM} as template DNA to remove the 3R region in addition to mutation of CiM and GiM. Brk^{NACM} and Brk^{NAGM} were generated by inverse PCR to delete the 3R region using brk^{CM} and brk^{GM} as template DNA, respectively. $Brk^{\Delta 4R}$ was generated by inverse PCR to remove the region lying between the CiM and the GiM (residues 383-453) and replace with a *Bgl*II site (RS). Lastly, Brk^{4M} was generated by inverse PCR to remove the CiM, GiM and the region lying in between (residues 377-464), using by brk^{NA} as the template DNA for removal of the 3R region.

Transgenic flies were generated by injection of pUAST plasmid DNA (final conc. 0.8μg/μl) containing each of the transgenes plus Turbo wingsclipped helper plasmid at a concentration of 1μg/μl into posterior of W^{1118} syncytial blastoderm embryos. Injected embryos were incubated at 18°C, then room temperature in a humidity chamber until larvae emerged from vitelline membrane. Larvae were collected and placed in food vials at 25°C until adults emerged. Each individual Go fly was mated to 3 W^{1118} flies of the opposite sex, and the F1 generation from each vial was screened for flies containing colored eyes indicative of transformation.

2.8.4 Clonal analysis and ectopic expression studies

Homozygous mutant clones were generated in wing imaginal discs by hs-flp/FRT-induced mitotic recombination (Golic and Lindquist, 1989). Clones were generated in the second or early third instar of larvae by heat shock for 1 hour at temperatures ranging from 33°C to 37°C.

hs-flp; FRT82B CtBP$^{l(3)87De\text{-}10}$ *gro*^{E48}/FRT82B Ubiq-GFP (and the same for single mutant clones) Clones in discs were identified by loss of GFP.

hs-flp: FRT82B CtBP$^{l(3)87De\text{-}10}$ *gro*^{E48}/FRT82B arm-LacZ (and the same for single mutant clones) Clones in discs were identified by loss of lacZ staining.

Ectopic expression of UAS-transgenes was achieved by independently crossing transformant lines to two Gal-4 expressing lines; en-Gal4 (expressed in the posterior of the wing) and C765 (ubiquitous expression in the wing) (See section 2.4.8.1 for criteria).

2.8.5 Reporter constructs

salE1 is a 471 bp fragment at the 3′end of the sal1.8S/E fragment of Kuhnlein et al. salGCNB is a 285 bp fragment comprising the 5′ half of salE1. salE1MB123 had the three putative Brk sites mutated from GGCGYY to GTCGYY using the Stratagene QuickChange Site-Directed Mutagenesis Kit. These fragments were cloned into the GFP reporter vector, pHSB, which is a modified version of the pH-Stinger vector (Barolo et al., 2000) in which two Brk

binding sites in the hsp70 promoter have been mutated by inverse PCR. UbxB is an *ultrabithorax* reporter construct, expressed in the embryonic midgut mesoderm (Thuringer et al., 1993).

2.8.6 Electrophoretic mobility shift assays (EMSAs)

brk^{NLS} PCR fragment was cut with *Bgl*II/*Not*I and cloned into *Bam*HI/*Not*I pET21a expression vector (Novagen). Plasmid DNA was obtained using Qiagen QIAprep spin Mini Prep (Catalog #27104) Kit. Protein was produced using Promega TNT quick Coupled Transcription/Translation System according to manufacturers instructions.

2.8.6.1 Probe Preparation

Equal molar ratios of UbxB oligos (25 pmol each) were mixed and annealed under the following conditions: 95°C 10 minutes; 65°C 15 minutes; 37°C 15 minutes, 23°C 15 minutes and 4°C for 1 hour to a final concentration of 50 pmol/μl. UbxB probe was radiolabeled by Klenow extension of 3′ ends using α-^{32}P CTP and unincorporated nucleotides were removed using BioRad P-30 gel spin columnsProbe sequences are as follows:

5′ GACTCTGGACTGGCGTCAGCGCCGGCGCTTCCAGCT 3′

5′ GGCAGCTGGAAGCGCCGGCGCTGACGCCAGTCCAGA 3′

The following buffer (Mad buffer) was used in binding reactions (Xu et al., 1998):

50 mM KCl, 20 mM HEPES, 1 mM DTT, 1 mM EDTA, 5 μg/ml poly[d(A-T)] , 10 0.25 mg/ml BSA, 4% Ficoll (To optimize Brk^{NLS} binding, final concentration of 100 mM KCl was added for some experiments).

2.8.6.2 EMSA reactions

Briefly, EMSAs were performed by incubating *in vitro* translated BrkNLS with 1 μl ^{32}P-labeled (10,000 cpm/μl) UbxB probe in 10 μl reactions. For competition experiments, 10X or 100X of unlabeled probe was added to reaction. Reactions were incubated for 20 min at Room Temp. The entire reaction was separated on a non-denaturing 4% polyacrylamide gel following standard protocol (Ausubel et al., 1993). For supershift experiment, 0.5 μl of anti-HA antibody was added to the reaction and allowed to incubate for an additional 10 minutes. The gels were dried and exposed to autoradiographic film.

2.8.7 Immunostaining, X-gal staining and analysis of adult wings.

Dissection and staining of wing imaginal discs was carried out by standard techniques. Omb expression was detected using a lac-Z enhancer trap. The following primary antibodies were used: anti-Sal (rabbit, 1:50) (Kuhnlein et al., 1994); anti-βgal (rabbit, 1:2000 Cappell) and chicken, 1:2000; Abcam); anti-HA (mouse, 1:1000; Covance). Secondary Antibodies included Alexa 488 (mouse, rabbit, rat) and Alexa 568 (minX) (rabbit) (Molecular Probes); and Cy2 (chicken) Cy5 (mouse) (Jackson). Embryos carrying the UbxB reporter were stained with X-gal by standard protocol; all embryos were fixed and stained along side each other. Wings from adult flies were mounted in GMM.

3 Chapter 3: Genome wide analysis of *Drosophila* protein kinases involved in nervous system development

3.1 Abstract

Protein kinases play important roles in many eukaryotic cellular processes including nervous system development. Nervous system development in *Drosophila* progresses through a series of events including neuroblast specification, neuroblast formation, neurogenesis, development of nerve cells/glia, axon outgrowth and pathfinding and formation of precise connections with target cells. The Drosophila genome contains approximately 13,000 genes and of these, there are approximately 229 protein kinase genes. The availability of the sequenced genome makes it possible to conduct a focused search for kinases involved in nervous system development. RNA interference (RNAi) was used to systematically knockdown the function of individual kinase genes and analyze the effect of each gene on the developing nervous system. Sequence specific primers were designed for 54 kinase genes and dsRNA corresponding to each of these genes synthesized. Flies expressing neuronal specific GFP were injected with dsRNA and screened for kinase genes causing aberrant CNS and PNS development. Of the 54 kinase dsRNA injected, 36 did not produce a nervous system phenotype, 13 produced a non-specific phenotype and 5 produced nervous system specific phenotypes. The five candidate genes included Epidermal Growth Factor Receptor (CG10079), Mushroom Bodies Tiny (CG18582), Cyclin dependent kinase 2 (CG5363), C-terminal Src kinase (CG17309) and p21 activated kinase3 (CG14895).

3.2 Introduction

The fruit fly, *Drosophila melanogaster* has been a valuable model organism for genetic studies for close to a century. Traditionally, the power of *Drosophila* genetics relied on forward genetic screens to identify genes that affect a given biological process (St Johnston, 2002). Forward genetic screens uncover many genes required for specific processes, but other genes can be missed due to limitations of the screening process. For example, embryonic screens may not uncover some mutations because the genes are expressed during oogenesis by the mother and mask a mutation in the zygotic gene (St Johnston, 2002). Patterning of the early embryo body axes is directed by mRNAs that are deposited in the oocyte by the mother and localized at the specific positions within the embryo (St Johnston and Nusslein-Volhard, 1992). The genes providing the mRNA are known as the maternal effect genes as they are expressed in the mother rather than the embryo. Recently, the availability of *Drosophila* genome sequences enables a reverse genetic approach to be utilized to determine the function of predicted classes of gene products. Since many developmental processes as well as known human disease genes are conserved between flies and vertebrates (St Johnston, 2002), studies in the fly may provide useful insights into understanding disease in humans.

I am interested in looking at how one class of proteins, the protein kinases, control development of the nervous system in *Drosophila*. First, I am going to briefly review the function of protein kinases and then provide a brief review of nervous system development in flies.

3.2.1 Protein Kinases

Protein kinases are enzymes that catalyze the transfer of a phosphate group from the nucleotides ATP or GTP onto serine, threonine or tyrosine residues of their substrate proteins (Morrison et al., 2000). Protein kinases play instrumental roles in controlling many eukaryotic cellular processes including cell cycle regulation, DNA replication, metabolism, growth and differentiation and signal transduction (Morrison et al., 2000). Kinases, whether receptor or cytoplasmic, control these processes by covalent attachment of a phosphate group to an amino acid side chain of their substrates (Johnson and Barford, 1993). Addition of the phosphate group containing two negative charges can induce a conformational change in the substrate protein, resulting in a modification of its activity (Johnson and Barford, 1993). This conformational change can be reversed by another class of enzymes, the protein phosphatases, which remove the phosphate (Morrison et al., 2000). Reversible protein phosphorylation is a key strategy used to control activity of proteins in complex networks of signaling pathways in eukaryotic cells (Morrison et al., 2000).

Based on sequence similarity, kinases from species as diverse as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and humans all share homology in the catalytic kinase domain (Manning et al., 2002). Moreover, protein kinases and their signaling pathways are evolutionarily conserved, further emphasizing their importance in eukaryotic organisms (Manning et al., 2002). There are numerous classes of kinases including AGC, Atypical, CAMK, CK1, CMGC, Ste, TK, TKL, RGC, PTK, OPK, and 'Other' based on kinase domain similarity (Manning et al., 2002; Morrison et al., 2000). Overall, kinase function serves critical roles in the regulation of enzymatic activity as well as signal transduction pathways in diverse organisms from yeast to man.

3.2.2 *Drosophila* Nervous System Development

Nervous system development in *Drosophila* progresses through a series of events including neuroblast specification, neuroblast formation, neurogenesis, development of nerve cells/glia, outgrowth and pathfinding of axons, and the formation of precise connections with target cells (Goodman and Doe, 1993). I am going to provide a brief review nervous system development in flies. First, central nervous system development in the early embryo will be discussed, followed by peripheral nervous system development and then axon pathfinding.

3.2.2.1 Central Nervous System Development

Signaling pathways provide cues for cells at all stages of neuronal cell differentiation. The central nervous system is derived from two regions that lie on either side of the ventral midline of the cellular blastoderm embryo, known as the neuroectoderm (Fig. 37Ai) (Goodman and Doe, 1993). The neuroectoderm is a region of the embryo within which cells have the ability to produce neuroblasts or progenitors of the epidermis, epidermoblasts (Campos-Ortega, 1993). Cells in the neuroectodermal region can take on either a neural or ectodermal fate depending on the cues obtained from their surrounding environment (Goodman and Doe, 1993). At the onset of gastrulation, the ventral-most mesoderm region invaginates into the interior of the embryo allowing the two lateral neuroectodermal regions to join at the ventral midline of the embryo (Fig. 37Aii). Neural specification in *Drosophila* occurs during the initial phase of germ band elongation (stage 8) (Fig 37Aiii), prior to the onset of mitotic activity in the ectodermal layer (Foe, 1989; Foe and Alberts, 1983; Hartenstein, 1985).

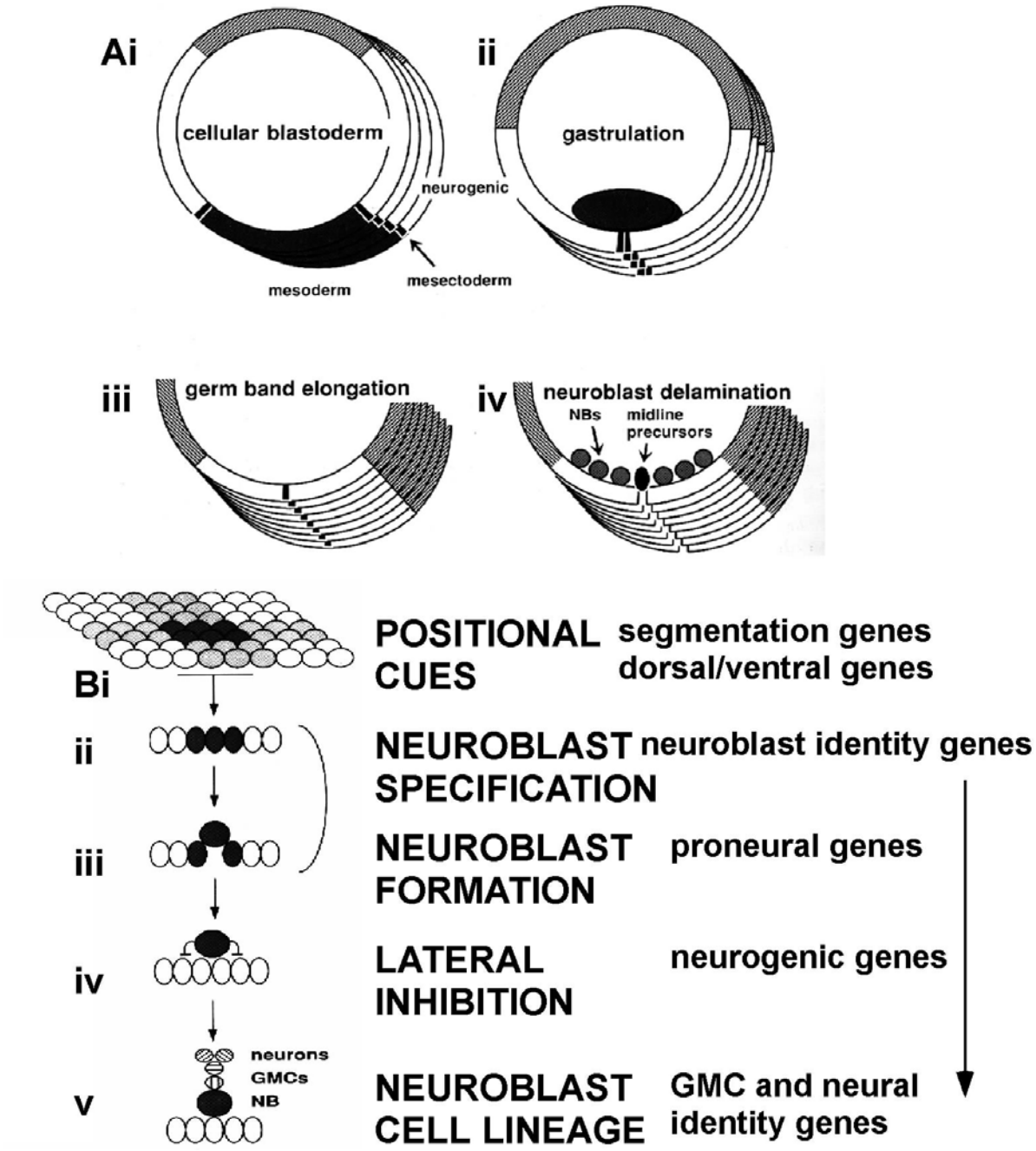

Figure 37. Formation of the neurogenic-midline region and neuroblasts in *Drosophila* embryos.

Figure 37. Formation of the neurogenic-midline region and neuroblasts in *Drosophila* embryos.

(Ai-iv) Schematic representation of cross sections from embryos at successive developmental stages, with dorsal side up and ventral down. (Ai) During cellular blastoderm stage, the presumptive neurogenic region lies on either side of the future mesoderm, with the midline precursor cells (mesectoderm) lying in between these regions. (Aii) During gastrulation, mesodermal cells invaginate into the embryo, bringing the presumptive midline cells and neurogenic region to the ventral side of the embryo. (Aiii) During germ band elongation, the midline precursors intermix, forming eight midline precursors per segment. (Aiv) Midline precursor cells and neuroblast cells delaminate and move inward. (Bi-v) Neuroblast formation. (Bi) Cells in the neuroectoderm receive positional cues from segmentation and dorsal/ventral genes. (Bii) Four to six cells are specified as neuroblasts. (Biii) Each of the neuroectodermal cells express one or more of the proneural genes that allow cells to be competent to differentiate as neuroblasts. (Biv) Only one cell in the cluster will enlarge and delaminate as a neuroblast, and will express some neurogenic genes that inhibit other cells in the cluster from becoming neuroblasts. Figures taken from (Goodman and Doe, 1993).

Positional cues from segmentation and dorsal/ventral axis specification genes produce a cluster of 4-6 cells which all have the potential to become neuronal precursors, but only one cell of each cluster takes on the neuronal fate (Fig. 37Bi) (Goodman and Doe, 1993). Individual clusters express one or more of the proneural genes (including genes of the *achaete-scute* complex) that give the neuroectodermal cell the potential to become neural precursors (Fig. 37Bii). The *achaete (ac)* and *scute (sc)* genes encode transcription factors that bind to target genes to initiate neural specification. Within the neuroectoderm, cells expressing these genes form groups known as proneural clusters, and one cell in each cluster, the neuroblast, expresses *achaete* at higher levels compared to neighboring cells (Campos-Ortega, 1993). All the cells in the cluster ubiquitously express Notch (a transmembrane receptor protein), but the cell that gives rise to the neuroblast express Delta (a transmembrane ligand) at higher levels compared to the other cells (Artavanis-Tsakonas et al., 1995; Campos-Ortega, 1993). Binding of Delta to its receptor Notch, leads to inhibition of the proneural genes and the concomitant loss of the cells ability to become a neuroblast, and these cells instead become epidermal cells (Goodman and Doe, 1993). Notch signaling activates expression of the E(spl)-Complex (Enhancer of split complex), and these gene products in combination with the transcriptional co-repressor Groucho, repress the expression of proneural genes *ac/sc* (Goodman and Doe, 1993). The levels of Delta and Notch signaling may be regulated by a feedback mechanism where high levels of Delta produce high levels of Notch signaling, and activated Notch protein can downregulate Delta expression (Artavanis-Tsakonas et al., 1999). Delta expression is negatively regulated by Notch signaling (Delta can only signal to adjacent cells). If one cell has a slightly higher level of Delta, it will activate higher levels of Notch signaling in surrounding cells that will then lower Delta

expression. Delta expression in the signaling cell will then go up because Notch signaling is down (Artavanis-Tsakonas et al., 1999).

Within the proneural cluster, only a single cell will enlarge and leave the neuroectodermal surface (delaminate) by moving to the interior of the embryo as a neuroblast (Fig. 37Biv) (Goodman and Doe, 1993). The neuroblast will continue to divide asymmetrically to form one neuron and three glial cells (Fig. 37Bv). In the absence of Notch signaling, all of the neuroectodermal cells develop as neuroblasts at the expense of epidermis (Campos-Ortega, 1993). In contrast, loss of function of any proneural genes results in loss of neuroblasts and increased numbers of epidermoblasts (Campos-Ortega, 1993). Therefore, the neural and epidermal progenitor cells are controlled by neurogenic and proneural genes, and these gene products are involved in a complex network enabling both neural and epidermal progenitors to be produced (Campos-Ortega, 1993).

3.2.2.2 Peripheral Nervous System Development

The Peripheral Nervous System (PNS) is composed of sensory and motor components. Sensory neuron cell bodies are present in the periphery and their axons extend toward the CNS, whereas the motor neuron cell bodies are located in the CNS and extend axons to the periphery (Jan and Jan, 1993). There are three types of sensory organs: a) external sensory organs containing external sensory structures designed to detect mechanical or chemical signals b) chordontonal organs located internally and c) multiple dendritic neurons, internal sensory organs functioning as touch receptors or proprioceptors (Jan and Jan, 1993). During embryonic development, formation of the external sensory organs and multiple dendritic neurons require expression of two proneural genes *achaete* and *asense* (Dambly-Chaudiere, 1987). Similar to

CNS development, the PNS also requires proneural and neurogenic gene expression for cells to take on the neuronal fate (Dambly-Chaudiere, 1987). In addition to the action of *Delta* and *Notch* in lateral inhibition, two other genes *shaggy* and *scabrous* are also involved. The *shaggy* gene product, a serine-threonine kinase, is required for cells of the proneural cluster to take on the epidermal fate (Heitzler and Simpson, 1991), whereas loss of *scarbrous* results in duplication of sensory bristles (Mlodzik et al., 1990). Neuronal precursors express genes of the Pan-neuronal precursor class that enable the neurons to acquire specific characteristics including the ability to extend axons and dendrites, act as transmitters, and develop receptors for intercellular communication (Jan and Jan, 1993). Ultimately, the specific sensory organ a neuronal precursor produces is controlled by the neuron type selector gene class (Jan and Jan, 1993). Overall, many of the genes required for CNS development also play roles in larval PNS as well as adult PNS development.

3.2.2.3 Axon Pathfinding

At the onset of axon outgrowth, sensory and motor axons must find the way to their targets in the CNS and muscles without any preexisting neuronal landmarks, but upon encountering each other they may use the other as a guide to their destination (Jan and Jan, 1993). Interestingly, axons extending to the distal periphery will begin to extend axons before those extending to more proximal targets, an order inverse to the distance they extend (Johansen et al., 1989). During axon outgrowth, the leading edge of the axon or growth cone detects environmental signals and converts the signals into movement towards a specific target (Lin and Forscher, 1993; Tanaka and Sabry, 1995). The signals guiding axon outgrowth may be secreted or membrane-bound and serve to either attract or repel the migrating growth cones (Culotti and

Merz, 1998; Guthrie, 1999; Tessier-Lavigne and Goodman, 1996). The ability of a growth cone to receive signals relies on receptors on its surface, and these signals are transduced via signaling pathways within the growth cone (Bashaw and Goodman, 1999; Hong et al., 1999; Ming et al., 1997). Precise connections with targets require growth cones via receptors to interpret different concentrations of signals as well as combinations of signals and to translate the information to control motility of the growth cone (Suter and Forscher, 1998). One important receptor in *Drosophila* is Dscam, involved in the Dock signal transduction pathway (Schmucker et al., 2000). Dscam detects axon guidance signals and transfers the signals into changes in the actin cytoskeleton via Dock and the protein kinase, Pak1 (Schmucker et al., 2000).

3.2.3 Role of Protein Kinases in Nervous System Development

Complete understanding of nervous system development requires the identification of all the molecules involved in this complex process. Although many genes involved in nervous system development have been identified, there are still many genes to be uncovered. Classical genetics in *Drosophila* has identified several kinases involved in nervous system development, but the availability of genomic sequences will reveal the entire spectrum of kinases present in the fly and enable determination of the roles of these kinases in nervous system development.

Protein kinases play instrumental roles in various processes of nervous system development in *Drosophila* as well as in vertebrates. For example, the cytoplasmic kinase, Shaggy is required in a cell autonomous manner for cells in the proneural cluster to become epidermal rather the neural progenitors (Heitzler and Simpson, 1991). In addition, other kinases such as Cdk5 and Pak1 are required for different aspects of axonal pathfinding (Connell-Crowley et al., 2000; Hing et al., 1999).

Cyclin-dependent kinase-5 (CDK5) plays a role in neuronal differentiation (Nikolic et al., 1996), axon guidance (Kwon et al., 1999), and possibly synaptic plasticity (Li et al., 2001) as well as neurodegeneration (Patrick et al., 1999). In *Drosophila*, Cdk5 and its activating partner p35 are involved in axon pathfinding, as disruption of Cdk5 function results in aberrant axon pathfinding (Connell-Crowley et al., 2000). In vertebrates, CDK5 is predominantly localized to mammalian post-mitotic neurons where it works in conjunction with nervous system specific activators p35 and p39 (Tsai et al., 1993). The CDK5/p35 complex is localized at the leading edges of growth cones and is involved in phosphorylation of neuronal microtubule-associated proteins (MAPs), thereby providing evidence for the role of CDK5 in regulation of axon extension in developing neurons (Maccioni et al., 2001). On the other hand, CDK5 has been implicated in neurodegeneration via tau hyperphosphorylation promoted by β-amyloid proteins (Maccioni et al., 2001). Therefore, changes in regulation of the CDK5/p35 complex may trigger neurodegeneration as seen in Alzheimers disease (Maccioni et al., 2001).

Another kinase, *Drosophila* Pak1 serine-threonine kinase (p21-activated kinase), is a downstream effector in the Dock pathway and regulates R-cell axon guidance in the ommatidia by controlling the actin cytoskeleton in growth cones (Hing et al., 1999; Newsome et al., 2000). The Drosophila compound eye is composed of approximately 800 identical photoreceptor organs known as ommatidia. Each ommatidia consists of 8 photoreceptor neurons (R1-R8), four cones cells and additional pigment cells (Bonini and Choi, 1995; Halder et al., 1995).

Studies by Allen et al. reveal that critical neuronal connections required for human cognitive function involve signal transduction via Pak3 and a mutation in Pak3 is present in patients with nonsyndromic X-linked mental retardation (Allen et al., 1998). Therefore, transduction of signals via Pak3 appears to be required for human cognitive functioning (Allen et

al., 1998). In general, Pak proteins seem to be effecter proteins linking Rho GTPases, Rac and Cdc42, to the actin cytoskeleton (Sells et al., 1997) and thus are integral components of the Rho GTPase/Rac signaling pathway controlling neuronal development.

Receptor tyrosine kinases have also been shown to be involved in various stages of nervous system development. In mice, Eph receptor tyrosine kinases and their ligands function in many aspects of nervous system development (Holder et al., 1998). More recently, it has been determined that receptor tyrosine kinases (RTK), including the Trk RTK, the Src family of non-receptor tyrosine kinases and the Eph RTK are involved in signaling cascades implicated in synaptic plasticity and long term memory (Purcell and Carew, 2003). Furthermore, invertebrate studies provide evidence that RTKs may be involved in memory (Dura et al., 1995).

Clearly, kinases have been shown to play key roles in many stages of neuronal development, thus the use of reverse genetics is likely to effectively uncover new roles for other kinases in the genome. It is plausible to hypothesize that kinases most likely function at all stages of neuronal development. Therefore, screening *Drosophila* kinases using a recently discovered reverse genetic tool, RNA interference, may prove to be an effective way to uncover additional kinases involved in nervous system development.

3.2.4 RNA interference (RNAi)

RNA interference (RNAi) is a mechanism for directly knocking down gene expression of genes with known sequence and has proven to be a powerful reverse genetic approach. RNAi was originally discovered in *Caenorhabditis elegans* when injection of double-stranded RNA (dsRNA) into the worm was shown to result in degradation of the corresponding mRNA and silencing of the gene product (Fire et al., 1998). Injection with dsRNA proved to be much more

potent in knocking down mRNA levels than injection with either sense or anti-sense RNA alone. In addition to injection of dsRNA, RNAi has been demonstrated in *C. elegans* by feeding on *E. coli* engineered to express dsRNA of a nematode gene (Timmons and Fire, 1998) and soaking the worms in a solution of dsRNA (Tabara et al., 1998). RNAi was also shown to be an effective and potent means of silencing genes in early *Drosophila* embryos (Kennerdell and Carthew, 1998). Subsequent studies have shown that RNAi is also effective in *Drosophila* cell culture enabling high throughput screening using RNAi to silence a plethora of genes (Clemens et al., 2000).

RNA interference refers to the use of double-stranded RNA (dsRNA) to target mRNAs for degradation leading to silencing of gene expression. The dsRNA sequence is utilized by RNA machinery to produce a protein-RNA complex that degrades the corresponding mRNA, thereby producing an extremely specific means of silencing genes, as other non-specific mRNAs are not affected (Zamore et al., 2000). It has been proposed that the biological roles of RNAi may be to maintain the integrity of the genome by suppressing mobilization of transposons or to prevent an accumulation of repetitive sequences in the germline (Aravin et al., 2001; Ketting et al., 1999; Ketting and Plasterk, 2000; Wu-Scharf et al., 2000). In addition, RNAi may be a defense against viral infections as well as a regulator of gene expression (Zamore, 2001).

3.2.4.1 Mechanism of RNA interference

RNAi is a two-step process (Fig. 38). The RNAi pathway is initiated by the processive, ATP-dependent cleavage of the dsRNA into small interfering RNAs (siRNAs) 21-25 nt in length

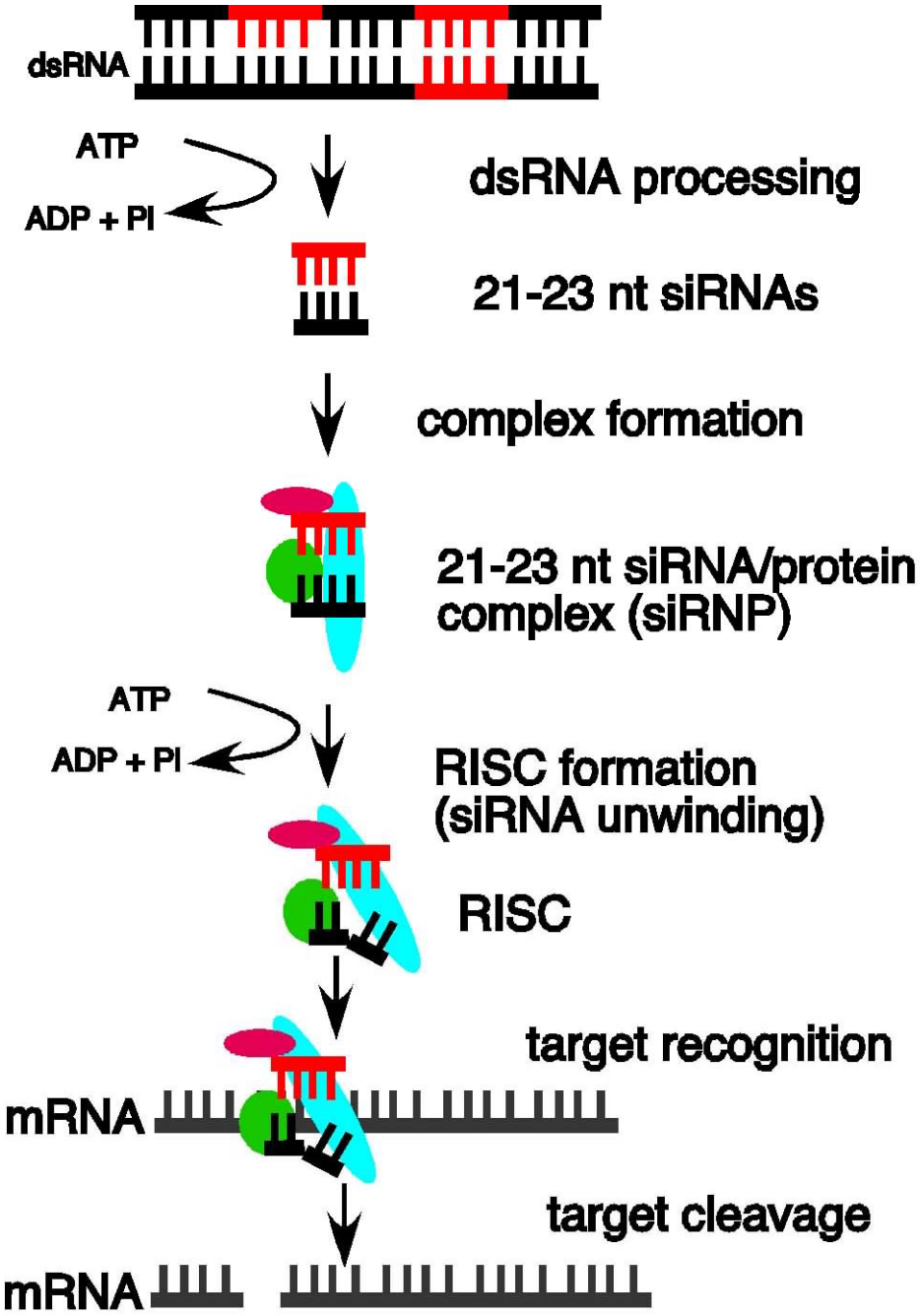

Figure 38. Mechanism of RNAi.

An ATP-dependent cleavage of the double-stranded RNA substrate into 21-23 nt siRNAs by Dicer, initiates the RNAi pathway. The siRNAs interact with a multi-protein complex that unwinds the siRNA in an ATP-dependent manner. Duplex unwinding produces a conformational change in the RNA-induced silencing complex (RISC), allowing recognition of target mRNA by the anti-sense strand of siRNA and subsequent cleavage of the mRNA. Figure taken from (Zamore, 2001).

by Dicer, a Ribonuclease (RNaseIII), (Bernstein et al., 2001; Elbashir et al., 2001; Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). Dicer is a member of the RNaseIII family of dsRNA specific endonucleases (Bernstein et al., 2001), and similar to other family members produces a siRNA product consisting of a 5′ phosphate and 3′ hydroxyl with two unpaired nucleotides at each end (Elbashir et al., 2001). The siRNAs then interacts with a multi-protein complex that is not able to mediate RNAi until the ATP-dependent unwinding of the siRNA duplex is initiated (Nykanen et al., 2001). Upon duplex unwinding, the complex undergoes a conformational change producing an active RNA induced silencing complex (RISC) (Hammond et al., 2000; Nykanen et al., 2001). The siRNA antisense strand of the activated RISC complex can recognize and hybridize with corresponding target mRNA leading to cleavage of the mRNA by the RISC complex (Hammond et al., 2001; Nykanen et al., 2001).

3.2.5 Utility of RNAi to silence gene function

The availability of genomic sequences from *C. elegans*, *Drosophila*, mouse and humans have provided a source of target sequences against which RNAi can be utilized as a reverse genetic tool to begin understanding the function of many genes with previously unknown function. RNAi has been effectively used as a tool to perform multiple screens in *C. elegans* (Gonczy et al., 2000), *Drosophila* embryos (Ivanov et al., 2004; Kim et al., 2004), and *Drosophila* cell culture (Bettencourt-Dias et al., 2004; Boutros et al., 2004; Goshima and Vale, 2003; Kiger et al., 2003; Lum et al., 2003; Somma et al., 2002) to identify genes involved in numerous biological processes, including cell cycle regulators, cell morphology, growth and viability and signaling pathways.

In the past, classical genetics identified mutations in many genes including those involved in nervous system development. Availability of cell type specific markers for many sensory organs has also enabled detailed study of the embryonic sensory nervous system. Furthermore, many genes are used at multiple developmental stages enabling phenotypes of lethal mutations to be analyzed in embryos, but there are drawbacks to analyzing embryonic phenotypes. For instance, some genes are expressed during oogenesis and the gene products contributed by the mother may be enough to mask a possible phenotype in the embryo even when the zygotic gene is non-functional (St Johnston, 2002). Therefore, classical genetics may be limited by its inability to identify zygotic and maternal effects mutations simultaneously, and many mutations were not uncovered. RNAi has been shown to reduce levels of both maternal and zygotic mRNA enabling further analysis of these previously uncharacterized gene products (Adams and Sekelsky, 2002). RNAi provides a powerful reverse genetic tool to silence gene function in *Drosophila* and we are particularly interested in those genes involved in central and peripheral nervous system development. We are interested in the protein kinase family because of their importance in signal transduction and regulation of cellular activity. In the present study, an RNAi screen of *Drosophila* kinases was completed to identify kinases involved in nervous system development.

3.3 Results

The *Drosophila* genome contains approximately 13,000 predicted genes and of these there are approximately 229 protein kinase genes (Kiger et al., 2003). Previous studies have identified kinase genes involved in nervous system development (Connell-Crowley et al., 2000; Heitzler and Simpson, 1991; Hing et al., 1999). The availability of the sequenced genome makes

it possible to conduct a focused search for kinases involved in nervous system development. In the present study RNA interference was used to systematically knock down the function of individual kinase genes and analyze the effect of each gene on the developing nervous system.

Upon release of the sequenced *Drosophila* genome in 2000 (Adams et al., 2000) it was possible to identify all of the kinases in the genome based on homology with the protein kinase catalytic core. A FlyBlast search using the kinase domain of serine/threonine kinase dPar1 (a kinase being studied in the lab) initially identified 165 *Drosophila* kinases to be silenced using RNAi. A subset of 60 kinases were chosen for testing due to availability of cDNAs, as Expressed Sequence Tags (ESTs), to use as template DNA. However, if a particular EST could not be used, then *Drosophila* genomic DNA (BAC clone) was used. Sense and antisense PCR primers were designed for each kinase gene with a 5′ T7 promoter sequence TAATACGACTCACTATAGGGAGACCAC for binding of T7 RNA polymerase in *in vitro* transcription reactions (Refer to Appendix B for a complete list of primer pairs). In order to ensure specificity, primers were designed to amplify exon regions adjacent to the kinase domain and if this was not possible, alternate exons were used. A Blast search was completed with each primer to determine that the sequence was indeed specific for that gene and would not also hybridize with other genes. PCR fragments were produced from cDNA (ESTs) or genomic DNA (BAC clones), and then used as template DNA in *in vitro* transcription reactions to synthesize both strands simultaneously for dsRNA production. The dsRNA products, ranging from 200 to 859 bp, were purified and injected into syncytial blastoderm embryos (Fig. 39A).

Initially, screening to identify kinases involved in nervous system development utilized a fly line expressing GFP under the control of a nervous system specific promoter (ELAV) allowing GFP expression exclusively in the CNS and PNS to be visualized in live embryos

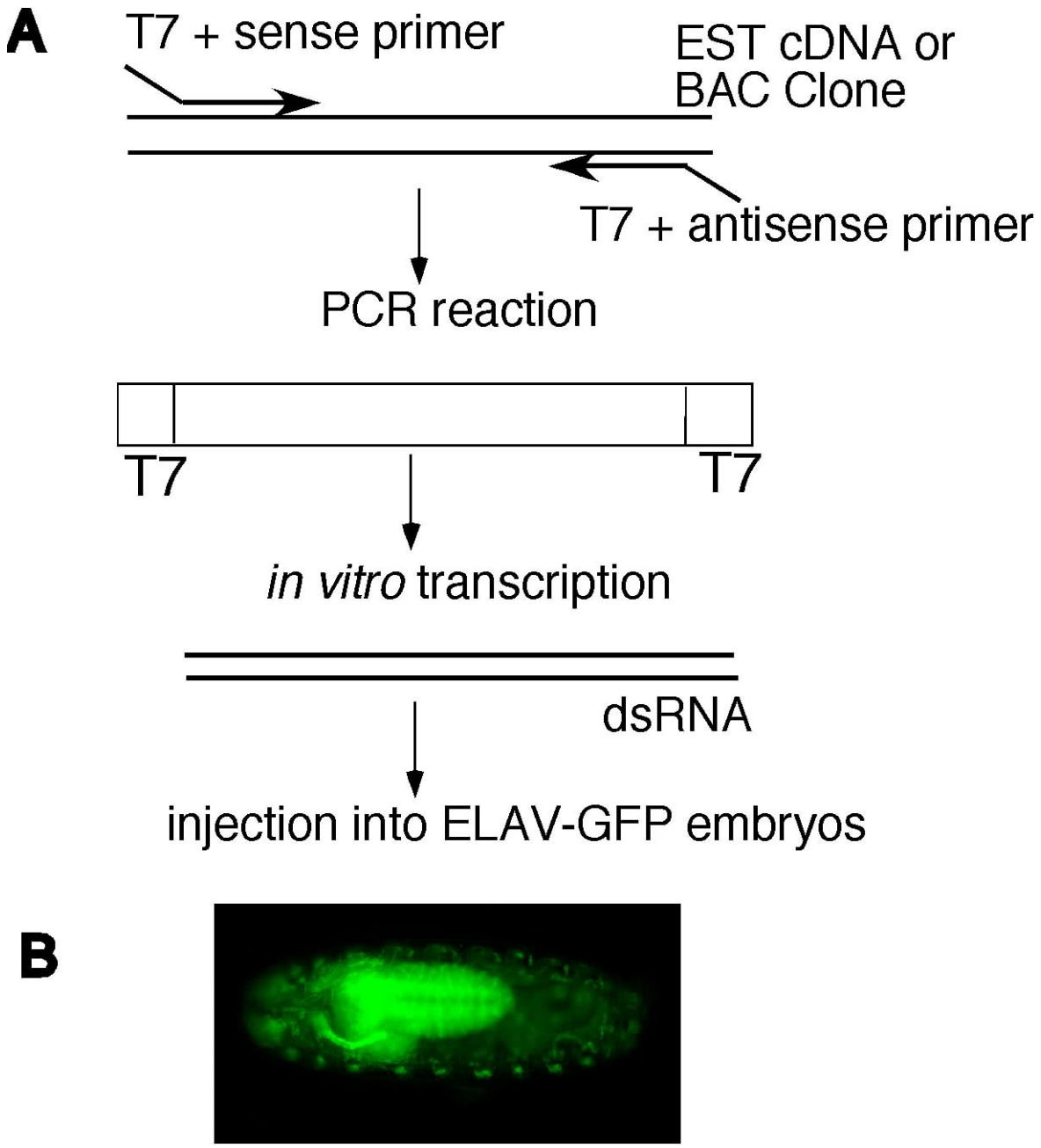

Figure 39. RNAi screening protocol.

(A) PCR primers specific for each protein kinase were designed with T7 promoter sequences, and used to produce PCR products from *Drosophila* genomic DNA (BAC clones) or cDNA (EST).PCR fragments served as template DNA for *in vitro* transcription reactions using T7 polymerase, to produce sense and anti-sense strands simultaneously. dsRNA products were purified and injected into embryos that expressed GFP under the control of an ELAV promoter. (B) Representative stage17 embryo showing GFP expression in the ventral nerve cord.

(Fig. 39B) (Lee and Luo, 1999). Embryos were injected with dsRNA, allowed to develop to embryonic stage 16-17, just prior to hatching, and visualized using epifluorescence to detect the presence of defects in the CNS or PNS. Moreover, viewing live embryos enabled assessment of lethality or the inability of embryos to progress beyond a particular stage of development that would have been impossible if embryos were fixed and stained. In order for a kinase gene to be considered a candidate, the injected embryos had to show disruption of the commissures or longitudinals of the ventral nerve cord or irregular patterning of the PNS such as an axon guidance defect or missing segmental neurons (Fig. 40A,B). In addition, the embryos had to have developed beyond dorsal closure (stage 15).

For actual phenotypes to be determined following injection with dsRNAs, it was necessary to complete injection buffer controls to ensure any observed phenotype was real and not an artifact of the injection procedure. At least 50% of the injection buffer control embryos had to look wild type to make any judgment on phenotypes observed in the experimental kinase dsRNA injections. A wild type CNS contains a distinct 'ladder-like' ventral nerve cord containing anterior and posterior commissures for each segment and longitudinal connectives on either side of the commissures along the A/P axis (Fig. 43C,D). The PNS contains axons that extend from the CNS towards the muscles and sensory axons extending from the sensory organ cell bodies to the CNS. Each individual segment has nerves and cell bodies confined within the segment and not crossing to adjacent segments.

Overall, an average of 62% of injection buffer controls survived and did not exhibit any phenotypes. In comparison, an average of 59% of the injected experimental embryos survived and showed no phenotype, an indirect, non-specific phenotype or a specific nervous system phenotype. Approximately 170-200 embryos were injected with each of the kinase dsRNAs and

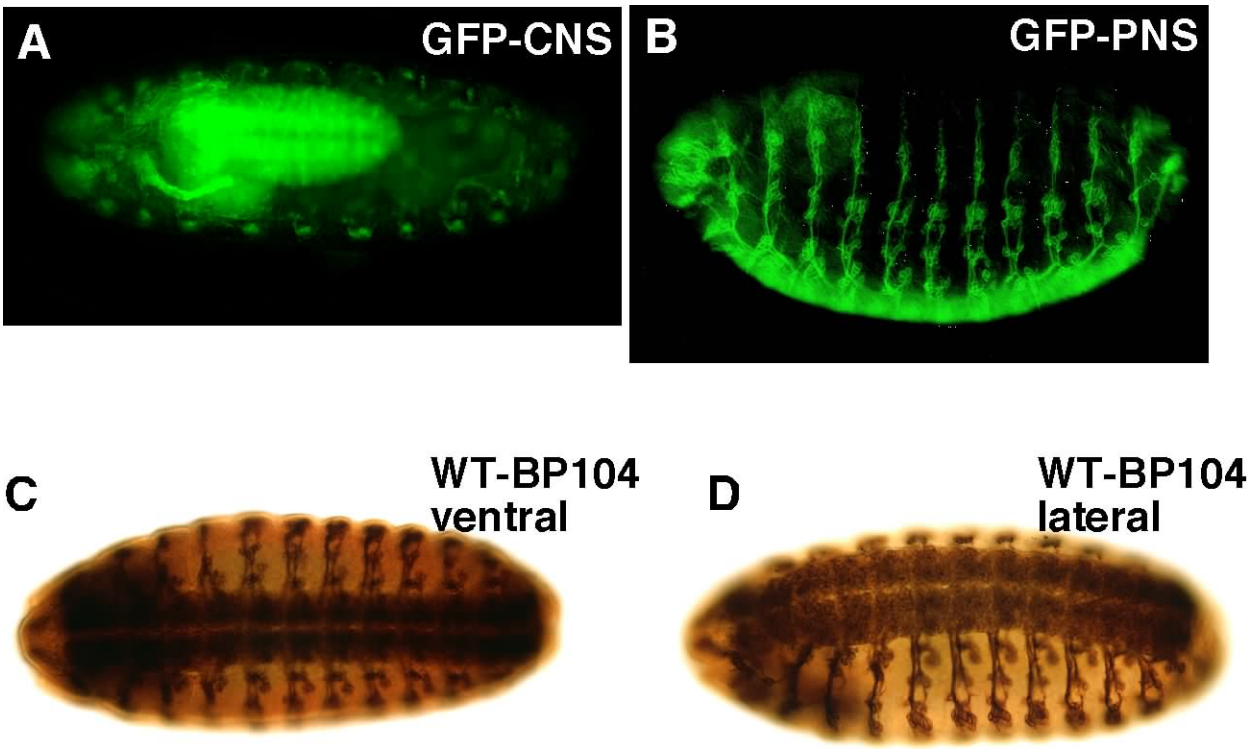

Figure 40. GFP and antibody stained control embryos.

(A and B) Embryos expressing GFP under the control of the ELAV promoter. An ELAV-GFP line of flies was utilized for initial screening of CNS and PNS defects, following injection of dsRNA corresponding to *Drosophila* kinase genes. (A) ventral view of stage 17 embryo showing ventral nerve cord and (B) lateral view showing PNS. (C and D) Embryos stained with BP104 antibody that recognizes a nervous system specific splice variant of neuroglian. (C) Ventral view of CNS (ventral nerve cord). (D) Lateral view of embryo showing continuous pattern of segmental neurons of the PNS within each segmental boundary.

~50% of the surviving embryos had to exhibit a nervous system phenotype to be considered as a candidate. Of the 54 kinase genes analyzed, 36 of the dsRNAs did not produce any nervous system phenotype upon visualization of injected embryos using epifluorescence (Tables 1,2).

Table 1. Overview of Drosophila Kinase RNAi Screen Results

Class of Phenotype	Number of kinase genes
Specific Nervous System Phenotype	5
Non Specific Nervous System Phenotype	13
No Nervous System Phenotype	36

Table 2. Kinase genes not producing a phenotype following injection

Gene Number	Gene Name
CG1362	Cdc2rk
CG1388	tak
CG2615	ik2
CG2621	sgg
CG2829	BcDNA:GH07910
CG2899	ksr
CG3051	SNF1A
CG3068	aur
CG3086	MAPk-2-Ak2
CG4224	CG4224
CG4268	Pitslre
CG4379	Pka-C1
CG4551	Smi35A
CG5125	ninaC
CG5179	Cdk9
CG5475	Mpk2
CG5680	bsk
CG6114	CG6114
CG6518	inaC
CG6551	fused
CG6620	ial
CG7001	Pkl17E
CG7177	CG7177
CG7719	greatwall
CG7826	mnb
CG9222	CG9222
CG9774	rok
CG11228	hippo
CG12306	polo
CG14992	Ack
CG15793	Dsor1
CG17161	grapes
CG17342	LK6
CG17998	Gprk
CG18069	CamKII
CG18247	shark

Injection of 13 kinase dsRNAs produced non-specific phenotypes (Table 3). For most of the kinase genes producing a non-specific phenotype, approximately 30% of the embryos exhibited a disorganized Central Nervous System (CNS), and in some cases a disorganized Peripheral Nervous System (PNS). However, these embryos also exhibited other developmental defects such as inability to complete germ band retraction or a failure to undergo dorsal closure, which did not allow them to progress to later stages of development. In some cases, the embryos had a disorganized CNS, but were also lethal (CG2049 and CG5072). These embryos did not develop beyond germ band extension, and were not considered as candidates. In addition, CG10033 showed a severe neurogenic phenotype (a majority of the epidermis was transformed into neuronal tissue) but the embryos also exhibited other morphological defects including the inability to progress past germ band retraction and this kinase was also not considered as a candidate. If earlier developmental stages were affected by knocking down levels of the kinase using RNAi, these kinases were not considered as candidates because any observed phenotype might have resulted from a defect in another developmental process that did not allow the embryos to progress beyond dorsal closure. In addition, we allowed these embryos to incubate longer to determine whether they would develop beyond dorsal closure, but they did not, so we classified these genes as producing an indirect, non-specific nervous system phenotype (Table 3).

Screening for kinases producing specific nervous system phenotypes resulted in five candidate genes including: Epidermal Growth Factor Receptor (Egfr, CG10079), Mushroom Bodies Tiny (mbt, CG18582), Cyclin-dependent kinase 2 (Cdc2, CG5363), C-terminal Src Kinase (Csk, 17309) and p21-activated kinase 3 (Pak3, CG14895) (Tables 1 and 3). Each of these candidate genes were further analyzed by re-injection of each dsRNA into W^{1118} embryos

Table 3. Kinase Genes producing Specific and Non-Specific Phenotypes following injection of dsRNA into embryos.

Gene Number	Gene Name	Phenotype	Phenotype Description
CG5363	Cdc2	Specific	CNS disorganized, PNS disorganized
CG10079	Egfr	Specific	Compressed CNS, PNS disorganized
CG14895	Pak3	Specific	PNS axon guidance defect
CG17309	Csk	Specific	Abnormal CNS, PNS defects
CG18582	Mbt	Specific	CNS somewhat abnormal, axon guidance defect
CG1210	Pk61C	Non-specific	Disorganized CNS, other developmental defects
CG1227	CG1227	Non-specific	Disorganized CNS, other developmental defects
CG1495	CamKI	Non-specific	Disorganized CNS, other developmental defects
CG2049	CG2049	Non-specific	Disorganized CNS, lethal
CG5072	Cdk4	Non-specific	Disorganized CNS, lethal
CG7186	SAK	Non-specific	Disorganized CNS, other developmental defects
CG8173	CG8173	Non-specific	CNS somewhat disorganized
CG8485	CG8485	Non-specific	Disorganized CNS, other developmental defects
CG8866	CG8866	Non-specific	Disorganized CNS, other developmental defects
CG10033	foraging	Non-specific	Neurogenic phenotype, other developmental defects
CG10579	Eip63E	Non-specific	Disorganized CNS, other developmental defects
CG14217	CG14217	Non-specific	Disorganized CNS, other developmental defects
CG16973	misshapen	Non-specific	Disorganized CNS,

and staining with monoclonal antibody (mAb) BP104 that recognizes a nervous system specific splice variant of neuroglian. Embryos were fixed at stages 15-17 post injection, stained and evaluated for phenotypes corresponding to those observed in live embryos using epifluorescence. In addition to injections with dsRNA to each of the five candidate genes, control embryos were injected with injection buffer and stained alongside the experimental embryos. Buffer control embryos had a distinct ventral nerve cord (CNS) with longitudinal connectives running from the anterior to the posterior of embryos and commissures situated perpendicular to the connectives forming the normal "ladder-like" structure of the ventral nerve cord. The PNS of each embryonic segment possessed continuous axons confined to their individual segments (Fig. 40C,D).

3.3.1 Epidermal Growth Factor Receptor (Egfr)

Injection of dsRNA corresponding to Egfr (CG10079) revealed a range of neuronal phenotypes of differing severity. Some embryos displayed a fusion of the longitudinal connectives into the midline resulting in a loss of distinct commissures between the longitudinal connectives and no clear midline (Fig. 41A,B,C). Other embryos contained a fusion of the longitudinal connectives as well as expansion of the CNS more laterally beyond the normal ventral nerve cord region (hyperplastic), encompassing a greater surface area of the embryo (Fig. 41C). Furthermore, some embryos contained a PNS that lost the clearly segmented nerves confined within an individual segment, being replaced by disorganized clumps of nerves (Fig. 41C). In addition, a few embryos that did not show a hyperplastic CNS contained axon guidance defects in the PNS, with an axon extending from segment 2-3 that was almost parallel with the ventral nerve cord (Fig. 41A).

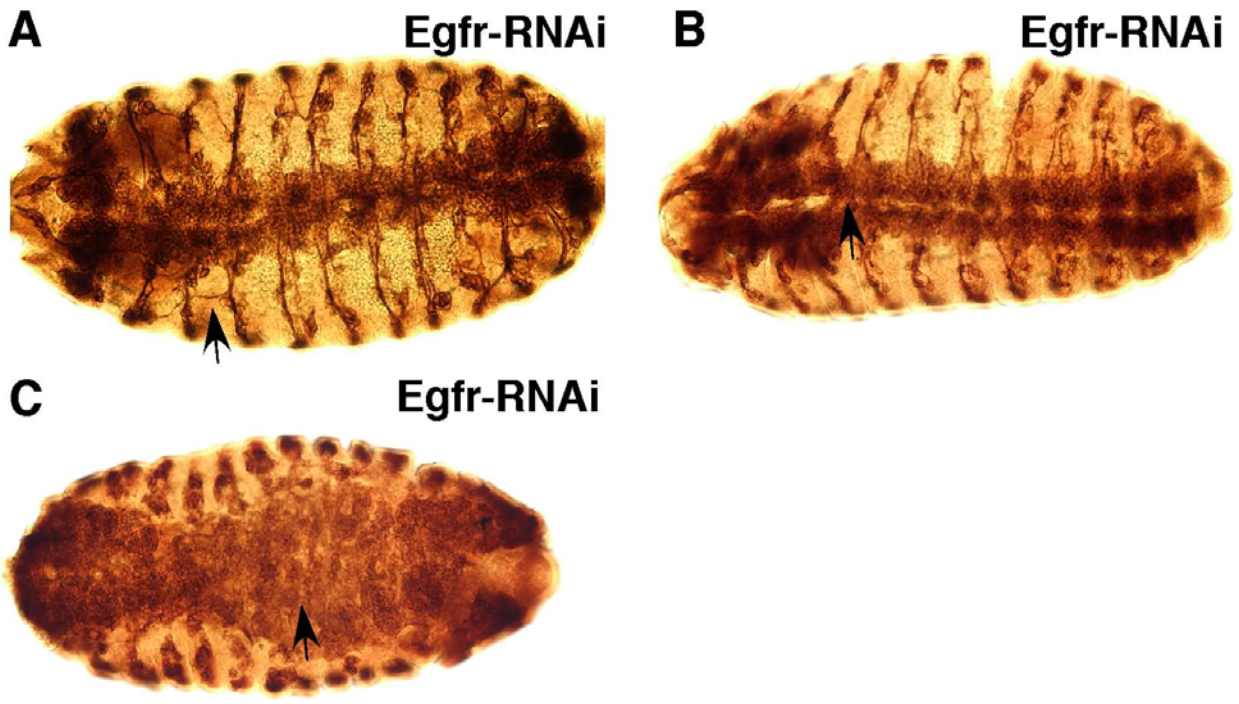

Figure 41. Phenotype of embryos injected with Epidermal growth factor receptor (Egfr) dsRNA.

Embryos were injected with Egfr dsRNA and stained with BP104 mAb. Embryos exhibit a range of phenotypes of differing severity. (A) Embryo has fusion of longitudinal connectives into the midline and loss of distinct commissures between the connectives. There is also an axon guidance defect between segmental neurons 2 and 3 (arrow). (B) Partial fusion of longitudinal connectives, but not as severe as in embryo (A). (C) Expansion of the ventral nerve cord into lateral regions of the embryo. Peripheral neurons are not continuous and form 'clumps' along each segment.

3.3.2 C-terminal Src Kinase (Csk)

C-terminal Src Kinase (Csk, CG17309), produced a phenotype with an abnormal CNS and PNS. The CNS appeared to have a gap along the midline and it has lost the commissures lying between the longitudinal connectives, thereby losing its "ladder-like" appearance (Fig. 42). In some embryos, the CNS appears segmented and almost continuous with the PNS, with breaks in the longitudinal connectives as well as loss of commissures positioned between the longitudinal connectives (Fig. 42). These embryos did not have distinct longitudinals extending continuously from the anterior to the posterior. Moreover, the axons of the PNS were confined within their individual segments, but they were not continuous along the segment with clumps of axons, then a space with no axons, then additional axons beyond the space (Fig. 42).

3.3.3 Cyclin-dependent kinase 2 (Cdc2)

Embryos injected with dsCdc2 (CG5363) lacked a clear "ladder-like" structure of the CNS and disorganized PNS. The ventral nerve cord had a wavy appearance compared to the wild type embryos (Fig.43A,B) In some embryos, the ventral nerve cord was pinched together in some regions, but other sections contained a space between the midline (Fig. 43B). In addition, the PNS was severely disorganized with several of the segments missing neurons completely or fusion of neurons from adjacent segments (Fig. 43A). For a majority of the embryos, it was not possible to distinguish the 10 distinct segments of peripheral nerves due to loss of segmental nerves or hypoplasia of the PNS neurons.

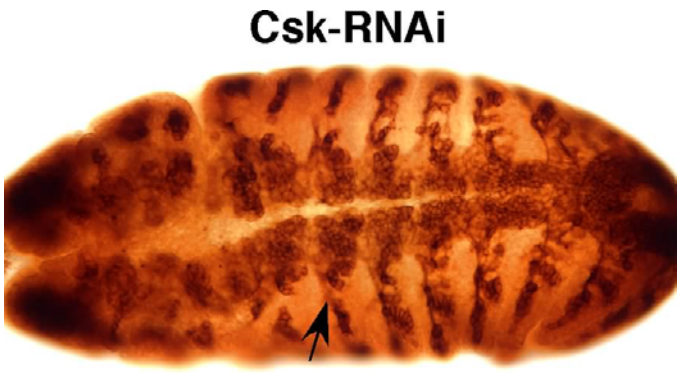

Figure 42. Phenotype of embryo injected with C-terminal Src Kinase (Csk) dsRNA.

Embryos were injected with Csk dsRNA and stained with BP104 mAb. and revealed phenotypes in the CNS and PNS. A separation exists along the midline of the embryo, and there are distinct gaps in the normally continuous A/P longitudinal connectives. Commisures are missing between the longitudinal connectives. The peripheral neurons appear to be continuous with the those of the CNS and form clumps rather than distinct individual neurons (arrow).

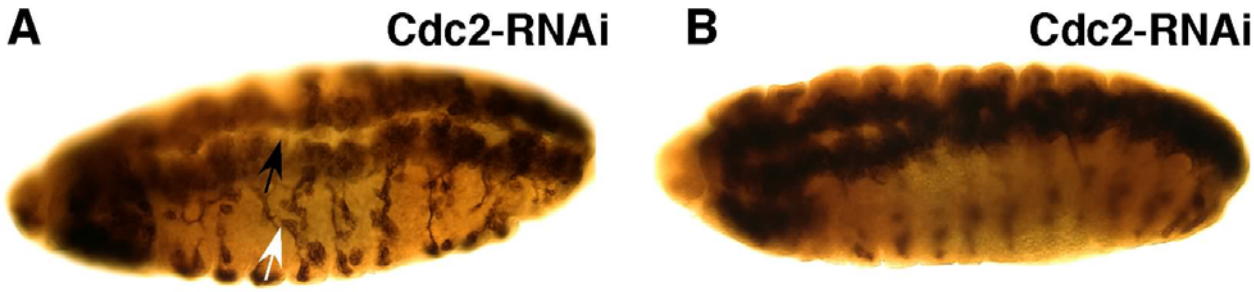

Figure 43. Phenotype of embryos injected with Cyclin-dependent kinase 2 dsRNA.

Embryos were injected with Cdc2 dsRNA and stained with BP104 mAb. (A) The embryo lacks a clear "ladder-like" structure of the normal CNS and contains an extremely disorganized PNS. Ventral nerve cord has a wavy appearance (black arrow). Many of the peripheral neurons are missing and/or are fused with those from adjacent segments (white arrow). (B) Ventral nerve cord is pinched together in some regions, but not in others.

3.3.4 Mushroom Bodies Tiny (mbt)

Injection of dsRNA corresponding to Mushroom Bodies Tiny (mbt, CG18582) resulted in an abnormal CNS as well as an axon guidance defect in the PNS. The commissures of the CNS are not distinct or are missing when compared to injection buffer control embryos that contain a typical ladder like structure. Interestingly, there was a subtle phenotype in the PNS corresponding to an axon guidance defect, where an axon from segment A2 crosses over and connects to neurons contained in segment A3 (Fig. 44A,B,C). PNS guidance in other segments appeared normal.

3.3.5 p21-activated kinase 3

Finally, injection of Pak3 (CG14895) also revealed a very subtle, but interesting phenotype of an axon guidance defect (Fig. 45A-D). Axons from one segment crossed over the segmental border and joined with neurons from an adjacent segment. There was some variation of the segments affected, but approximately 80% of the embryos exhibited crossing over from segment A1 to segment A2. The CNS appears to be normal with commissures between the longitudinal connectives. The validity of the Pak3 RNAi axon guidance defect was further demonstrated using a Pak3 loss of function mutant line (EF1191), containing a P-element insertion in the Pak3 gene. Staining of EF1191 embryos with mAb BP104 revealed an axon guidance defect consistent with the RNAi phenotype (Fig. 45E).

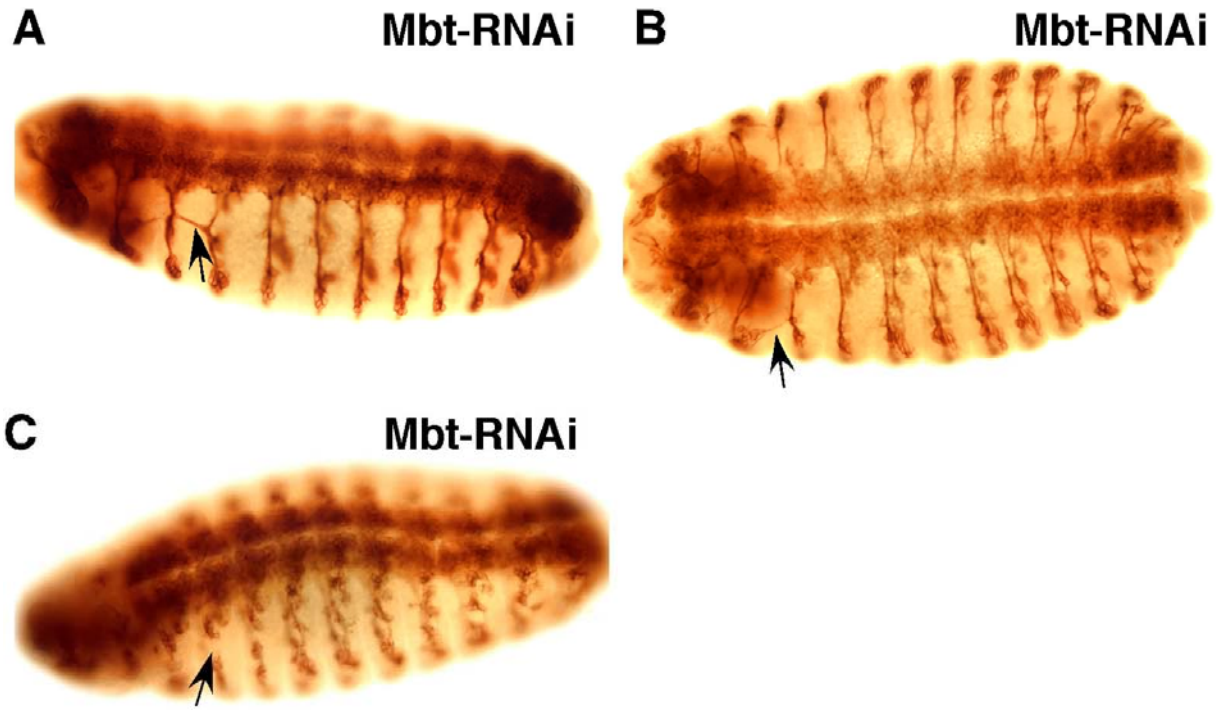

Figure 44. Phenotype of embryos injected with Mushroom Bodies Tiny (mbt) dsRNA.

Embryos were injected with mbt dsRNA and stained with BP104 mAb. (A) Embryo shows a peripheral axon guidance defect between segments 2 and 3 (arrow). (B) This embryo also has a peripheral axon guidance defect, but it is between segments 1 and 2 (arrow). (C) Some of the peripheral nerves are not continuous within the segment (arrow), leaving a gap.

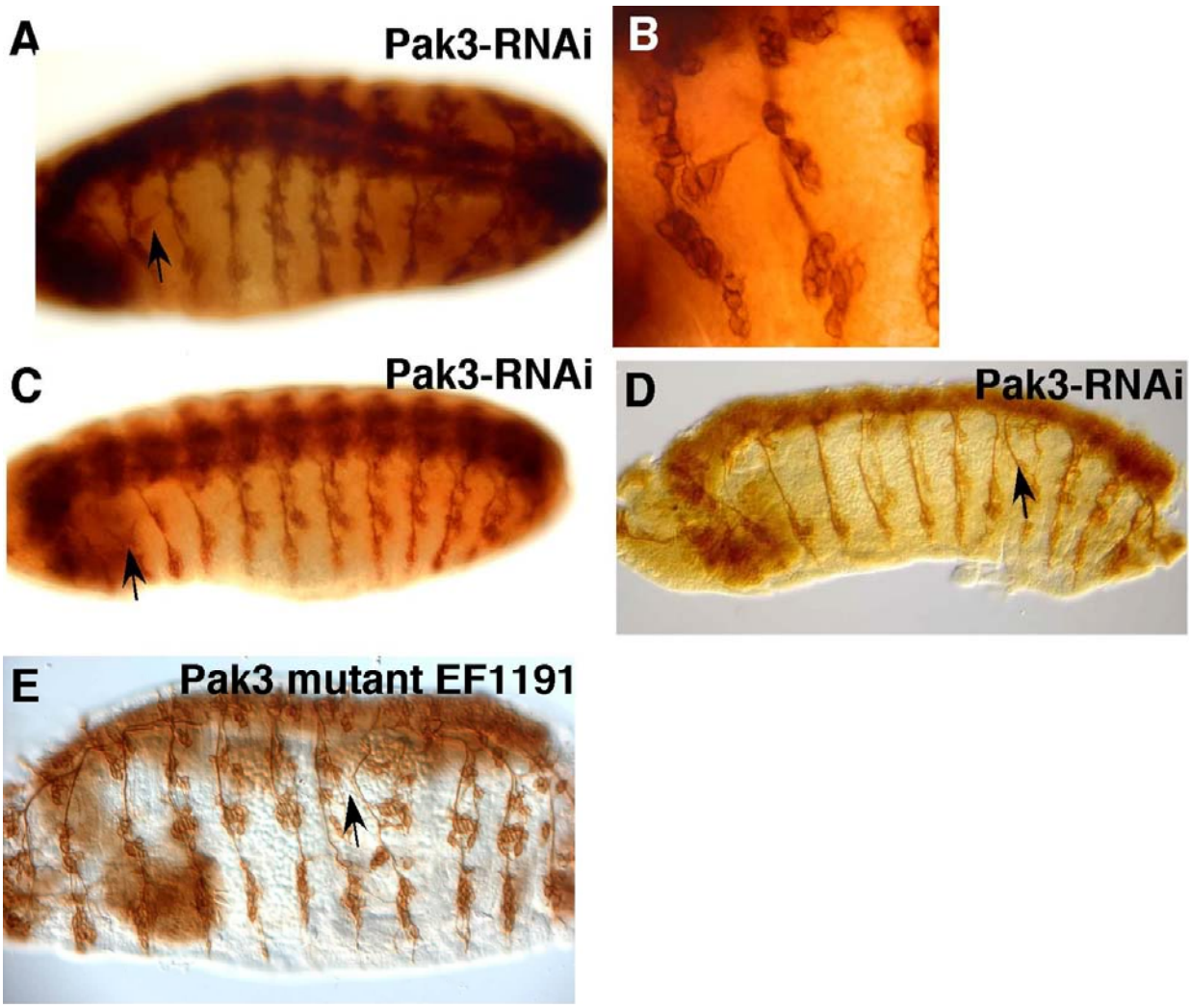

Figure 45. Phenotype of embryos injected with p21-activated kinase3 (Pak3) dsRNA.

Embryos were injected with Pak3 dsRNA and stained with BP104 mAb. (A) Axon guidance defect is present between segments 2 and 3 (arrow). There is also an axon guidance defect between segments 8 and 9. (B) 10X magnification of defect in A. (C) Another embryo showing an axon guidance defect between segments 2 and 3. (D) Axon guidance defect between segments 7 and 8. (E) Pak3 mutant containing a P-element insertion in the Pak3 gene. Pak3 mutant embryo exhibits an axon guidance defect similar to the RNAi phenotype.

3.4 Discussion

Analysis of 54 Drosophila kinase genes using RNAi produced specific nervous system phenotypes for five of the genes: Egfr (CG10079), Csk (CG17309), Cdc2 (CG5363), Mbt (CG18582) and Pak3 (CG14895). Indirect, non-specific phenotypes were observed with injection of dsRNA corresponding to 13 kinase genes and injection of dsRNAs to 36 kinase genes did not produce any nervous system phenotype (Table 1).

3.4.1 Kinases affecting Drosophila Nervous System Development

3.4.1.1 Epidermal Growth Factor Receptor (Egfr)

RNAi silencing of Egfr (CG10079) resulted in a nervous system phenotype with a distinct loss of commissure tracks across the longitudinal connectives along the length of the ventral nerve cord leading to a fusion of the midline (Fig. 41A,B,C). Moreover, the CNS was extremely disorganized and extended laterally beyond the normal region of the ventral nerve cord. In addition, the normally segmented neurons of the PNS were replaced by clumps of random neurons (Fig. 41C). Identification of a nervous system phenotype following injection of dsEgfr was encouraging, as numerous studies have implicated Egfr in nervous system development. To begin with, an Ethyl methanesulphonate (EMS) screen for mutations affecting commissure formation in the CNS of the embryo resulted in eleven different Egfr alleles (Hummel et al., 1999). It was also determined that excess Egfr signaling can override lateral inhibition in the proneural cluster, enabling adjacent cells to become Sensory Organ Mother Cell (SMC's) and sensory organs (Culi et al., 2001). In addition, Egfr signaling is critical for

interactions in the early ectoderm, the region where neuroblasts and glial cell lineages delaminate, to determine fates of neuronal and glial cell lineages (Scholz et al., 1997). At later stages of embryonic development, Egfr function is required in the midline glial cells (MG) and in its absence, the MG cells fail to differentiate or die producing a fusion between the commissures (Scholz et al., 1997). Egfr signaling induces formation, patterning and fate specification of early forming neuroblasts along the dorsal-ventral axis of the embryo (Skeath, 1998). Furthermore, Egfr signaling helps specify the fate of medial neuroblasts and promotes neuroblast formation in the intermediate column (Skeath, 1998). Egfr signaling plays an instructive role in CNS patterning and exerts differential effects on dorsal-ventral subpopulations of neuroblasts (Udolph et al., 1998). In addition to the role of Egfr in nervous system development, this kinase also plays roles in many other developmental processes. Overall, the RNAi phenotypes observed in the present study appears to be consistent with the role of Egfr in nervous system development reported previously.

3.4.1.2 C-terminal Src Kinase

The RNAi induced phenotype of Csk (CG17309) results in both abnormal CNS and PNS. There is a large separation between the longitudinal connectives of the ventral nerve cord on the ventral surface of the embryo (Fig. 42). Furthermore, the commissures between the two longitudinal connectives are completely missing, with a loss of the normal “ladder-like” appearance of the CNS. Abnormalities are also present in the PNS of the Csk RNAi embryos. The axons of the PNS are not continuous along their individual segments, but rather there are breaks of continuity within the segment. Studies in mice demonstrated that animals homozygous for a knockout of the Csk gene were embryonic lethal at mid gestation and exhibited neural tube

defects including a failure to close their cephalic folds, a wavy and disorganized neural tube (Imamoto and Soriano, 1993). Moreover, histological sections at the optic vesicle revealed cephalic folds that were interrupted by a layer of neuroectodermal cells (Imamoto and Soriano, 1993). In rats, Csk is expressed at high levels in the CNS throughout embryonic development and the levels are reduced in the adult brain (Kuo et al., 1997). In *Drosophila*, Csk has been implicated in regulation of cell numbers by inhibiting cell proliferation (Pedraza et al., 2004). Additional analysis is required to further explore the nature of this phenotype.

3.4.1.3 Cyclin-dependent kinase 2 (Cdc2)

A nervous system specific phenotype was also observed when dsRNA corresponding to Cdc2 (CG5363) was injected into embryos. The ventral nerve cord had a wavy appearance and lacked its normal "ladder-like" appearance compared to wild type embryos (Fig. 43A,B). Some of the embryos exhibited a fusion of the ventral nerve cord along the midline (Fig. 43B). In addition to the abnormal structure of the CNS, the PNS was also abnormal. In some of the segments, the neurons were completely missing, in others they were fused with the neurons from adjacent segments (Fig. 43A). It was not possible to determine exactly which neurons were missing with the antibody used, and additional antibodies would have to be used to identify if sensory or motor neurons were affected following RNAi. The observed RNAi phenotype is validated by more recent studies in which Cdc2 has been linked to neural progenitor asymmetric divisions (Tio et al., 2001). Segregation of cell fate determinants into one of the two daughter cells mediates asymmetric cell divisions (Tio et al., 2001). Asymmetric division in *Drosophila* neuroblasts and progeny ganglion mother cells (GMCs) are mediated by preferential localization of cell fate determinants to either the apical or basal side of the cortical complex (Tio et al.,

2001). Improper functioning of Cdc2 results in abnormal asymmetric divisions due to the improper localization of asymmetric components during mitosis and the failure of daughter cells to acquire the correct fates (Tio et al., 2001). Therefore, when levels of Cdc2 are knocked down using RNAi, the process of asymmetric divisions may be compromised leading to the observed phenotypes. It has been demonstrated that Par/aPKC complex is required for proper asymmetrical division and mutation in components of the complex led to mislocalization of fate determinants, Prospero and Numb (Kuchinke et al., 1998; Petronczki and Knoblich, 2001). Therefore, antibody staining using anti-Prospero or anti-Numb antibodies could be completed in embryos following injection with Cdc2 dsRNA to determine localization of these proteins and whether they are properly localized.

3.4.1.4 Mushroom Bodies Tiny

Mushroom bodies tiny (Mbt, CG18582) shares homology with Pak1 related kinase in humans, p21-activated kinase 3 in rats and Cdc42/Rac effector kinase in mice. Mbt is a group II PAK, containing a C-terminal kinase domain and an N-terminal binding domain for Rho family of small GTPases (p21-binding domain, PBD), but lacks a C-terminal auto-inhibitory domain (AID) typical of group I PAKs (Dan et al., 2002; Jaffer and Chernoff, 2002; Pirone et al., 2001). RNAi with *mbt* resulted in a distinct axon guidance defect in the peripheral nervous system of injected embryos (Fig. 44A,B). In addition, there were peripheral nerves missing in some embryos (Fig 44C). Mutations in the *mbt* gene perturb the survival or generation of Kenyon cells in the adult mushroom body therefore implicating Mbt's involvement in adult CNS neurogenesis (Melzig et al., 1998). Moreover, photoreceptor cells in the eye or neurons in the brain are frequently missing in *mbt* mutants suggesting a function of Mbt in cell proliferation,

differentiation, or survival (Schneeberger and Raabe, 2003). In contrast, *mbt* mutants in other studies did not exhibit axon guidance defects (Hing et al., 1999; Melzig et al., 1998). It is interesting to have such a contradiction between *mbt* mutant studies and the RNAi phenotype showing an axon guidance defect. One possible explanation for such a discrepancy is the analysis in the embryo following injection of *mbt* dsRNA compared to analysis in adults, as *mbt* may have different functions in nervous system development at different developmental stages. However, it is not known whether all the neurons have obtained their proper fate or survived in the embryos following RNAi, and this possibility would also have to be considered.

3.4.1.5 p21-activated kinase 3

The final candidate is p21-activated kinase 3 (Pak3, CG14895). Pak3 is a group I Pak, sharing homology with other group I Pak kinases including mammalian Pak1-3 and *Drosophila* Pak1 (Dan et al., 2001; Jaffer and Chernoff, 2002; Pirone et al., 2001). Group I Pak kinases, contain an auto-inhibitory domain (AID) which negatively regulates its activity via interaction with the kinase domain. Function of the AID is abolished upon binding by activated Cdc42 or Rac, resulting in autophosphorylation and activation of the kinase domain (Jaffer and Chernoff, 2002).

Pak3 injected embryos exhibited a very subtle, but interesting axon guidance defect phenotype. The peripheral axons from one segment crossed over into another segment, connecting with neurons of the adjacent segment (Fig 45A-D). The CNS of injected embryos was normal and produced the typical ladder-like structure of commissures as “rungs” along the longitudinal connectives of the ventral nerve cord. Since injection of Pak3 dsRNA produced an axon guidance defect and Pak1 has been implicated in axon guidance, it was necessary to

confirm the observed phenotype using dsRNA to a different region of the Pak3 gene. Injection of the alternate Pak3 dsRNA produced an identical phenotype to the original Pak3 dsRNA. In mice, transcripts for both Pak1 and Pak3 are almost exclusively expressed in the brain and spinal cord but not in other tissues tested (Burbelo et al., 1999). These findings propose a role for both PAK proteins in control of signaling or cellular architecture in the mouse CNS. Furthermore, Pak1 acts as a downstream effector of Rac in the process of dendrite initiation in cortical neurons (Hayashi et al., 2002). Studies in *Drosophila* suggest Pak1 acts downstream of Dock (an SH2/SH3 adaptor protein) and Trio (a guanine nucleotide exchange factor) in a pathway regulating R cell axon guidance to spatially restricted domains within growth cones and control direction of axon extension (Hing et al., 1999; Newsome et al., 2000). The axon guidance defect observed following RNAi with Pak3 appears to be in line with the previously described function of Pak 1 as a downstream effector of Cdc42/Rac signaling to regulate R cell axon guidance. Further verification of the Pak3 RNAi axon guidance defect was demonstrated using a Pak3 mutant fly line (EF1191), containing a P-element insertion in the Pak3 gene. Staining of EF1191 embryos with mAb BP104 revealed an axon guidance defect consistent with the RNAi phenotype (Fig. 45E).

3.4.2 Comparison of results to another RNAi screen.

A recent paper by Ivanov et al., 2004 described screening of 25% of the Drosophila genome using RNAi to identify genes required for nervous system development (Ivanov et al., 2004). The results describe the identification of two kinase genes, greatwall (gwl, CG7719) and *Sak* (CG7186), as having nervous system phenotypes (Ivanov et al., 2004). Greatwall plays a role in motor axon guidance and synaptogenesis in larvae (Kraut et al., 2001), whereas injection

of dsRNA into embryos resulted in an absence of commissures and disruption of longitudinal connectives (Ivanov et al., 2004). In comparison, dsRNA corresponding to *gwl* (CG7719) did not produce a phenotype in the present study (Table 2). It is not clear why this discrepancy occurred, but one possible explanation could be cross-reactivity of the dsRNA with another kinase gene. Synthesis of dsRNA in the Ivanov study used general primer sequences corresponding to the vectors containing the cDNA, rather than primers designed specifically for each kinase gene. Therefore, the dsRNA product may have corresponded to the kinase domain and was not specific for *gwl*. Based on the results obtained by Ivanov et al., it would be necessary to determine the sequence of the dsRNA they used to verify its specificity, as well as to reevaluate our own result for the *gwl* gene. Additionally, Ivanov et al. observed varying mutant phenotypes following injection of *Sak* (CG7186) ranging from early developmental defects to more specific nervous system phenotypes including reduced numbers of neurons, lack of longitudinal connectives and abnormal ventral nerve cord (Ivanov et al., 2004). These findings are consistent with those observed in our study, but we eliminated this candidate based on observing earlier developmental defects.

Overall, the screen successfully identified five candidate kinase genes involved in nervous system development. Among the five candidates was Egfr, whose involvement in nervous system development has been verified by numerous studies. Moreover, Cdc2, Csk, Mbt and Pak3 or their homologs have all been shown to be involved in some process relating to nervous system development in other organisms. Further studies will be required to determine the exact role each of these kinase genes plays in the development of the nervous system in *Drosophila*.

3.5 Future Studies

Since the role of Egfr has been studied in *Drosophila* nervous system development, it would not be logical to pursue additional studies on this candidate. However, additional studies would be completed on the other four candidates, with particular focus on the role of Pak3 in axon guidance. To determine the expression of each of the four candidates, *in situ* hybridization would be completed on stage 14-17 embryos. The expression would be analyzed to ensure that the genes are highly expressed in the CNS or PNS. In addition, antibody staining would be completed with various markers for cells in both the CNS and PNS following injection of dsRNA to Cdc2 and Csk. For example, mAb BP102 stains the anterior and posterior commissures and longitudinal connectives of the ventral nerve cord. It would be interesting to examine the extent of disruption in the nerves of the CNS due to the RNAi phenotype visualized using BP104 that stains both CNS and PNS. Further analysis would utilize mAb 22C10 that stains PNS neurons (sensory) (Zipursky et al., 1984), and a subset of neurons in the ventral nerve cord as well as mAb 1D4 (anti-FasII) (Vactor et al., 1993) to stain motor neurons and their axons. Staining using mAb 22C10 and mAb 1D4 would be completed on embryos following injection with dsRNA to *mbt* and *Pak3* to determine whether sensory or motor neuron axons are exhibiting the observed axon guidance defect.

As stated above Pak3 would be the focus of further analysis, so the remaining experiments would be completed exclusively on Pak3. *Drosophila* Deficiency lines spanning the region of the *Pak3* gene would be analyzed to determine if each line displays a similar phenotype to RNAi embryos. The *Pak3* mutant fly line (EF1191), containing a P-element insertion in *Pak3* would be analyzed to determine the exact insertion site of the P-element, and attempts could be made to rescue the axon guidance defect phenotype by precise excision of the P-element. Since

a P-element exists in Pak3, P-element mobilization may generate additional alleles and possibly produce more severe phenotypes.

Attempts to determine the function of a particular kinase in a signal transduction pathway controlling a specific process during development requires an understanding of what controls the activity of the kinase as well as identification of downstream targets of the kinase. A genetic screen to identify genes that function upstream and targets downstream could be completed.

3.6 Materials and Methods

3.6.1 Identification of *Drosophila* Kinases and Primer Design

A FlyBlast Search was used to identify *Drosophila* kinases based on homology with protein kinase catalytic core (dPar1) and those defined by Morrison et. al. (Morrison et al., 2000). Each primer pair was designed to span exon regions ~400-900 bp, not including the kinase domain, but this was not possible in all cases due to small exon size in some genes or absence of cDNA for the kinase. If possible, the region chosen would be adjacent to the kinase domain in the same exon. FlyBase and Berkley Drosophila Genome Project (BDGP) databases were utilized for EST and genomic sequence data. Primer sequences were selected using Primer 3 program (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) to be 20-24 nt in length with a $T_{M.}$ of 60°C. The 27 nt T7 promoter sequence TAATACGACTCACTATAGGGAGACCAC was added to the 5′ end of each primer. A complete list of kinase genes, corresponding primer pairs, source of template DNA and size of PCR product/dsRNA can be found in Appendix B. The average length of dsRNA is 478 bp.

3.6.2 Synthesis of dsRNA

PCR was used to amplify region of genomic or cDNA for each gene and to add the T7 promoter sequence. PCR products were analyzed by 1.5% agarose gel electrophoresis to verify the products were the correct sizes and purified using Qiagen QIAquick PCR Purification Kit (Catalog #28104). Concentration of products was determined using a Spectrophotometer. Sense and antisense RNA was synthesized simultaneously using 1 μg of PCR template DNA and T7 RNA Polymerase (NEB) and incubated 1 hour 30 minutes at 37°C. Annealing of sense and antisense strands occurs during the synthesis reaction. Following synthesis, template DNA was removed using RNase Free DNase (RQ-1, Promega) for 20 min at 37°C. RNA products were Phenol-chloroform extracted and precipitated with ethanol and NH_4OAc. dsRNA was dissolved in TE buffer and concentration determined by A_{260}/A_{280} using a spectrophotometer. 5 μg of dsRNA was analyzed on 1.5% agarose gel to ensure RNA migrates as a single band. Aliquots of dsRNA were stored as NaOAc/EtOH precipitate at -80°C until use.

3.6.3 Preparation of dsRNA and injection apparatus

Aliquots of dsRNA were spun for 10 min at 14,000 rpm, dissolved precipitate in injection buffer (0.1 mM NaPhosphate pH 7.8, 5 mM KCl) (Rubin and Spradling, 1982) to a final concentration of 5 μM. Injection needles are borosilicate capillaries (World Precision Instruments TWF100-4) that were baked prior to use to remove RNases. Needles were pulled using Narishge needle puller Model PP830. Needles were sharpened against the edge of a slide at a 45° angle to the needle, enabling the fused tip to be opened allowing liquid flow. Embryos were injected with ~85pL (range 65-110pL) of volume corresponding to ~0.2 fmoles of dsRNA

per embryo using a pneumatic picopump (Picopump-World Precision Instruments). Site of injection corresponded to the lateral side of the embryo, random from anterior to posterior.

3.6.4 Injections for screening

A line of flies expressing GFP under the control of a NS promoter ELAV (Lee and Luo, 1999) were used during the initial screening process so live animals could be visualized using epifluorescence on a Nikon E800 microscope.

Eggs were collected on agar molasses plates for 45 min at 25°C, dechorionated for 2 minutes in 50% Bleach, aligned on coverslips containing tape glue (double stick tape ~5cm in 1ml heptane), desiccated for 12-15 minutes in a sealed container containing Drierite stones (W.A. Hammond Drierite Company, Ltd), then covered with halocarbon oil (Halocarbon Products, Inc). Syncytial blastoderm embryos were injected and older embryos were destroyed. Injected embryos were incubated at 18°C for 2 days (stage 16-17) in a humidity chamber and analyzed for nervous system phenotypes using epifluorescence.

3.6.5 Immunohistochemical analysis of potential candidates

Any potential candidate dsRNAs were reinjected into W^{1118} embryos and analyzed using mAb BP104 (Developmental Studies Hybridoma Bank) that recognized a nervous system specific splice variant of neuroglian. Follow same protocol as indicated above, but embryos were incubated under halocarbon oil until stage 15-16. Prior to fixing embryos, excess oil was removed from around the embryos using a razor blade. The embryos were removed from the tape glue using heptane, allowing the loose embryos to collect in 60mm Petri dish with heptane and transferred to a glass vial of fix solution (10:3:7 (v/v) n-heptane/37% formaldehyde/PEM-

vortexed to ensure saturation) using a P-1000 with a cut tip, Embryos were fixed at room temperature for 30 minutes, then were removed from fix/heptane interface using P-1000 with a cut tip, and placed onto fine mesh basket stuffed with Kim wipes allowing the embryos to remain on the mesh. Embryos were gently picked using double stick tape and placed embryo side up into a 60mm Petri dish, then covered with PBS. Embryos were manually devitellinized using a needle to nudge the embryos from the membrane and transferred to PBS + 0.1% Triton X-100 (PBT).

3.6.6 Antibody Staining

Embryos were blocked in PBT + 10% goat serum for 20 minutes. Blocking agent was removed, and primary antibody mAb BP104 added at a 1:3 dilution in PBT + 5% goat serum. Embryos were incubated in primary antibody overnight at 4°C. Primary Ab was removed from embryos, and they were washed in PBT, then a biotinylated secondary Ab goat αmouse IgG (Vector Laboratories) was added at a 1:200 dilution in PBT + 5% goat serum, and incubated for 30 min at room temperature. Secondary antibody was removed, embryos were washed, and Avidin/Biotin Complex (AB Complex) that was preformed on ice for 30 minutes was added to embryos. Embryos were incubated with AB complex for 15 minutes at room temperature, and then washed with PBT. Staining was completed using 0.5 mg/ml diaminobenzidine (DAB) + 0.003% H_2O_2 in PBS. Staining reaction was stopped with PBS. Embryos were equilibrated in a glycerol series to a final 85% glycerol/PBS concentration. Embryos were mounted and viewed using Nikon microscope E800.

APPENDIX A

Primers used to generate UAS-brk constructs

construct	Primer Name	Primer sequence
brk53	M13 Forward	gtaaaacgacggccagt
	brk53	gatcgaattcATTGTTGGCCACCGATGA
brkS	M13 Forward	gtaaaacgacggccagt
	brkS	GATCGAATTCTGGGAGTAGTTGCTGCTG
brkNLS	M13 Forward	gtaaaacgacggccagt
	brkNLS	GATCGGATCCGACCTTCCTCTTTTTCTTCGGGGGCTGTTGCTGATTGTTGGC
brkNLSW	M13 Forward	gtaaaacgacggccagt
	brkNLSW	gatcgaattcccatggtcgccaGGATCCGACCTTCCTCTT
brkStop1	M13 Forward	gtaaaacgacggccagt
	brkStop1	gatcgaattcGGCTGGTGTGGCAACCGC
brkA	M13 Forward	gtaaaacgacggccagt
	brkA1	gatcggatccGTGTGGAACCATGCCATT
brkA2	M13 Forward	gtaaaacgacggccagt
	brkA6	gatcagatctGTGATGATGATGGGCGGC
brkC	M13 Forward	gtaaaacgacggccagt
	brkC	gatcgaattcCAGCTGATGGAACACCTT
brkStop1DA17	brkA7	gatcagatctCACCATCATGCCGCCCAC
	brkA6	gatcagatctGTGATGATGATGGGCGGC
brkStop1NA	brkA2	gatcagatctAATGGCATGGTTCCACAC
	brkA3	gatcagatctCAACTGGTGCACCAGCGG
brkEA	brkEiFbgl	gatcagatctCACGCCGCCGCGGTGGGT
	brkstop1B(bgl)	gatcagatctGGCTGGTGTGGCAACCGC
brkEC	brkE1Fbgl	gatcagatctCACGCCGCCGCGGTGGGT
	brkE1Rbgl	gatcagatctCTCCTTCTGATGCTGCAT
brkED	brkE2Fbgl	gatcagatctGCAGCCAATGGCATGGTT

	brkstop1B(bgl)	gatcagatctGGCTGGTGTGGCAACCGC
brkEF	brkE3Fbgl	gatcagatctGAGACACCTGCAACCATT
	brkstop1B(bgl)	gatcagatctGGCTGGTGTGGCAACCGC
brkStop1NAC	M13 Forward	gtaaaacgacggccagt
	brkStop1Ct	gatcgaattcGGATCCGAGAGAGAGGTC
brkCM	brkMA2	gagctagcggccgcTCTGGCTTCCTCTGCCCGCCGTCAA
	brkMA1	gagctaagcggccgcCATTGCGGCTGGTGTGGCAAGCGC
brkGM	brkGro1	agctaagcggccgcAAATAGCTTGACCTGTTT
	brkGro2	agctagcggccgcTTTGCTGGACGATGATGAG
brkGD	brkMB2	agctgcatcgatGAGGAGCAGGATCATCAT
	brkMB1	agctgcatcgatCAGCTTGTGCTCCTCCGG
brkCMGM	see CM/GM	
brkNA	brkA2	gatcagatctAATGGCATGGTTCCACAC
	brkA3	gatcagatctCAACTGGTGCACCAGCGG
brkNACM	brkA2	gatcagatctAATGGCATGGTTCCACAC
	brkA3	gatcagatctCAACTGGTGCACCAGCGG
brkNAGM	brkA2	gatcagatctAATGGCATGGTTCCACAC
	brkA3	gatcagatctCAACTGGTGCACCAGCGG
brk3M	brkA2	gatcagatctAATGGCATGGTTCCACAC
	brkA3	gatcagatctCAACTGGTGCACCAGCGG
brkS2NACM1	M13 Forward	gtaaaacgacggccagt
	brkStop5	gatcgaattcTTGACGGCGGGCAGAGGA
brkS2NACM2	M13 Forward	gtaaaacgacggccagt
	brkStop6	gatcgaattcCTCCAGCTTACTAATCTT
brkS2NACM3	M13 Forward	gtaaaacgacggccagt
	brkStop7	gatcgaattcCTCCACGTCCACCTCCAC
brkNLS4R	brk4RA	gatcagatctGGATCCTCTGCCCGCCGT
	brkStop2E	gatcagatctCAGCTTGTGCTCCTCCGG
brkD4R	brk4RDA	gatcagatctGAGAGAGAGGTCCATGGG

	brk4RDB	gatcagatctCCCTCTAAACAGGTCAAG
brk4M	brk4MN1	gatcgctagcGAGGAGCAGGATCATCAT
	brkStop1N	gatcgctagcGGCTGGTGTGGCAACCGC

APPENDIX B

Primer sequences, PCR product sizes and template used to generate dsRNA products

Gene	Gene Name	Primer Left	Primer Right	PCR (bp)	PCR template
CG1210	Pk61C	agcaactccgatgaagacgat	ccaacgcctaattctgcacta	653	BACR48E09
CG1227	CG1227	aattcaccctacgatcccatc	ctacaagcgaccctccagttt	216	GH27612
CG1362	Cdc2rk	gagatagcacagctggacatga	cttgcttttcaggcactcttc	234	SD02166
CG1388	tak	ccacacagttaacaccgacaac	gacagatccgtggtcatcatc	500	SD06739
CG1495	CaMKI	atggaaaagctgtcgatgtgt	ctactgcgatgctgcatttgt	557	GH14309
CG2049	Pkn	ttataccacagttggggaagc	gtctcgagtcctccagattca	346	BACR10M14
CG2615	ik2	cgagaatggaccaattgaatg	tgcatctgcgagatcacatc	774	SD10041
CG2621	shaggy (sgg)	gatcaggcggaactcacagt	ggactgtgttctggccaattt	768	BACR30B01
CG2829	BcDNA:GH07910	ccttcggtcacaatcagtcg	ttcggtatcggtggctgaat	202	LD38852
CG2899	ksr	gaaaggatccccttggtaaa	agtctccgtcttccgttgaat	638	BACR24024
CG3051	SNF1A	caatgtgatcgacacctacgc	ttagcgagccagttgaatgat	859	GH12596
CG3068	aurora (aur)	gctcccttctactcgaagaact	gctgcctctacgtttcacttc	457	BACR03N24
CG3086	MAPk-2-Ak2	aaccacggcctagccatatc	gtcgcattggccatgtataac	407	LD17156
CG4224	CG4224	gtatgccaacaatctggagga	gcggttgtttcagtgaggag	770	GH09326
CG4268	Pitslre	aagatctggccaggatacacc	gttattccagcgttcaggaca	467	LD39519
CG4379	Pka-C1	ggaggggctgttacagttgtt	ggattgcgatcttccaaaag	350	BACR02I05
CG4551	Smi35A	taaggtgctcatatcggttgc	ccaccttctgcatgctattgt	533	GM04027
CG5072	Cdk4	tacaacagcaccgtggacat	aggggttcctgctgaaagtaa	329	LD31205
CG5125	ninaC	ggccatgttccagatcattc	gcggaaattcctgcttgatt	452	BACR01E19
CG5179	Cdk9	cagctatgcggctcctttac	taccaaacccggtcaatcata	392	GH21935
CG5363	Cdc2	cgatatctggtccattggatg	atttcgaactaagcccgattg	337	LD38718
CG5475	Mpk2	cgccgaatttttgaagaaga	tctccagatatggatgggaaa	200	BACR29F06
CG5680	basket (DJNK)	cgtttatgcaacggttacagc	catccacttcctcagcatcat	258	HL02677
CG6114	CG6114	atcgcccacaacagttacctc	caatggtcggacctgagttg	413	BACR18K24
CG6518	inaC	aagggtctcgaaatggtcaat	taaaggcatccacacagggta	799	GH24781
CG6551	fused (fu)	atcatcttgagaccgatgtgg	gcttttcctcattggtggaat	495	BACR17H16
CG6620	ial	gaacagcacggagagcaccta	tcgttacacaatgcaaactcg	260	LD07127

CG7001	PKl17E	accaaccagccaacacagtc	tcgttcttgctggtctcctta	275	LD21956
CG7177	CG7177	ccggaatcatcgacaacatta	aataactcgcaccaggcaact	475	GH11386
CG7186	SAK	atgctcaaatggtggacactc	ggacggcgtctttagtagctt	799	LD32344
CG7719	greatwall (gwl)	gatgagtggcatcaacatgaa	ccggtcatgaactcgtagaaa	744	LD35132
CG7826	minibrain (mnb)	tacatccagtcgcgcttctac	ctgcactggatgaggagacc	646	BACR29H04
CG8173	CG8173	atgatgacacgaaggagaacg	atcgctttctgtcgcaacatt	230	BACR42I14
CG8485	CG8485	gtgaggaggacgaagaggagt	actgcgattgtccctcttttt	627	GH25405
CG8866	CG8866	actctgcaaaaggccattgat	aatccggttccagcatttct	352	GH23955
CG9222	CG9222	tcgcctatgatcctttcatgt	ataccaagggtcctccttgac	242	GH07192
CG9774	rho-kinase (rok)	tgcgtcaacacaactacaagg	ttgttcgcgacacatagtacg	470	BACR03P14
CG10033	foraging (for)	catttcttcggctgtcgtatc	acgtgattcgtttgtctttgg	657	GH10421
CG10079	Egfr	attcccggggataagttcac	cgatggtctgggtgggtatc	511	LP11484
CG10579	Eip63E	ccaaaattagagaagcagcaga	ttattgttgctggtgttgtgc	554	LD46554
CG11228	hippo	agttcatacgcaacgccaag	aagttcgactccagctccac	415	LD11983
CG12306	polo	gcgacgagaacacagatcct	ccgttggtcaaatgcataact	394	LD02473
CG14217	CG14217	ttgcagctttgttgaactgtg	ctgactactggctgccgatac	334	LD42442
CG14895	Pak3	accagtaccgcccaagaaat	gttcccttgggtcatctgaat	308	GH15507
CG14992	Ack	ggcacgagctgaaattgataa	agggatctcgctcccttacag	753	BACR48M07
CG15793	Dsor1	ccaccctggagtcgatattc	gtattacgctttggcgtcgat	316	LD41207
CG16973	misshapen (msn)	ctctggagatggctgagtcac	agcccgagtagcgatagtcct	306	SD05170
CG17161	grapes (grp)	ttcctatgacctggtggactc	gtgggtcctttaagcacgata	636	LD14845
CG17309	Csk	cacagcatactgtccacgttt	atggcgttcagtttgacctc	530	BACR11G22
CG17342	LK6	ctggcgatgcaattgaagat	ctaccactgccactgcttcc	760	SD09050
CG17998	Gprk2	cagcgacatcaatcacaagaa	agctctcaaactcccggaac	338	LD20566
CG18069	CaMKII	ttcttggtaaaaactgcaaagc	ttttggggtataaaatcgaatg	256	GM04335
CG18247	shark	cattgatgacggtccctactg	ctgatccaacagggatagcac	703	BACR21B04
CG18582	mbt	caattttgagcatcgtgtgc	cgctactagctccacctactcc	500	BACR48E20

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