Understanding the mechanisms behind BMP-mediated growth in
_Drosophila_ wing precursors

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Declaration

I Ruta Ziukaite confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract

The Bone Morphogenetic Protein 2/4 (BMP2/4)-like ligand Decapentaplegic (Dpp) is a morphogen that specifies positional information in Drosophila wing precursors. However, it has also been shown to play a vital role in regulating the growth of this tissue. The spatial and temporal requirements of Dpp for normal cell proliferation have been the subject of debate, which has intensified recently with the suggestion that the endogenous stripe of Dpp is not required for growth during the third larval instar. To address this claim and other questions pertaining to Dpp and growth, we have developed an HA-tagged conditional allele of dpp and a V5-tagged conditional allele of brk. These tools enable us to remove gene expression in a precise spatial and temporal manner with GAL4-driven expression of Flp recombinase. Contrary to a recent report, I show that deletion of the characteristic ‘stripe’ of dpp expression along the A/P compartment boundary is detrimental to wing disc growth. Furthermore, I find that continued dpp expression is essential throughout larval development for normal growth. I also show that graded levels of Dpp are not required to promote cell proliferation. Instead, I argue that Dpp has an indirect role in growth control and confirm that its primary function is to suppress brk expression in the medial regions of the wing disc. Overall, my results suggest that the endogenous stripe of dpp expression generates a gradient that specifies cell fates, but also promotes cell proliferation by ensuring that Dpp signalling stays above a certain threshold.
Impact Statement

As part of the updated 2016/2017 UCL academic guidelines, all theses submitted for the award of MPhil/PhD are required to include a short statement on the impact of their research.

Developmental biologists have long been fascinated by the concept of morphogens, signalling molecules that are secreted by a particular group of cells and spread to form a concentration gradient. This gradient, which provides each cell with positional information, gives rise to intricate patterns of gene expression in a developing tissue. Ultimately, morphogens are responsible for producing the intricate patterns that we see in nature today. Examples of these include the stripes on a zebra or the venation pattern on a butterfly wing. Decades of research has identified the mechanisms that morphogens utilize throughout development to give rise to these patterns. However, studies have also shown that morphogens, like Decapentaplegic (the Drosophila BMP2/4 homolog), regulate growth and cell proliferation. However, it is not understood how morphogen gradients regulate tissue size.

Decapentaplegic, also known as Dpp, is a morphogen that is secreted from a narrow band of cells in the centre of a developing fruit fly (Drosophila melanogaster) wing. In this study, I characterise where and when this morphogen must be produced to give rise to a perfectly-patterned adult wing with the correct number of cells. These experiments involved utilizing CRISPR/Cas9 technology to generate a conditional allele, which allowed me to prevent the production of Dpp in specific regions of the developing wing. Ultimately, my results show that Dpp production in this ‘central stripe’ region is required for the wing to grow. However, my study shows that the characteristic gradient of Dpp is not necessary to promote cell proliferation. Further work is needed to understand how Dpp signalling regulates growth in different compartments and regions of the developing wing. These studies will shed light on how morphogens regulate the size of organs. Moreover, further research will provide insight into how growth and patterning are coupled throughout development.
Acknowledgement

First and foremost, I would like to take this opportunity to thank my supervisor, Jean-Paul Vincent, for giving me the opportunity to pursue my PhD in his laboratory. Thank you for taking a chance on me, for giving me the freedom to pursue my own ideas, and for your invaluable advice throughout the past four years. I also want to sincerely thank Cyrille Alexandre, who guided me through my first year in the lab (truthfully, I still run to him whenever an experiment goes wrong). Thank you for teaching me the A-Z of molecular biology and for being a constant source of laughter. I would not be the scientist I am today without your support.

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### Abbreviations

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<tr>
<td>AEL</td>
<td>After egg laying</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td><em>ban</em></td>
<td><em>bantam</em></td>
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<tr>
<td>Brk</td>
<td>Brinker</td>
</tr>
<tr>
<td>Ci</td>
<td>Cubitus interruptus</td>
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<tr>
<td>CtBP</td>
<td>C-terminal binding protein</td>
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<tr>
<td>Dad</td>
<td>Daughters against Dpp</td>
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<td>DLP</td>
<td>Dally-like protein</td>
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<td><em>ds</em></td>
<td><em>dachsous</em></td>
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<td>DSRF</td>
<td>Drosophila Serum Response Factor</td>
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<td>Dpp</td>
<td>Decapentaplegic</td>
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<td>DP</td>
<td>Disc proper</td>
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<td>En</td>
<td>Engrailed</td>
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<td><em>esg</em></td>
<td><em>escargot</em></td>
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<td><em>fj</em></td>
<td><em>four-jointed</em></td>
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<td>FLP</td>
<td>Flippase</td>
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<td>FRT</td>
<td>Flippase recombination target</td>
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<td>Gbb</td>
<td>Glass bottom boat</td>
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<td>Hh</td>
<td>Hedgehog</td>
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<td>Heparan-sulfate proteoglycans</td>
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<td>Homothorax</td>
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<td>LV</td>
<td>Longitudinal veins</td>
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<td>Medea</td>
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<td>Mtv</td>
<td>Master of thickveins</td>
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<td>Optomotor blind</td>
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<td>Pentagone</td>
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<td>PH-3</td>
<td>Phospho-histone H3</td>
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<td>PM</td>
<td>Periopdial membrane</td>
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<td>Put</td>
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<td>Abbreviation</td>
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<td>Rn</td>
<td>Rotund</td>
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<td>Spalt</td>
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<td>Saxophone</td>
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<td>Scw</td>
<td>Screw</td>
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<td>SE</td>
<td>Silencer elements</td>
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<td>Shnurri</td>
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<td>Tkv</td>
<td>Thickveins</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
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<td>UAS</td>
<td>Upstream activation sequence</td>
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<td>ubx</td>
<td>ultrabithorax</td>
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<td>Vg</td>
<td>Vestigial</td>
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<td>Wg</td>
<td>Wingless</td>
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<tr>
<td>Yki</td>
<td>Yorkie</td>
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<td>Zfh2</td>
<td>Zinc finger protein 2</td>
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Chapter 1. Introduction

Throughout development, tissue patterning and growth must be tightly coupled to ensure the formation of correctly-patterned appendages with the right number of cells. If growth is terminated prematurely, then certain cell fates or territories are not specified. Conversely, patterning events trigger the growth of new domains. The intimate relationship between patterning and growth can be exemplified in gain-of-function experiments with axolotls, where small grafts of connective tissue can induce the formation of ectopic, normally-patterned limbs (Lee et al., 2013). In the past decade, the wing imaginal disc has also emerged as a popular system to study the interplay between patterning and growth. Multiple growth regulators have been identified in wing imaginal discs. These can be broadly grouped into two main categories: 1) systemic signals (i.e. Insulin-like peptides) that scale the size of multiple tissues within a developing organism and 2) patterning signals that regulate growth locally within a growing tissue. Decapentaplegic (Dpp), the Drosophila homolog of vertebrate BMP2/4, is one of the main patterning signals in the wing disc, expressed in a narrow stripe along the anterior-posterior (A/P) boundary. From this domain, Dpp spreads into both the A and P compartments to form a long-range gradient, which is heavily involved in specifying the position of longitudinal vein (LV) proveins in the wing disc. However, it has also been shown that Dpp signalling is required for growth and cell division. Since the analysis of cell cycle patterns in the wing imaginal disc suggest that proliferation is uniform throughout the tissue (Milan et al., 1996), scientists in the field have been prompted to ask: how can graded levels of a patterning signal promote uniform rates of cell proliferation in the wing disc? This is one of the main questions that we attempt to address in our study. Before outlining the proposed models that try to resolve this paradox, the following sections will provide an overview on wing disc development, patterning by morphogens, and the Dpp signalling cascade.
1.1 The specification and subdivision of the wing imaginal disc

Limb primordia, including the wing, haltere, and leg imaginal discs, are thought to originate from a ventrally-located cluster of cells in the embryo that express Distalless (Dll) (Cohen et al., 1993, Cohen et al., 1989). A subset of these cells migrate dorsally and begin to express vestigial (vg), a developmental marker that specifies wing and haltere fates (Cohen et al., 1993, Goto and Hayashi, 1997, Williams et al., 1991). Initially, both imaginal discs have the potential to develop into wings. However, the expression of ultrabithorax (ubx) in the third thoracic (T3) segment confers haltere identity to the imaginal disc in this region. The wing primordium is specified in the second thoracic (T2) segment, where ubx expression is absent. The wing imaginal disc is often characterized as an epithelial sac with two sides: 1) the disc proper (DP), which is a columnar pseudostratified epithelium and 2) the periopdial membrane (PM), a squamous epithelium consisting of stretched and flat cells. The DP will eventually give rise to the wing blade, the mesothorax and the hinge, a structure that connects the wing blade to the body wall (Figure 1.1). Although the PM does not contribute to the formation of adult structures, it is still important for several developmental processes, including wing disc eversion (Pastor-Pareja et al., 2004, Tripura et al., 2011).

During larval stages, the DP is subdivided into distinct compartments or units along three major axes: anterior-posterior (A/P), dorso-ventral (D/V), and proximo-distal (P/D). The first subdivision, between the A and P compartments, is inherited from the embryonic ectoderm and is defined by the restricted expression of engrailed (en) and invected (inv) in P cells (Kornberg, 1981, Kornberg et al., 1985, Simmonds et al., 1995). The subdivision of the wing disc into D and V compartments happens later in development, during the second larval instar. This occurs through the restricted expression of another selector gene, apterous (ap), in D cells (Cohen et al., 1992). Importantly, these subdivisions trigger the expression of signalling molecules at each compartment boundary. For example, engrailed induces the expression of dpp at the A/P boundary through the activity of hedgehog (hh) (Zecca et al., 1995). In addition, apterous (through Notch signalling), induces the expression of wingless (wg) at the D/V boundary (Diaz-Benjumea and Cohen, 1993, Diaz-Benjumea and Cohen, 1995). Together, these
signalling molecules provide an internal coordinate system for cells in the developing wing disc. Although the P/D axis is only revealed after wing disc eversion (a process that involves the folding and apposition of dorsal and ventral surfaces), it is subdivided into three main territories throughout larval development: the notum, the hinge, and the wing pouch (the precursor of the adult wing blade). In the wing imaginal disc, the most central structures, such as the pouch, will give rise to distal structures in the adult wing (Figure 1.1).

Figure 1.1 – Proximodistal patterning in the wing imaginal disc

(A) The wing imaginal disc is subdivided along the P/D axis into several different territories: the pouch or the prospective wing blade (green), the hinge (yellow), the pleura (red), and the notum (purple regions). (B–C) The notum regions and the pleura gives rise to the adult mesothorax. After metamorphosis, the pouch region will give rise to the adult wing blade. The hinge is a structure that connects the wing blade to the body wall (adapted from Hatini et al., 2013).
1.2 Cell proliferation and apoptosis in the wing disc

After egg hatching, the wing primordia in a first-instar larva consist of roughly 11 to 38 cells (Bryant, 1970, Garcia-Bellido and Merriam, 1971, Lawrence and Morata, 1977, Mandaravally Madhavan and Schneiderman, 1977). These cells begin to proliferate in an exponential manner at the end of the first larval instar (roughly 40 hours after egg laying), eventually giving rise to a structure that contains anywhere between 30,000 to 50,000 cells (Mandaravally Madhavan and Schneiderman, 1977, Garcia-Bellido and Merriam, 1971, Martin et al., 2009). As a result, the wing imaginal disc has been widely used to study the control of cell proliferation and apoptosis. Although the wing disc undergoes extensive cell division, the amount of cell death observed is low. There are a few exceptions, including a noticeable increase in dying cells at the border between the notum and the hinge in late third instar wing discs (Milan et al., 1997). In flies, apoptosis is induced via the pro-apoptotic genes *reaper (rpr)*, *head involution defective (hid)*, and *grim*, which inhibit the *Drosophila* inhibitor of apoptosis protein 1 (dIAP1) (Goyal et al., 2000, Ryoo et al., 2002). Inhibition of dIAP1 activates the expression of caspases, including *Drosophila* Interleukin-1 Converting Enzyme (DrICE) and Dcp-1 (Death Caspase-1), which promote cell death by cleaving a number of different substrates (Mills et al., 2005). It is important to note that in many cases, apoptosis can also induce proliferation. For example, caspases have been shown promote the release of mitogenic signals from dying cells, thereby inducing division in surrounding cells (Fan and Bergmann, 2008, Morata et al., 2011).

Cell division is primarily driven by the action of the Cdk family of serine-threonine protein kinases, which associate with Cyclins (Cyc) to promote advancement through different stages of the cell cycle (Follette and O'Farrell, 1997). Cyclins are divided into two major groups: Cyclin D and Cyclin E promote the progression from G1 to S phase, while Cyclins A/B are involved in the initiation of mitosis (Thompson, 2010). In *Drosophila*, Cdks and Cyclins are regulated by a host of different factors, most notably E2F and String/Cdc25 (Figure 1.2). For example, by activating the expression of Cyclin E, E2F promotes the G1/S transition (Dynlacht et al., 1994). Meanwhile, String/Cdc25 is critical for the formation of CycA/Cdk1 and CycB/Cdk1 complexes, both of which
initiate the G2/M transition (O'Farrell et al., 1989). Remarkably, perturbations in the cell cycle do not seem to have a significant effect on the final size of the *Drosophila* wing imaginal disc. For example, the removal of String/Cdc25 function relatively late in development gives rise to wing discs that contain fewer, but larger cells. Despite this, the overall size and morphology of the wing disc does not change. Similar experiments have confirmed that cell proliferation and cell growth, although closely linked, are controlled through different mechanisms. In this thesis, I closely examine how Decapentaplegic (Dpp), a well-characterised morphogen, regulates cell proliferation and influences the overall size of the wing imaginal disc.
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Figure 1.2 – The cell cycle in *Drosophila melanogaster*

The cell cycle is subdivided into four main phases: G1, S, G2, and mitosis. In *Drosophila melanogaster*, the CDK2/CycE complex is rate-limiting for progression from G1 to S phase. This process is controlled by a host of transcription factors, including E2F, which activates the expression of Cyclin E. On the other hand, CDK1/CycA and CDK1/CycB complexes initiate mitosis. These complexes are phosphorylated and activated by the String/Cdc25 phosphatase (adapted from Yuan et al., 2016).
1.3 Morphogen gradients in developmental biology

Alan Turing is credited for coining the term morphogen to describe “the idea of a form producer” or a molecule that organises biological patterns (Turing, 1990). Morphogens remained theoretical until revolutionary advances in molecular biology facilitated the identification of several signalling proteins, which seemed to function as morphogens. In the field of developmental biology, a morphogen is defined as a chemical substance that spreads from a localised source to establish a concentration gradient, which in turn influences the gene expression and cell fate decisions of surrounding cells (Figure 1.3). The existence of morphogens was and is still controversial, in part because it has been difficult to show that they act directly on cells and not through a signal relay mechanism. In the wing imaginal disc, the significance of morphogens has been debated, especially after Alexandre et al. showed that a membrane-tethered version of Wingless (Wg) could still produce flies with normally patterned appendages (Alexandre et al., 2014). Although the spread of Wingless may be dispensable for patterning in the wing disc, evidence does suggest that Dpp acts as a bona-fide morphogen in this tissue. Studies have shown that cells, which express ectopic Dpp, induce the expression of target genes in overlapping and surrounding areas. In contrast to this, cells that express a constitutively active version of the type I receptor Tkv (Tkv^{QD}) are not able to activate target gene expression in surrounding cells, suggesting that Dpp does indeed spread and act as a morphogen in the wing disc (Lecuit et al., 1996, Nellen et al., 1996). This was recently confirmed by Harmansa et al., who used a membrane-tethered anti-GFP nanobody (termed morphotrap) to capture GFP-Dpp on the surface of secreting cells (Harmansa et al., 2015). In contrast to Wg, these experiments showed that patterning and growth is perturbed in the absence of Dpp spreading in the wing imaginal disc. Despite strong evidence that Dpp acts as a morphogen, it is still unclear how many threshold responses are set by its gradient. Furthermore, it is not known precisely how the Dpp morphogen gradient is formed in the wing disc.
Figure 1.3 – Morphogen gradients specify multiple cell fates in a field of cells

(A) According to Lewis Wolpert, the position of each cell is defined by the concentration of a morphogen. (B) Target genes are activated by different concentration thresholds, giving rise to defined patterns of expression in a tissue.
1.4 An overview of the BMP signalling pathway

Decapentaplegic (Dpp) was first described by William M. Gelbart et al. in 1982 as a gene complex, which if mutated, produced phenotypes in one or more of the fifteen imaginal discs found in Drosophila larvae (Spencer et al., 1982). A few years later, in 1987, the dpp locus was sequenced and it was predicted that the gene encoded a member of the TGF-β family of signalling molecules (Padgett et al., 1987). This family of proteins is highly conserved and is found in all organisms, from Cnidaria to humans. There are four signalling pathways that comprise the TGF-β superfamily – TGF-β, Bone Morphogenetic Protein (BMP), Activin, and Nodal. The BMP subfamily is further divided into four groups – BMP2/4, the growth and differentiation (GDF) 5/6/7 group, BMP 5/6/7/8, and BMP 9/10. In Drosophila melanogaster, three different BMPs have been cloned and characterized. By far the most widely studied, Dpp is the Drosophila homologue of vertebrate BMP 2/4 (Padgett et al., 1987). The other BMPs found in Drosophila include Glass bottom boat (Gbb), which is the homologue of vertebrate BMP 5/6/7/8 and Screw (Scw), which encodes a novel BMP-like member of the TGF-β superfamily (Doctor et al., 1992, Arora et al., 1994). Each ligand is first synthesized as a pro-peptide, consisting of an N-terminal prodomain and a C-terminal mature ligand domain. After the formation of homodimers or heterodimers in the endoplasmic reticulum, these precursor proteins travel to the Golgi body, where they are proteolytically cleaved and processed by Furin and/or other convertases (Cui et al., 1998, Constam and Robertson, 1999, Kunnapuu et al., 2009). After being secreted into the extracellular space, BMP dimers bind tetrameric complexes of type I and type II serine/threonine kinase receptors (Yamashita et al., 1994). Unlike TGF-β ligands, BMP ligands have a higher affinity for type I receptors (Koenig et al., 1994). In Drosophila melanogaster, two type I BMP receptors have been identified – Thickveins (Tkv) and Saxophone (Sax) (Nellen et al., 1994, Brummel et al., 1994, Penton et al., 1994, Xie et al., 1994). Upon ligand binding, the constitutively active type II receptor Punt (Put) phosphorylates the corresponding type I receptor in the GS domain, a stretch of glycine and serine residues near the membrane spanning region (Wieser et al., 1995). This activates the kinase activity of the type I receptor and at the same time, creates a binding pocket for receptor-regulated Smads (R-Smads). Smad proteins, which are involved in signal transduction, are generally divided
into three different classes: receptor-regulated Smads (R-Smads), common-mediator Smads (co-Smads) and inhibitory Smads (I-Smads). R-Smads and co-Smads contain an N-terminal MH1 domain that can bind DNA and a C-terminal MH2 domain, which is involved in receptor interaction, the formation of Smad complexes, and nuclear import (Shi and Massague, 2003). Although the linker region separating the MH1 and MH2 domains is divergent among Smads, it contains multiple phosphorylation sites that allow crosstalk between different signalling pathways. Unlike the R-Smads and co-Smads, the I-Smads are only characterized by the presence of a MH2 domain, the only region that is conserved amongst all Smad proteins. In *Drosophila*, only one I-Smad has been described to date, Daughters against Dpp (Dad) (Tsuneizumi et al., 1997). Dad is activated by Dpp signalling, but also antagonizes the signalling pathway by competing with Mad for receptor binding (Tsuneizumi et al., 1997). After ligand binding, R-Smads are phosphorylated by type I receptors at an SXS motif in their C-terminal domain (Macias-Silva et al., 1996). Following this, phosphorylated R-Smads form a complex with Smad4 (co-Smad) via the MH2 domain (Wu et al., 2001) and translocate into the nucleus.

In *Drosophila* wing imaginal discs, it is thought that three different ligand dimer pairs exist: Dpp-Dpp homodimers, Gbb-Gbb homodimers, and Gbb-Dpp heterodimers. These dimers induce the phosphorylation of Mothers against Dpp (Mad), the *Drosophila* R-Smad involved in Dpp signalling (Figure 1.4). Phosphorylated Mad then forms a complex with *Drosophila* Smad4, Medea (Med). As described in more detail below, the pMad/Medea complex associates with different activators and repressors to regulate the expression of target genes. BMPs regulate a wide variety of target genes, which control numerous biological processes throughout development, including cell proliferation, differentiation, cell-fate determination, and morphogenesis (Spencer et al., 1982, Ferguson and Anderson, 1992, Wharton et al., 1993, Chang et al., 2003). However, BMPs are also vital for tissue homeostasis and stem cell maintenance (Xie and Spradling, 1998).
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Figure 1.4 – Schematic of the Dpp signalling pathway

BMP ligands travel through the extracellular space as homo- or heterodimers and signal through a tetrameric set of receptors (two type I and two type II receptors). Ligand binding induces a phosphorylation cascade, which ultimately results in the phosphorylation of R-Smads (Mad). Phosphorylated R-Smads form a complex with co-Smads (Medea) and translocate into the nucleus where the complex associates with a number of co-factors to activate or repress the expression of target genes. For example, Mad/Medea interact with Schnurri (Shn) to abolish the transcription of *brinker (brk).*
1.5 Decapentaplegic (Dpp) signalling in the wing imaginal disc

In the wing imaginal disc, *engrailed* (*en*) activity in the posterior (P) compartment prompts cells to secrete Hedgehog (Hh), a morphogen that diffuses into the anterior compartment to activate the expression of *dpp* (Basler and Struhl, 1994). Cells in the P compartment do not respond to Hh because the transcription factor required for Hh signalling, *cubitus interruptus* (*ci*), is only expressed in the anterior (A) compartment. Ultimately, this results in a narrow stripe of *dpp* expressing cells that runs along the A/P compartment boundary. From this stripe, Dpp spreads into both the A and P compartments to activate the nested expression domains of several genes, including *spalt* (*sal*) and *optomoter blind* (*omb*) (Lecuit et al., 1996, Nellen et al., 1996). In the wing disc, Dpp preferentially binds to the type I receptor Tkv (Haerry et al., 1998) and by doing so, induces the phosphorylation of Mothers against decapentaplegic (Mad) (Sekelsky et al., 1995). Once phosphorylated, pMad forms a complex with Medea (Smad4), which translocates to the nucleus and interacts with various co-factors to either activate or repress transcription (Das et al., 1998, Hudson et al., 1998, Inoue et al., 1998, Wisotzkey et al., 1998).

The characterization and identification of *brinker* (*brk*), a transcriptional repressor, turned out to be a vital step in understanding the Dpp signalling pathway in *Drosophila melanogaster*. Brk is a nuclear protein that contains an N-terminal DNA binding domain and binding motifs for co-repressors, including C-terminal binding protein (CtBP) and Groucho (Jazwinska et al., 1999, Hasson et al., 2001, Zhang et al., 2001). In addition to recruiting co-repressors, Brk also represses target genes by competing with transcriptional activators, such as Smads, for binding to DNA motifs (although the extent to which this occurs is unclear). For example, the Mad binding site, GGCGYC, is also a Brk binding motif (the consensus sequence for Brk binding is GGCGYY) (Sivasankaran et al., 2000, Zhang et al., 2001, Weiss et al., 2010). Thus, Brk is a major antagonist of the Dpp signalling pathway and must be inactivated in order to induce the expression of many Dpp target genes. This is achieved at the transcriptional level via Mad/Medea (Figure 1.5). Dissection of the *brk* cis-regulatory region revealed the presence of a short silencer element (SE) with a GRCGNC(N)\textsubscript{5}GTCTG consensus
sequence (Muller et al., 2003, Pyrowolakis et al., 2004). Further studies showed that this SE binds a trimeric complex – two phosphorylated Mad proteins bind the GRCGNC sequence, while the GTCTG sequence is bound by one molecule of Medea (Gao et al., 2005). However, this itself does not inactivate the expression of *brk*. Once Mad and Medea are bound to the SE, transcriptional repression of *brk* is only achieved after the recruitment of Schnurri (Shn), a large zinc finger protein (Figure 1.5). Shn can only bind to the SE if the two Smad binding sites are separated by exactly five nucleotides and the Medea binding site (GTCTG) contains a T in nucleotide position 4 (Pyrowolakis et al., 2004). If these requirements are not met, Shn is unable to interact with the complex and transcriptional repression is abolished.
Figure 1.5 – Dpp signal transduction in the wing imaginal disc

In the absence of Dpp signalling, Brk is expressed at high levels. Brk binds and represses many Dpp target genes via the GGCGYY sequence. In the extracellular space, Dpp will preferentially bind the type I receptor, Thickveins (Tkv). Upon ligand binding, the constitutively active type II receptor, Punt, phosphorylates Tkv at a GS-rich domain. In turn, this results in the phosphorylation of Mad, the Drosophila R-Smad involved in Dpp signalling. Phosphorylated Mad forms a complex with Medea (co-Smad), which translocates to the nucleus and binds specific DNA motifs. In order to repress brinker, the Mad/Medea complex binds to a silencer element in the *brk* cis-regulatory region. Two Mad proteins (shown in red) bind the GRCGNC sequence and one Medea protein (shown in blue) binds the GTCTG sequence. If the two sites are separated by exactly 5 nucleotides, the zinc-finger protein Schnurri is recruited to the complex and represses the transcription of *brinker*. Mad/Medea can also associate with other co-factors to either activate or repress the transcription of certain genes (Affolter and Basler, 2007).
1.5.1 How does Dpp signalling activate the expression of target genes?

After the characterization of *brinker* and *schnurri*, it became important to understand whether target genes are directly activated by pMad/Medea or if they are indirectly regulated via the derepression of *brk*. This was partly answered by the mutational analysis of *optomotor-blind* (*omb*) and *spalt* (*sal*), transcription factors that are activated by Dpp signalling in nested expression domains (Figure 1.6) (Lecuit et al., 1996, Nellen et al., 1996). Although Brk is important in setting the boundary of *spalt* expression, the actual transcription of the gene depends in part on Dpp signalling and in part on unidentified factors. This was nicely demonstrated by Barrio and Celis, who removed Brk binding sites from the *cis*-regulatory region of *spalt*. In turn, the expression domain of the gene was broadened. On the other hand, the inactivation of SMAD-binding sites in this regulatory region did not completely abolish the expression of *spalt* and did not change its expression domain (Barrio and de Celis, 2004). In contrast to *spalt*, *omb* transcription does not require direct activation by Smad proteins. This was demonstrated by comparing *brk* mutant and *brk/tkv*⁷ double mutant clones, which express *omb* at similar levels (Campbell and Tomlinson, 1999, Jazwinska et al., 1999). Altogether, there seems to be three different mechanisms that Dpp utilizes to regulate the expression of genes. In some instances, Dpp signalling is not directly required to induce gene expression, but it does so indirectly via the inactivation of *brk*. In most cases, it seems that gene activation does require some input from Mad/Medea. For example, *spalt* and *zen* (in the embryo) are both repressed by Brk and activated by Dpp signalling (Barrio and de Celis, 2004, Rushlow et al., 2001). However, there are some instances where genes are activated directly by Mad/Medea and this is completely independent of Brk. For example, this is the case for *Race* (*Related to angiotensin converting enzyme*), a gene that is expressed in the amnioserosa of the early embryo, where peak levels of Dpp signalling are found (Rusch and Levine, 1997, Ashe et al., 2000, Wharton et al., 2004, Xu et al., 2005). It is important to note that such genes, which are activated by Mad/Medea independently of Brk, have not yet been described in the wing disc.
1.5.2 How does Brk repress *omb* and *sal* at different concentrations?

Studies suggest that the differential expression boundaries of *omb* and *spalt* are determined by their relative sensitivities to Brk (Muller et al., 2003, Moser and Campbell, 2005). However, it is not known precisely how Brk represses these genes at different concentrations. In the wing disc, there is evidence to suggest that Brk uses alternative mechanisms to repress different target genes. It possesses at least three independent repression domains: the CtBP DNA motif, the Groucho DNA motif, and 3R (Hasson et al., 2001, Zhang et al., 2001, Winter and Campbell, 2004). Mutational analysis of Brk has shown that the 3R domain is sufficient for the repression of *omb*, but not *sal*. In order to fully repress *sal* expression, Brk must recruit either CtBP or Groucho (Winter and Campbell, 2004). Overall, this data suggests that it is not solely the number of binding sites in an enhancer that determines the sensitivity of a certain gene to a transcriptional repressor. Indeed, Brk seems to utilize completely different mechanisms to repress *omb* and *sal*. Further studies involving the specific repression domains of Brk will be needed to fully elucidate how it exerts its repressive activity on different genes. At the moment, no Brk homolog has been identified in vertebrates. Thus, it is not possible to make inferences from its activity in different model organisms.
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Figure 1.6 – The Dpp morphogen gradient activates genes in nested expression domains

In the wing imaginal disc, dpp is expressed in a narrow stripe of cells that is located just anterior to the A/P compartment boundary. Dpp forms a long-range gradient in the wing disc and activates the expression of target genes, such as omb and sal, in nested domains. This is achieved primarily through the inactivation of brinker, a transcriptional repressor that is thought to form an inverse gradient to pMad in the wing disc (adapted from Schwank, Basler 2010).

Figure 1.7 – The dpp locus contains three furin-recognition sites

BMP ligands, including Dpp, are first synthesized as inactive pro-proteins, which must be sequentially cleaved by convertases to generate active, C-terminal mature proteins. The dpp locus contains three furin-recognition sites and cleavage at these different sites gives rise to two mature forms of Dpp – a 26-kDa and a 23-kDa form. First, cleavage occurs at S2 (to generate an intermediate form of Dpp), followed by subsequent cleavage at either S1 or S3.
1.5.3 Post-translational processing and cleavage of Dpp ligands

Before Dpp is secreted from cells and able to engage with its receptors, it is first processed by certain proteases in the Golgi body. As previously described, BMPs are first synthesized as inactive precursor proteins that are proteolytically cleaved and activated by furin-like convertases to generate C-terminal mature proteins (Cui et al., 1998, Cui et al., 2001, Degnin et al., 2004). In *Drosophila* Dpp, three different furin-recognition sites have been identified (Figure 1.7). Studies indicate that sequential cleavage produces two mature forms of Dpp – a 26-kDa form and a 23-kDa form, both of which show the same signalling capacity *in vitro*. The first cleavage occurs at FSII, which produces an intermediate form of Dpp. This is subsequently cleaved at either FSIII or FSI to produce the two mature forms of the protein (Kunnapuu et al., 2009). Currently, it is not clear why two different ligands are produced. However, there is data suggesting that the larger form binds more efficiently to extracellular modulators of Dpp, like collagen IV, in comparison to the N-terminally truncated form (Sawala et al., 2012). Interestingly, the *Drosophila* Dpp pro-protein is cleaved in a completely different manner compared to vertebrate BMP4, which only contains two furin-recognition sites and is produced as a single, mature form (Cui et al., 2001, Degnin et al., 2004). Thus, throughout evolution, the furin-recognition sites in BMP2/4-type proteins have been diversified.
1.6 Dpp gradient formation involves many feedback mechanisms

In the blastoderm embryo, the Dpp gradient is established by an active transport mechanism that involves the activity of several extracellular proteins: Short gastrulation (Sog), Twisted gastrulation (Tsg), Tolloid (Tld), and Screw (Scw) (Arora et al., 1994, Francois et al., 1994, Shimell et al., 1991, Ross et al., 2001). In the embryo, Sog and Tsg act as inhibitors, binding to Dpp/Scw heterodimers and preventing their interaction with receptors at the cell surface. However, these molecules also shuttle BMP ligands towards the dorsal midline (the direction of transport is determined by high ventral-lateral expression of Sog), where peak levels of Dpp signalling are seen (Ross et al., 2001, Ashe and Levine, 1999, Decotto and Ferguson, 2001, Eldar et al., 2002, Shimmi et al., 2005b). In order to activate signalling at the dorsal midline, the complex is cleaved by Tld, a protease that facilitates the release of the ligands (Shimell et al., 1991, Marques et al., 1997). Similar sophisticated mechanisms are also seen in other Drosophila tissues, such as the pupal wing, where Dpp is transported from the longitudinal veins to the posterior crossvein by Sog and a Tsg paralog, Crossveinless (Shimmi et al., 2005a, Matsuda and Shimmi, 2012).

In contrast to the blastoderm embryo, it is not known precisely how the Dpp morphogen gradient is established in the wing imaginal disc. In this tissue, there is little evidence to suggest that either Sog or Tsg plays a vital role in gradient formation. In fact, the overexpression of either gene in the wing disc has little effect on patterning (Yu et al., 2000). However, it is well-known that Dpp gradient formation in the wing disc involves heparan sulphate proteoglycans (HSPGs), cell surface and extracellular matrix proteins that consist of covalently attached heparan sulphate chains (Fujise et al., 2003, Belenkaya et al., 2004). In Drosophila, one of the proteoglycans in the glypican subgroup, Dally, is thought to stabilize and concentrate Dpp molecules at the cell surface. Studies have shown that Dally binds to Dpp (Kirkpatrick et al., 2006, Akiyama et al., 2008) and that dally-overexpression clones exhibit significantly elevated levels of pMad (Fujise et al., 2003). Although this data suggests that Dally sequesters Dpp ligands, it is also thought that HSPGs promote lateral diffusion, most likely by binding to Dpp and passing it to another glypican on a neighbouring cell. For example, in dally mutant wing discs, the
characteristic gradients of pMad and Dpp-GFP are no longer detectable, suggesting that Dally does indeed promote the spread of Dpp into the lateral regions of the wing disc (Fujise et al., 2003). Ultimately, a fine balance of HSPGs needs to be achieved in order to facilitate the formation of a long-range gradient.

Another important extracellular modulator of Dpp is Pentagone (Pent), a novel protein that was discovered due to the presence of four silencer elements (SE) near its transcription start site (please refer to section 1.2 for a description of how Dpp represses transcription via SEs). Indeed, pent is repressed by Dpp signalling and consequently, it is expressed in a graded fashion, with the highest levels seen in the peripheral regions of the wing disc. The phenotype seen in pent mutant wing discs resembles the phenotype caused by the medial over-expression of dally – the characteristic gradient of pMad expression is lost and cells in the lateral regions of the pouch seem to be depleted of Dpp signalling. This results in flies with slightly smaller wings and patterning errors, primarily the loss of the fifth longitudinal vein (Vuilleumier et al., 2010, Vuilleumier et al., 2011). Thus, low levels of Pentagone in the medial regions of the wing disc seem to promote the distribution of Dpp ligand. It is also interesting to note that Pentagone is a secreted molecule that associates with glypicans, such as Dally, on the cell surface (Vuilleumier et al., 2010). However, the relevance of this interaction remained unclear for some time. A new study suggests that Pentagone induces the internalisation of both Drosophila glypicans, Dally and Dally-like protein (DLP) (Norman et al., 2016). This data suggests that Pentagone adjusts the levels of glypicans in the wing disc, thereby promoting the spread of Dpp throughout the tissue (Figure 1.8).

In addition to HSPGs and Pentagone, one of the most important determinants of the Dpp gradient is the expression pattern of the BMP type I receptor, thickveins. The expression of tkv is negatively regulated by Dpp signalling (Lecuit and Cohen, 1998, Tanimoto et al., 2000). Thus, like pent, it is expressed at high levels in the peripheral regions of the wing disc and at low levels in medial areas (Figure 1.9). In addition to this, tkv is also tightly regulated by master of thickveins (mtv), a transcription factor whose expression is controlled by both Hedgehog (Hh) and Engrailed (En). In the domain of dpp expression, Hh signalling upregulates the expression of mtv, thereby inducing the
repression of \textit{tkv} (Tanimoto et al., 2000). In the P compartment, \textit{mtv} is repressed by the activity of En, resulting in higher basal levels of Tkv in the posterior (P) compartment compared to the anterior (A) compartment (Figure 1.9). This contributes to a steeper gradient of pMad in the P compartment and it explains why Dpp is able to diffuse further in the A compartment. Overall, the expression pattern of \textit{tkv} is vital for establishing the complex Dpp activity gradient in the wing imaginal disc. There are also countless other molecules that are involved in BMP gradient formation, such as the type IV collagen proteins Viking and Dcg1. For an in-depth review on the spatial regulation of BMP activity, please see Ramel and Hill, 2012.
**Figure 1.8 – The role of HSPGs and Pentagone in Dpp gradient formation**

(A) In the absence of Pentagone, high levels of HSPGs bind Dpp, resulting in its accumulation at the cell surface. Although this promotes signalling, it also inhibits the diffusion of Dpp to lateral regions in the wing disc. (B) Pentagone binds to HSPGs, such as Dally, and induces their degradation. By adjusting the levels of Dally in the wing disc, Pentagone promotes long-range gradient formation and inhibits the sequestration of Dpp by HSPGs (adapted from Norman et al., 2016).
Figure 1.9 – The expression pattern of thickveins establishes the Dpp activity gradient

The expression of the type I receptor, thickveins (tkv), is negatively regulated by Dpp signalling. Ultimately, this results in high levels of Tkv in the lateral regions of the wing disc, which diminish in the central regions. The expression of tkv is also regulated by another morphogen, Hedgehog (Hh). Hh diffuses across the A/P compartment boundary to activate the expression of dpp in a narrow stripe of cells. At the same time, it also induces the expression of master of thickveins (mtv), a transcription factor that represses tkv. The hyper-repression of tkv in this domain produces a dip in p-Mad levels near the A/P compartment boundary. Finally, it is also important to note that the basal levels of Tkv are higher in the P compartment compared to the A compartment. This is a result of engrailed (en) activity in the P compartment, which represses mtv. Ultimately, this hinders the diffusion of Dpp and results in a much steeper gradient of p-Mad in the P compartment (Tanimoto et al., 2000).
1.7 Dpp-dependent models of growth regulation in the wing disc

The Dpp gradient is indispensable for patterning and specifies the positions of several longitudinal vein (LV) proveins in the late third-instar wing disc (Figure 1.10). In *Drosophila melanogaster*, there are five LVs (L1-L5) that run proximodistally and two main crossveins: the anterior and posterior crossveins, which connect L3-L4 and L4-L5 respectively. In the wing imaginal disc, Dpp is primarily responsible for specifying the positions of L2 and L5. Indeed, extensive studies have shown that the positioning of L2 is dependent on the expression domain of *spalt*, while the position of L5 is specified by inputs from *spalt*, *optomotor blind*, and *brinker* (de Celis et al., 1996, Sturtevant et al., 1997, de Celis and Barrio, 2000, Cook et al., 2004). Although the role of Dpp in specifying cell fates in the wing disc has been well-characterized, there are still many questions surrounding the mechanisms that it utilizes to regulate growth. It is known that hypomorphs, which lack Dpp signalling in the developing wing disc, give rise to adults with small wings (Figure 1.10). In addition, clones of cells over-expressing *dpp* seem to induce proliferation in surrounding cells (Zecca et al., 1995). In some cases, if the clone arises at a distance from the A/P boundary, this can induce the formation of an ectopic winglet (Figure 1.10). These early studies generated many interesting questions. For example, how do graded levels of a morphogen give rise to a homogenous proliferation of cell proliferation in the wing disc? In order to address this question, many new models of growth regulation were developed by different laboratories. In this section, we review these models in depth.
Figure 1.10 – Altering the levels of Dpp in the wing disc produces growth defects

(A) The Dpp gradient specifies the position of LV proveins 2 and 5 in late third-instar wing imaginal discs by setting the expression domains of sal and omb (proveins are marked by the expression of argos or the loss of DSRF). (B) A lack of dpp expression in wing imaginal discs gives rise to adults with small wings (dpp^{dB}/dpp^{10} mutant adult wings). (C-D) Clones of cells expressing Tubal>dpp in the wing disc induce proliferation in surrounding cells, sometimes leading to the formation of an ectopic winglet (depending on the position of the clone). Panel A taken from Blair, 2007 and panels B-D are taken from Zecca et al., 1995.
1.7.1 The slope model and the temporal rule model

Studies have shown that cell proliferation occurs at approximately uniform rates in all areas of the wing imaginal disc (Garcia-Bellido and Merriam, 1971, Milan et al., 1996). This observation prompted many developmental biologists in the field to ask the same question: how can a signalling gradient influence homogenous cell proliferation? Day and Lawrence proposed that cells read the local steepness of a gradient, and that this information is used to determine whether or not a cell undergoes division (Day and Lawrence, 2000). The slope model was attractive at first, especially since it also invoked a mechanism for size determination. Day and Lawrence predicted that the highest and lowest levels of Dpp (in the central and peripheral regions of the wing disc) are fixed (Figure 1.11 A). Thus, growth would extend the gradient until the local steepness falls below a certain threshold, thereby abrogating the growth of the tissue (Day and Lawrence, 2000). However, uniform levels of Dpp (imposed by UAS-Dpp) have been shown to trigger significant growth, especially in the lateral regions of the wing disc (Capdevila and Guerrero, 1994, Lecuit et al., 1996, Nellen et al., 1996). Thus, the slope model was later refined by Rogulja and Irvine in 2005. The authors show that uniform levels of Dpp in the wing disc inhibit the proliferation of medial cells, but also increase the proliferation of cells in the lateral regions of the disc (Rogulja and Irvine, 2005). Thus, Rogulja and Irvine argue that two different cell populations exist in the wing disc: medial cells that proliferate in response to differences in Dpp pathway activity and lateral cells, which can proliferate even in response to low, absolute levels of Dpp. There are certain drawbacks to the slope model – for example, it is not clear how cells in the wing disc would be programmed to respond differently to Dpp signalling. Furthermore, neither of these groups address how uniform cell proliferation is achieved in dpp source cells, which are exposed to high, uniform levels of Dpp ligand.

Another model, coined the temporal rule model, suggests that changes in the absolute levels of Dpp drive cell proliferation in the wing disc (Figure 1.11 B). In their study, Wartlick et al. carefully quantify Dpp concentration (using a Dpp-GFP fusion protein) and signalling levels throughout the third-larval instar. They find that while the shape of the gradient stays the same in the P compartment, the absolute levels of Dpp increase constantly during development. The authors correlate the relative increases in
Dpp concentration and signalling levels (measured with \textit{dad-nRFP}) with the estimated cell doubling time. This correlation suggests that cells undergo mitosis only after Dpp signalling levels have increased by approximately 50% since the beginning of the last cell cycle (Wartlick et al., 2011). This model is simple and it accounts for the uniform growth seen throughout the wing imaginal disc. However, it also has its downsides – how do cells in the lateral regions of the wing disc, where Dpp is either absent or present at extremely low levels, measure a changeable increase in Dpp signalling levels? Thus, it seems unlikely that this model can account for the growth seen in the peripheral regions of the wing disc.

The slope model and the temporal rule model are both instructive models of growth regulation, where Dpp plays a direct role in influencing the rate of cell proliferation throughout the wing disc. These models also both address a paradox – graded levels of Dpp influence and promote homogenous growth throughout the tissue. However, it is important to note that it is not entirely clear whether uniform growth does indeed occur at all stages of larval development in the wing disc. BrdU labelling in wing discs does seem to indicate a more-or-less uniform pattern of cells in S-phase. However, studies involving the quantification of clonal growth rates suggest that cells in the center of the pouch proliferate slightly faster than those found at the edges of the pouch (Mao et al., 2013). Further studies with novel tools (i.e. a cell cycle counter) will be needed to precisely quantify changes in the rate of cell proliferation in different regions of the wing disc.

\subsection*{1.7.2 The growth equalisation model}

The models described above, which suggest that relative and/or absolute levels of Dpp directly control cell proliferation, have been challenged by studies on \textit{brinker}. One of the most important functions of Dpp in the wing disc is to establish an inverse gradient of Brk, which subsequently aids in establishing the expression domains of \textit{sal} and \textit{omb}. However, studies also show that \textit{brk} is a potent growth repressor. Martin et al. show that the over-expression of \textit{brk} in the pouch severely inhibits proliferation, giving rise to adults with small wings (significant levels of apoptosis were not observed in these discs) (Martin
et al., 2004). Thus, considering that Dpp controls the expression of several genes via the inactivation of brk, does it also regulate growth through the same mechanism? Indeed, it has been shown that the extrusion of Mad double-mutant clones from the epithelium can be prevented by the simultaneous elimination of brk activity (Marty et al., 2000). Furthermore, the overgrowth phenotype (an over-proliferation of cells in the lateral regions) seen in brk mutant wing discs is the same phenotype seen in discs with constitutive Dpp signalling (Capdevila and Guerrero, 1994, Lecuit et al., 1996, Nellen et al., 1996). In order to test whether or not Dpp regulates growth exclusively by repressing brk, Schwank et al. compared the volume of TkvQD and brkM68 clones in different regions of the wing disc. No size differences were observed between TkvQD or brkM68 clones in either the medial or the lateral regions of the wing disc. Indeed, both TkvQD and brkM68 clones overgrew in the lateral regions, but did not exhibit any significant size differences in the medial regions (when compared to wildtype clones) (Schwank et al., 2008). This data suggests that the activation of the Dpp signalling pathway is equivalent to the loss of brk activity. This hypothesis was further supported by the behaviour of TkvQD/brkM68 double mutant clones, which did not overgrow in the medial regions of the wing disc. Finally, Schwank et al. use hypomorphic alleles of dpp and brk to show that discs lacking the activity of both genes also exhibit the pattern of uneven growth seen in brk mutant wing discs. Quantification of PH-3 density indicates that cells in mutant wing discs proliferate at higher rates in lateral regions and at lower rates in the medial regions (Schwank et al., 2008). Thus, the authors suggest that Dpp and Brk are involved in a growth-modulatory system, evening out regional proliferative differences. More precisely, Dpp signalling prevents the expression of brk in the central regions of the wing disc, thereby promoting cell proliferation in this area (Figure 1.11 C). However, high levels of Brk are required in the lateral regions of the wing disc to curb proliferation rates, although it is unclear why these cells would proliferate more in the absence of brk expression. How do proliferative differences arise in the absence of Dpp and Brk? Some speculate that cells in the lateral region have the ability to proliferate at higher rates because they are closer to the periopdial membrane (an epithelium composed primarily of squamous cells), which might secrete a growth factor into the disc proper (Schwank et al., 2008). Another plausible explanation for the origin of these differences is the disruption of mechanical forces. It has been proposed that cells in the center of the wing
disc experience increased compression throughout disc growth, while cells in the peripheral regions become stretched (Aegerter-Wilmsen et al., 2007). If we assume that stretched cells have a higher propensity to divide, then a potent growth repressor (Brk), would be needed to slow the proliferation of these cells to rates seen in the medial regions of the wing disc. At the moment, this is pure speculation. New methods will need to be established in order to alter mechanical forces in the wing disc and assess the effect that these alterations have on growth and cell proliferation.
Figure 1.11 – Different models of Dpp-dependent growth regulation in the wing disc

(A) The slope model states that cells in the wing imaginal disc read the local steepness of the Dpp gradient (i.e. neighbouring cells are able to compare relative levels of Dpp). This information instructs cells whether or not to undergo division. Since the highest and lowest points of the concentration gradient are fixed, growth extends the gradient until it is no longer able to stimulate cell proliferation. (B) The temporal rule model proposes that the absolute levels of Dpp are the main determinant of growth. A cell undergoes mitosis only after a 50% increase in Dpp signalling levels. (C) The growth equalisation model posits that Dpp and Brk act to even out proliferative differences arising in the disc. In the absence of both, lateral cells over-proliferate and medial cells divide at a lower rate. Image sources: (A) Schwank and Basler, 2009 (B) Harmansa et al., 2015 (C) Schwank et al., 2008.
1.7.3 Akiyama and Gibson’s model

In 2015, a paper that was published in Nature generated a lot of controversy in the field – Akiyama and Gibson suggested that the characteristic stripe of dpp expression, running along the A/P compartment boundary, was dispensable for growth during the third larval instar. In order to control Dpp activity, the authors generated an untagged, conditional dpp allele (in this thesis, the allele is referred to as dpp\textsuperscript{FRT-TA}) by deleting an essential exon and replacing it with a rescuing fragment flanked by FLP recombination target (FRTs). Using a disc-specific dpp-gal4 driver and UAS-FLP, they inactivated their allele at the A/P compartment boundary (Figure 1.12 A). Surprisingly, they found that this manipulation had no effect on the morphology or growth of the wing disc (Akiyama and Gibson, 2015). To show that inactivation of their allele was efficient, the authors stained wing imaginal discs with an antibody against the pro-domain of Dpp and with an antibody against phosphorylated Mad. Wing imaginal discs dissected at 72, 96, and 120 hours AEL showed a loss of pro-Dpp and p-Mad immunoreactivity along the A/P boundary, but some residual expression was still seen in the posterior and ventral-anterior hinge regions. At 120 hours AEL, brk de-repression was also observed, although the authors did not look at the expression of Brk at earlier stages of development (Akiyama and Gibson, 2015). This is a key point, since many studies have shown that Brk is a potent growth repressor and that its expression in the wing pouch significantly reduces cell proliferation. Unfortunately, the authors do not address how substantial growth could still occur in the presence of Brk. They attribute the continued growth seen in the absence of the Dpp stripe to low levels of Dpp, originating from the anterior compartment. Indeed, when they inactivate their allele with ci-gal4 and UAS-FLP, the resulting wing discs exhibit a severe reduction in overall size (Figure 1.12 B). Thus, the authors hypothesize the existence of another source of Dpp in the anterior compartment, which cannot be detected by available antibodies, but can function to promote growth throughout the entire wing disc.
Figure 1.12 – Akiyama et al. claim that the stripe of Dpp is dispensable for growth

(A) Inactivation of a conditional dpp allele with a disc-specific dpp-gal4 driver and UAS-FLP gives rise to wing discs with no apparent morphological or growth defects. One way that the authors demonstrated efficient excision was by staining discs with an antibody against the prodomain of Dpp. (B) Inactivation of dpp with ci-gal4 (domain of expression is shown in dark grey) and UAS-FLP does produce severe growth defects. The authors speculate that a previously uncharacterized source of Dpp exists in the anterior compartment. Akiyama et al. suggest that this source cannot be detected with available antibodies, but it can promote growth in the entire wing disc (Akiyama and Gibson, 2015).
1.7.4 Does Dpp regulate growth via the Fat-Hippo signalling pathway?

There are many core signalling pathways in the wing imaginal disc that regulate growth and cell proliferation. Studies, primarily performed by Ken Irvine’s group, suggest a link between the Fat-Hippo signalling pathway and the Dpp signalling pathway. Fat is a large atypical cadherin protein that also seems to function as a transmembrane receptor for a signalling pathway that regulates planar cell polarity and growth (Bennett and Harvey, 2006, Cho et al., 2006, Cho and Irvine, 2004, Fanto et al., 2003, Mao et al., 2006, Matakatsu and Blair, 2006, Silva et al., 2006, Willecke et al., 2006). Fat activity is primarily regulated by two genes, four-jointed (fj) and dachsous (ds), the latter of which seems to associate with Fat and might act as a ligand for the receptor (Yang et al., 2002, Matakatsu and Blair, 2006). In contrast, fj is a protein kinase that phosphorylates the cadherin domains of both Fat and Ds in the Golgi body (Strutt et al., 2004, Ishikawa et al., 2008). In the wing imaginal disc, Fat signalling is influenced by the graded expression of its regulators: uniform expression of Fj and Ds activates Fat signalling around the entire circumference of a cell, which inhibits growth (Rogulja et al., 2008). This is thought to occur because Fat signalling promotes Warts stability and activity, which in turn antagonizes Yorkie (Yki), an oncoprotein in the Hippo pathway that induces the expression of pro-growth and anti-apoptotic genes (Cho et al., 2006).

In 2008, Rogulja et al. suggested that the Dpp morphogen gradient controls growth by regulating the expression and localization of Fat pathway components, including dachsous. The authors propose that Ds preferentially concentrates on the membrane of cells with less Dpp pathway activity (when it contacts a cell with higher Dpp pathway activity) (Rogulja et al., 2008). However, this model was disproved by Schwank et al., who showed that the characteristic gradients of ds and fj and the proximo-distal polarization of Ds were not lost in wing imaginal discs with uniform Dpp activity. Moreover, the gradients of Ds and Fj appeared normal in wing discs mutant for both dpp and brk, suggesting that Fat pathway activity is not dependent on the Dpp morphogen gradient (Schwank et al., 2011). Schwank et al. argue that both signalling pathways promote cell proliferation and growth in a complementary manner. In a previous paper, the authors argue that the function of the Dpp-Brk system is to curb proliferation in the lateral regions of the wing disc (Schwank et al., 2008). On the other hand, inhibition of
the Fat signalling pathway triggers overgrowth in the medial regions of the wing disc (Schwank et al., 2011), indicating that it functions to repress growth in this area (Figure 1.13). Altogether, these results suggest that the combined activities of the Fat and Dpp-Brk systems produce the uniform profile of cell proliferation seen in wildtype wing discs.

Since Yki (the major downstream effector of the Hippo pathway) cannot bind DNA itself, it regulates gene expression by interacting with DNA-binding proteins, such as Scalloped (Sd) and Homothorax (Hth) (Goulev et al., 2008, Wu et al., 2008, Zhang et al., 2008, Peng et al., 2009). In a relatively recent paper, Oh and Irvine argue that the Dpp and Fat-Hippo signalling pathways are interlinked not only because the Dpp gradient regulates the expression of Fat pathway components, but because Yki and Mad directly interact with each other to form a complex, which regulates the expression of different target genes. Although the expression of activated-Yki does not have an effect on the levels of *omb, sal, or dad* in the wing imaginal disc, manipulations in either signalling pathway affect the expression of *bantam* (*ban*), a miRNA that regulates growth. Notably, clones over-expressing Brk (*UAS-Brk*) upregulate their expression of *bantam*. By performing chromatin immunoprecipitation (ChIP) experiments, Oh and Irvine show that Mad and Yki colocalize to the C12 enhancer region of *ban* in vivo (Oh and Irvine, 2011). The results described provide compelling evidence that *ban* is indeed a downstream target of the Dpp and Hippo signalling pathways. However, it is not clear whether *ban* is independently regulated by Yki and Mad or whether it requires activation via a Yki/Mad complex.
Figure 1.13 – Dpp and Fat act in a complementary fashion to promote uniform growth

The following images represent cell proliferation maps generated from EdU incorporation data (the highest values are shown in red, and the lowest values are shown in blue/transparent). In wildtype wing imaginal discs, proliferation is more-or-less homogenous. Uniform levels of Dpp disrupt this homogenous pattern by repressing brk throughout the entire wing disc, resulting in a dramatic increase in proliferation in the lateral regions. By an unknown mechanism, this also leads to decreased proliferation in the medial regions of the disc. The converse is true upon removal of Fat signalling – increased levels of proliferation are seen in the medial regions of the disc. Medial cells curb proliferation in lateral areas via an unknown mechanism. These results suggest that in wildtype discs, the Fat and Dpp-Brk systems complement each other, ultimately resulting in uniform proliferation along the A/P axis (Schwank et al., 2011).
### 1.8 Searching for target genes that regulate growth and proliferation

After decades of research, we still do not fully understand how the Dpp signalling pathway regulates growth in the wing imaginal disc. Most likely, as the growth equalisation model argues, this occurs indirectly via the repression of brk (Schwank et al., 2008). Unfortunately, the target genes regulating growth downstream of Brk have not been well characterized. In 2013, Doumpas et al. performed ChIP-Seq on wing imaginal discs with a highly sensitive antibody against Brk, which identified 2,547 peaks (corresponding to 1,671 genes) that were enriched compared to normalized input control. Previously reported binding sites were identified in genes, such as *dad*, *sal*, and *omb*. Peaks were also found in the following genes: *d-myc*, *foxo*, *ds/ft*, *stg*, *vg*, and *wg* (Doumpas et al., 2013). Doumpas et al. decided to focus on *d-myc*, which contains four putative binding sites for Brk. Moreover, the authors show that Brk and Myc are expressed in complementary domains in late third-instar wing imaginal discs. This provides compelling evidence that Brk might indeed restrict the expression of *d-myc*. In fact, *brk* mutant clones or clones expressing activated Thickveins (TkvQD) display elevated levels of d-Myc in the lateral regions of the wing disc. Furthermore, overexpression of Brk in the patched domain causes a reduction in d-Myc protein levels in this region (Doumpas et al., 2013). Despite this, the authors find that the overexpression of d-Myc (UAS-DMyc) in the pouch only partially rescues the size of *nub>brk* adult wings – ‘rescued’ wings are 15% larger relative to the size of a wildtype wing (Figure 1.14). Since it was proposed that *ban* also regulates growth downstream of Brk (Rogulja et al., 2008), Doumpas et al. decided to rescue the size of *nub>brk* adult wings by co-expressing *ban*, in addition to d-Myc. The combined expression of these two factors rescues the size of *nub>brk* adult wings to a larger extent – ‘rescued’ wings are almost half the normal wing size (Doumpas et al., 2013). When analysing the results of these experiments, it is important to note that the sole expression of UAS-DMyc gives rise to adult wings that are smaller compared to wildtype, suggesting that this transgene might trigger cell death and apoptosis in the pouch (Doumpas et al., 2013). Overall, this study indicates that there must be other target genes that regulate growth and cell proliferation downstream of Brk. It will be interesting to identify these genes and to see if different signalling pathways converge to regulate the expression of similar pro-growth, anti-apoptotic genes.
Figure 1.14 – *bantam* and d-Myc are potential downstream targets of Brk

Wing imaginal discs that express *UAS-Brk* under the control of *nub-gal4* (a pouch driver) give rise to adult wings that are severely decreased in size compared to wildtype. This phenotype can be partially rescued by the co-expression of *bantam* and d-Myc, either alone or in combination. The combined over-expression of the two genes in the pouch (in a *nub>brk* mutant background) gives rise to adult wings that are nearly half the size of wildtype wings.
1.8.1 What is the role of sal and omb in growth control?

It is well known that Dpp signalling controls patterning through the regulation of sal and omb. Both of these genes are transcription factors that are involved in specifying the position of the longitudinal vein (LV) proveins in the wing imaginal disc (Sturtevant et al., 1997, de Celis and Barrio, 2000, Cook et al., 2004). Do they also regulate growth and cell proliferation? In Drosophila, there are two redundant genes with similar expression patterns, spalt major (salm) and spalt-related (salr) (de Celis and Barrio, 2009). In the wing disc, strong reductions in salm and salr expression give rise to adults with small wings (the central region between veins L2 and L4 is almost completely lost) (Organista and De Celis, 2013). Organista and de Celis suggest that this phenotype arises because spalt promotes the progression from G2 to mitosis and that cells with reduced levels of salm/salr accumulate in the G2 phase of the cell cycle (Figure 1.15 A-B). Indeed, wing imaginal discs mutant for both genes show reduced levels of PH-3 in the domain of sal expression. Interestingly, the expression of salm can also partially rescue the small wing phenotype that is caused by the ectopic expression of Brk in the pouch (Figure 1.15 C) (Organista and De Celis, 2013). These results support the notion that Sal proteins regulate cell proliferation in the central regions of the pouch. This function seems to be independent of the Dpp-Brk system, which limits proliferation in the lateral regions of the wing disc, because TkvQD clones still overgrow in the peripheral regions of wing discs mutant for salm/salr (Organista and De Celis, 2013).

Experiments carried out by Zhang et al. have also linked Omb to growth control, although some of these results are contentious in the field. In their paper, Zhang et al. note that uniform expression of Omb induces over-proliferation in lateral cells and suppresses proliferation in medial areas (similar to the phenotype seen in wing discs with uniform Dpp activity). Moreover, RNAi-mediated reduction of Omb with dpp-gal4 induces proliferation in the domain of dpp expression (Zhang et al., 2013). From these results, the authors conclude that omb suppresses proliferation in the medial regions of the wing disc. However, if it is expressed in the peripheral regions, it will promote cell division. This differential activity is attributed to regional control of bantam expression. Zhang et al. show that the ectopic expression of Omb in the hinge (with 30A-gal4) induces the expression of ban in lateral regions of the wing disc. On the other hand, RNAi-
mediated removal of Omb from the A/P boundary (with dpp-gal4) induces high levels of ban in dpp source cells (Zhang et al., 2013). It is unclear how Omb could differentially regulate the levels of bantam in the medial regions and the lateral regions of the wing disc. In their experiments, Zhang et al. also show that the expression of UAS-Brk or UAS-Dad with omb-gal4 increases the levels of BrdU incorporation in medial cells (Zhang et al., 2013). The authors take this as evidence that Dpp signalling generally supresses, not promotes, proliferation in the central regions of the wing disc. Their results are surprising because previous work has shown that brinker is a potent growth repressor and must be removed from the medial regions of the wing disc in order for growth to occur (Martin et al., 2004). Zhang et al. recognize that their results contradict the data from Martín et al. – however, they claim that the loss of proliferating cells in nub>brk wing discs can be attributed to severe cell death. Indeed, Zhang et al. observe some Caspase-3 expression in nub>brk wing imaginal discs. However, the size of the pouch is not rescued by co-expression of P35, a suppressor of apoptosis (Zhang et al., 2013). Thus, it does not seem likely that the growth defects seen in nub>brk wing discs can be attributed to cell death.

In conclusion, it is still unclear how omb precisely regulates growth in the wing disc and whether or not Dpp signalling can actually have a negative effect on cell proliferation in the medial regions of the disc.
Figure 1.15 – Spalt proteins regulate the growth of the wing blade

(A-B) Reductions in *salm* and *salr* activity give rise to small adult wings, which are characterized by the loss of the central region between L2 and L4. (C) The expression of *salm* is able to partially rescue the small wing phenotype associated with the ectopic expression of *brinker* in the pouch. Both of these experiments show that Spalt proteins, which are heavily involved in patterning the wing disc, also regulate growth.
1.9 Aims of the Study

Many patterning signals or morphogens are also involved in regulating growth throughout the development of multi-cellular organisms. However, the functional relationship between patterning and growth still remains unclear. To investigate how these processes are coordinated throughout the development of the wing imaginal disc, I set out to understand the mechanisms that Decapentaplegic (Dpp) utilizes to regulate cell proliferation and growth. Indeed, it is still unclear how a graded signal, such as Dpp, could generate a homogenous pattern of proliferation in a tissue. To resolve this paradox, many different models of growth regulation have been proposed. According to the slope model and the temporal rule model, Dpp plays a direct role in growth control. Conversely, the growth equalisation model argues that Dpp regulates growth indirectly through the repression of brinker. However, these models have not been rigorously tested and verified/refuted. Furthermore, very little is known about the target genes that influence growth control downstream of Dpp signalling and/or Brinker. In this thesis, I embark on several aims:

1) Create tagged, conditional alleles of dpp and brk that allow us to manipulate signalling in a precise spatial and temporal manner.

2) Use these new tools to rigorously test different models of growth regulation.

3) Test the role of d-myc and vestigial in growth control downstream of the Dpp signalling pathway and/or Brinker.

4) Better understand how patterning and growth are coordinated throughout the development of the wing imaginal disc.
Chapter 2. Materials & Methods

2.1 Drosophila strains and maintenance

Flies were raised at 25°C (unless otherwise specified) on standard Drosophila medium containing 4% glucose, 55 g/L yeast, 0.65% agar, 28 g/L wheat flour, 4 ml/L propionic acid and 1.1 g/L nipagin. For experiments with GAL80, flies were kept at 18°C and then transferred to 29°C in order to relieve GAL80-mediated inhibition of GAL4.

For timed experiments at 25°C, larvae were collected at three different time-points: 72, 96, and 120 hours after egg laying (AEL). Larvae undergoing a second molt were identified and transferred to a new food plate (the larvae of Drosophila molt twice before they pupate). The mandibular hooks of the mouth, characteristic in shape and size for each larval instar, were used as a criterion for molting. Since the second larval molt occurs between the second and third larval instars (~72 hours AEL), larvae were either dissected immediately or kept in a 25°C incubator for another 24 or 48 hours.
All the stocks used in this study (and their sources) are listed in the table below:

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<td>Generated for this study by R. Ziukaite</td>
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2.2 Molecular Biology

Cloning tandem gRNA expression vectors:
As described on the CRISPR fly design website, I used Gibson Assembly (available as a kit from NEB) to introduce two protospacer sequences into the CFD4 vector.

1. Choose CRISPR target sites and design primers (use the CFD4 cloning protocol on www.crisprflydesign.org).
2. Using forward and reverse primers, run a PCR with the CFD4 vector as a template (in combination with the Q5 PCR Master Mix from NEB).
3. Digest the CFD4 vector with BbsI.
4. Run the PCR reaction and the digested vector on a 1% agarose gel and cut out the DNA bands (PCR: 600bp; Backbone: 6.4kb).
5. Gel purify insert and backbone.
6. Assemble the plasmid using Gibson Assembly (available as a kit from NEB).
7. Transform into competent bacteria. Plate on Ampicillin plates.
8. Prepare DNA from positive colonies using a Pure-Link Maxi Prep Kit (Life Technologies).

Cloning homology arms into the PTV3 vector:
1. Run a PCR with forward and reverse primers (use BAC or genomic DNA as a template). Please note that genomic DNA is extracted from flies with the ChargeSwitch gDNA Mini Tissue Kit (Invitrogen).
2. Digest the PTV3 vector using the appropriate restriction enzymes.
3. Run the PCR reaction and the digested vector on a 1% agarose gel and cut out the DNA bands.
4. Gel purify insert and backbone.
5. Ligate fragments using T4 DNA Ligase and 10x Ligase Buffer (~1 hour, RT).
7. Prepare DNA from positive colonies using a Pure-Link Maxi Prep Kit (Life Technologies).
2.2.1 Generation of \textit{dpp}^{FRT-CA} and \textit{brk}^{FRT}

In order to engineer tagged, conditional alleles of Dpp and Brk, we used CRISPR/Cas9 to first generate a knockout (KO) of each gene by replacing an essential exon with an att\text{P} site (the att\text{P} site then acts as a landing platform for the reintegration of DNA fragments that contain tagged and altered isoforms of the gene of interest). A KO was first generated by co-injecting the PTV3 vector (Figure 3.1 A) and the CFD4 vector (Figure 3.1 B) into the posterior region of \textit{Drosophila} embryos that express Cas9 in the germline under the control of the \textit{nanos} promoter. The PTV3 vector (generated by Cyrille Alexandre) contains 5’ and 3’ homology arms that facilitate homology directed repair, an att\text{P} site, and a \textit{pax}-Cherry selection marker that allows us to easily identify potential candidates. The CFD4 vector expresses two different gRNAs, which bring the Cas9 enzyme to the correct sites in the genome. Following successful generation of the KO, embryos from this stock were then injected with our re-integration vector (RIV) (Figure 3.1 C). This vector contains an att\text{B} site, a MCS surrounded by FRT sites, and a \textit{pax}-GFP selection marker. Please note that the DNA sequences, which were re-inserted into the att\text{P} sites of KO mutants, can be found in the Appendix.

\textbf{Pertinent Sequences for Dpp KO (PAM sequence in red)} –

\textbf{CRISPR Target 1:} GCTTGGAGCAAAGAAATCATAGG  
\textbf{CRISPR Target 2:} GATGCCGTGGTATGGTGCGATGG  

\textbf{5’ ARM PRIMERS:}  
NotF: GATCCGGGCCGCGAAACCCCAAGGTTATAG  
NheR: GATCAGCTAGGCACACCCCCATCTTTTTAAGG  

\textbf{3’ ARM PRIMERS:}  
SpeF: GATCACTAGCTATAACCGCCATCCACTCAAC  
ApaR: GATCGGGCCCTCCCCTCACCAGTAATCCGTAG  

\textbf{Pertinent Sequences for Brk KO (PAM sequence in red)} –

\textbf{CRISPR Target 1:} GCTGCTGCTATCCATGATTAGG  
\textbf{CRISPR Target 2:} GCCCACAACCTGACCTCGTGG  

\textbf{5’ ARM PRIMERS:}  
NotF: GATCGCGGCGCCGGCAAAACCAGGGGATATAG  
NheR: GATCAGCTAGGCAGTAGTAGGTAGTAAG  

\textbf{3’ ARM PRIMERS:}  
PacF: CGATCGTTAATTAAACAAACACACACACACACATAGA  
AgeR: GATCACCAGGGCGACACAAAATAACCGACACAC
2.2.2 Generation of Myc-GFP with 929CHE vector

Since the antibody used to visualize d-Myc is not available anymore, I decided to make Myc-GFP, a C-terminal insertion of GFP into the d-myc locus. This was done by using the 929CHE vector obtained from Yohanns Bellaïche (group leader at the Institut Curie, Paris). This vector contains the sequences for GFP and pax-Cherry, which is used as a selection marker in this case and is flanked by LoxP sites to enable removal upon successful identification of candidates. There are also multiple cloning sites, which allow for the insertion of 5’ and 3’ homology arms (Gibson Assembly, NEB kit). After successful cloning of the 929CHE vector, it was injected into nos-cas9 embryos together with the CFD4 vector (containing the sequences for our gRNAs).

CRISPR Target 1: AACGAATCCGCTCGGTTAGTG
CRISPR Target 2: ATAGTGTTGCTCATACTACG

5’ ARM PRIMERS:
F: CCCGGGCTAATTATGGGGTGTCGCCCTTCGATTATGTTTTCGGATATCGATGATATCCGG
R: CCCGGGCTAATTATGGGGTGTCGCCCTTCGATTATGTTTTCGGATATCGATGATATCCGG

3’ ARM PRIMERS:
F: GTATAATGTATGCTATACGAAGTTATGGCAGTGTTGTCTCATACTATCGGCTTAAAGCG
R: GCCCTTGAACTCGATTGACGCTCTTCGACCTTTTCTCCTCCAAGGATGCCTCTC

2.2.3 Generation of brkGAL4 using the Brk KO

The brkGAL4 allele was generated by injecting RIV-GAL4 (Figure 5.1 B) into brk KO embryos containing an attP site. Following attP/B-mediated recombination, larvae were screened for proper insertion by observing pax-GFP expression. Once candidates were identified, they were properly balanced with FM7-GFP.
2.3 Immunohistochemistry and Image Acquisition

Imaginal discs (dissected in PBS) were fixed in 4% paraformaldehyde (in PBS) for approximately 30 minutes and stained with primary antibody overnight at 4 degrees Celsius. The next day, samples were washed 3x 15 minutes in 0.5% PBS Triton and stained with secondary antibody (1:500) at room temperature for 2 hours. Following incubation with secondary antibodies, discs were washed again 3x 15 minutes in 0.5% PBS Triton. Samples were mounted on glass slides with Mowiol. All images were acquired using a Leica SP5 microscope.

In this study, Alexa-conjugated secondary antibodies were used (Thermo Scientific Waltham, MA; 1:500). The following table lists all of the primary antibodies used in this study and their corresponding sources.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Species</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-Brinker</td>
<td>Hilary Ashe</td>
<td>Guinea Pig</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-Brinker</td>
<td>Aurelio Teleman</td>
<td>Guinea Pig</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-HA</td>
<td>Cell Signalling</td>
<td>Rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-Homothorax</td>
<td>Richard Mann</td>
<td>Rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-Phospho-Histone H3</td>
<td>Abcam; [HTA28]</td>
<td>Rat</td>
<td>1:5000</td>
</tr>
<tr>
<td>anti-Phospho-Smad1/5</td>
<td>Cell Signalling; 41D10</td>
<td>Rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-DSRF</td>
<td>Active Motif; 39093</td>
<td>Rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-Omb</td>
<td>Gert Pflugfelder</td>
<td>Rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-Vestigial</td>
<td>Sean Carroll</td>
<td>Rabbit</td>
<td>1:10</td>
</tr>
<tr>
<td>anti-Zfh2</td>
<td>Chris Q. Doe</td>
<td>Rat</td>
<td>1:400</td>
</tr>
<tr>
<td>anti-V5</td>
<td>Invitrogen; 2F11F7</td>
<td>Mouse</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-GFP</td>
<td>Abcam; ab290</td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
2.4 qRT-PCR Protocol

Third instar larvae were heat shocked for 30 minutes at 102 hours AEL and wing discs were dissected in PBS at 120 hours AEL, before being transferred to PBS-Tween 20. Samples were spun down, and the pellets were snap-frozen in liquid nitrogen, stored at -80 degrees Celsius or processed immediately. RNA from the dissected discs was extracted with the Macherey-Nagel NucleoSpin RNA isolation kit, and cDNA was obtained with the Roche Transcriptor high fidelity cDNA synthesis kit. Quantitative PCR was performed in triplicates using the MESA Green qPCR Mastermix Plus for SYBR assay. All measurements were normalized to actin-5c, alpha-tubulin and TATA box binding protein mRNA levels.

Forward primer for qRT-PCR: CGCCGGCAAATCCAAAAATG
Reverse primer for qRT-PCR: TGCTGTTGCTGATTGTGGC

2.5 Data Analysis

Every experiment was repeated at least once. All images were analysed in Fiji (ImageJ) and data was plotted using either GraphPad Prism or ggplot2 functions in R Studio. Error bars in plots denote the standard deviation (SD) unless stated otherwise, and the statistical tests used to evaluate significance are described in each figure legend. Statistical significance is denoted as follows: ns: p>0.05, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

2.5.1 Defining growth in our model system

This thesis closely examines how Decapentaplegic (Dpp) signalling affects the overall growth of the wing imaginal disc. Two assumptions are made: the wing imaginal disc is a two-dimensional epithelium and perturbations in Dpp signalling do not affect the final size of a cell. Thus, we can define growth as follows –

\[ \text{Growth} = \frac{\text{cell size} \times \text{cell number}}{\text{time}} \]
Since cell size is presumed to be constant, growth is attributed to the change in the number of cells over time (the sum of cells generated by division minus the number of cells lost to apoptosis). As mentioned previously, the amount of cell death in the wing disc is negligible (Milan et al., 1997). Thus, in our case, the size of a wing disc should reflect the amount of cell proliferation that occurs. As stated previously, many different growth factors affect cell proliferation in the wing disc. However, this study examines how patterning signals, also known as morphogens, regulate growth throughout development.

2.5.2 Quantification of growth and cell proliferation

The surface area of all wing discs and adult wings was measured using Fiji (ImageJ). In Figure 4.3, PH-3 density was quantified using the following script, which was developed in Fiji (ImageJ):

```java
waitForUser("Define oval");
run("Measure");
area=getResult("Area", 0);
run("Duplicate");
run("8-bit");
run("Gaussian Blur", "sigma=1");
setAutoThreshold("Li dark");
run("Convert to Mask");
run("Watershed");
run("Analyze Particles", "clear add");
n=roiManager("count");
mit_index=n/area;
setResult("mitotic index",0,mit_index);
```

In Figure 4.8, PH-3 density was calculated in different regions of wing imaginal discs using a program written with Python (available upon request). This program reads and processes PNG images. For every image, the program applies a Gaussian filter to remove any noise and then it applies a threshold to generate the outline of the wing disc. After identifying the contour, the program splits the wing imaginal disc into three different regions: the left (lateral), medial, and right (lateral) regions. Following this, the program counts all of the PH-3 “spots” in the wing disc and assigns them to the corresponding regions. Finally, the surface area of each region is calculated. Thus, all of our quantifications are normalized to surface area. A more detailed explanation of this program can be found in section 4.4.1.
Chapter 3. Results

Exploring the spatial and temporal requirements of Dpp signalling for normal growth and cell proliferation

3.1 Chapter Introduction

In order to visualize Dpp protein, several labs have generated Dpp-GFP expressing transgenes, which are expressed using the UAS/GAL4 system (Entchev et al., 2000, Teleman and Cohen, 2000). Although these transgenes can rescue Dpp loss-of-function alleles, they do not allow visualization of the endogenous protein. Furthermore, there is still no suitable antibody against the mature secreted protein. With these issues in mind, a senior investigator in our laboratory (Cyrille Alexandre) set out to use CRISPR/Cas9 technology to generate a tagged, conditional allele of Dpp that would not only allow us to visualize endogenous protein, but would enable us to manipulate Dpp signalling in a precise spatial manner. This new allele was constructed by deleting an essential exon and replacing it with a rescuing fragment flanked by Flp Recombination Targets (FRTs). This conditional allele makes it possible to remove Dpp from a specific region of interest in the wing disc, such as the pouch, and to assess the effect on patterning, growth, or proliferation. In this section, I use this new tool to answer three important questions:

1. What is the expression pattern of endogenous Dpp in the wing disc?
2. Is the central stripe of Dpp, secreted along the A/P boundary, required for growth during the third larval instar?
3. Is Dpp signalling in the wing disc continuously required throughout larval development?

It is worth noting that a large portion of the work in this section addresses a controversial claim made in a recent paper in Nature (Akiyama and Gibson, 2015). As part of this study, Akiyama et al. have also created a conditional allele of Dpp (herein referred to as \( dpp^{FRT-TA} \)) by deleting an essential exon and replacing it with a rescuing fragment flanked by FRT sites. They found that excision of this allele within the normal domain of \( dpp \)
expression (with dpp-gal4) had no adverse effect on growth. Inactivation was confirmed using an antibody against the prodomain of Dpp and an antibody against pMad. In contrast to the conclusions made by this study, I argue that the removal of the central stripe of Dpp, even during the third larval instar, has a deleterious effect on growth. Furthermore, I also provide data that suggests that the inactivation of dpp\textsuperscript{FRT-TJ} is not efficient and could lead to misleading results.

3.2 Generating HA-tagged dpp\textsuperscript{FRT-CA} and V5-tagged brk\textsuperscript{FRT}

In order to engineer a tagged, conditional allele of Dpp, we used CRISPR/Cas9 to first generate a knockout of the gene by replacing an essential exon with an attP site. This site then acts as a landing platform for the reintegration of DNA fragments that contain tagged and altered isoforms of the gene of interest. For example, a knockout of Dpp was first generated by co-injecting the PTV3 vector (Figure 3.1 A) and the CFD4 vector (Figure 3.1 B) into the posterior region of Drosophila embryos that express Cas9 in the germline under the control of the nanos promoter. The PTV3 vector (generated by Cyrille Alexandre) contains 5’ and 3’ homology arms that facilitate homology directed repair, an attP site, and a pax-Cherry selection marker that allows us to easily identify potential candidates. The CFD4 vector expresses two different gRNAs, which bring the Cas9 enzyme to the correct sites in the genome. In order to generate a Dpp knockout, we removed the second coding exon (including 100 base pairs before the splice acceptor) and replaced this entire region with an attP site. Following successful generation of the knockout, embryos from this stock were then injected with our re-integration vector (RIV) (Figure 3.1 C). This vector contains an attB site, a MCS surrounded by FRT sites, and a pax-GFP selection marker. In the case of Dpp, we re-inserted the pro-domain, the second coding exon with two hemaglutinin (HA) tags, and the 3’ UTR (Figure 3.1 D). It is important to note that the HA tag is located downstream of all three furin cleavages sites, thereby allowing us to visualize the distribution of mature protein.

In order to be able to manipulate the expression of brk in different regions of the wing imaginal disc, I also used the same strategy to generate brk\textsuperscript{FRT}. Since brk only has one coding exon, this fragment was removed and replaced with an attP site in order to
generate a knockout. Following this, the RIV vector was used to re-insert the cDNA that was removed (including the 3’ UTR of Brk) with a V5 tag (Figure 3.1 E). In the following sections, I will describe how these conditional alleles have been used to manipulate signalling in a precise manner and how they have allowed us to test current models of growth regulation.

3.2.1 Visualizing endogenous levels of Dpp and Brk in wing imaginal discs

To visualize the expression of Dpp and Brk, I stained homozygous dpp\textsuperscript{FRT-CA} and brk\textsuperscript{FRT} wing imaginal discs at three different time-points in larval development: 72 hours AEL (L2/L3 molt), 96 hours AEL (mid-L3) and 120 hours AEL (late L3). Dpp immunoreactivity was visualized with an antibody against HA. At all time-points, Dpp expression was detected in a narrow stripe (4-5 cells) at the A/P boundary (Figure 3.2 A-C). It is interesting to note that the width of the stripe does not seem to change from the beginning to the end of the third larval instar. Brk immunoreactivity was visualized with an antibody against V5. Brk is first detected in wing discs at 72 hours AEL, at the onset of the third larval instar (Figure 3.2 D). The first cells to express Brk are located in the lateral regions, at the edge of the wing imaginal disc. At later stages of larval development, the pattern of expression is refined and maintained in the lateral regions (Figure 3.2 E-F). Brk is never detected in the medial regions of the wing disc. Altogether, these results suggest that before the third larval instar, Dpp signals throughout the entire wing disc. In other words, before L3, there are high enough levels of Dpp throughout the entire wing disc to suppress brk expression. However, as the wing disc grows, the levels of Dpp at the edge of the wing disc decrease. Eventually, a certain threshold is reached and brk is de-repressed.
Chapter 3. Results

A. 5' Homology Arm
   - MCS A
   - attP
   - Lox P
   - Pax CHE
   - Lox P
   - MCS B
   - Amp R

B. U6:1
   - gRNA
   - U6:3
   - gRNA
   - Amp R

C. attB
   - FRT
   - MCS
   - FRT
   - Lox P
   - Pax GFP
   - Amp R

D. HA
   - FRT
   - pro-domain
   - Dpp
   - 3' UTR
   - FRT

E. V5
   - FRT
   - Brk
   - 3' UTR
   - FRT
Figure 3.1 – The cloning strategy behind $dpp^{FRT-C4}$ and $brk^{FRT}$

(A) PTV3 vector used to generate knockouts for both Dpp and Brk. The vector contains 5’ and 3’ homology arms for homology directed repair, an attP site, and a $pax$-Cherry selection marker. (B) CFD4 vector that expresses two different gRNAs. (C) Reintegration vector (RIV) that contains an attB site, a MCS surrounded by FRT sites, and a $pax$-GFP selection marker. (D) Schematic showing the strategy behind the generation of $dpp^{FRT-C4}$. Non-coding exons are shown in grey, coding exons are shown in brown, and mature protein is shown in green. The second coding exon was removed and replaced with an attP site. This site was then used to re-insert the pro-domain of Dpp, the second coding exon with an HA tag and the 3’UTR of Dpp. (E) Schematic showing the cloning strategy behind $brk^{FRT}$. The coding exon is shown in pink. This entire region was removed and replaced with an attP site. This site was then used to re-introduce the cDNA with a V5 tag and the 3’ UTR of Brk.
Figure 3.2 – Visualizing endogenous levels of Dpp (HA) and Brk (V5)

(A–C) Homozygous dpp\textsuperscript{FRT-CA} wing discs were stained for HA (Dpp) at 72, 96, and 120 hours AEL. Dpp is expressed from a narrow stripe of cells at the A/P boundary of the wing imaginal disc. (D–F) Homozygous brk\textsuperscript{FRT} wing discs were stained for Brk (V5) at 72, 96, and 120 hours AEL. Brk is first expressed in several cells at the lateral edges of the wing disc. It is important to note that Brk expression could not be detected before the third larval instar.
3.3 Removal of Dpp in the prospective wing blade disrupts growth

As described in previous sections, our conditional allele allows us to inactivate Dpp in any region of interest by using a specific GAL4 driver to express FLP recombinase. Thus, in an attempt to clarify the role of Dpp in the growth of the pouch, I first used rn-gal4 in combination with UAS-FLP to inactivate Dpp signalling in the prospective wing blade. In this experiment, wing imaginal discs were dissected at both 96 and 120 hours AEL in order to observe growth defects at different developmental time points (Figure 3.3 A-D’). Since our allele is tagged, I confirmed proper gene inactivation by staining for HA. At both 96 and 120 hours AEL, no HA immunoreactivity was detected in the pouch (not shown). Furthermore, immunostainings with anti-Brk showed that brk expression was de-repressed throughout the entire prospective wing blade. In these experiments, growth was markedly impaired and pouch size was assessed using an antibody against Homothorax (Hth). At both 96 and 120 hours AEL, mutant wing discs (\textit{dpp^{FRT-CA}; rn-gal4, UAS-FLP}) exhibited a 59% reduction in pouch size when compared to control (homozygous \textit{dpp^{FRT-CA}}) wing discs (Figure 3.3 E-F). Although the pouch of experimental discs was significantly smaller than that of control discs, it was not completely eradicated. One possible reason for this is the late activity of our driver (rn-gal4), which is first expressed only at the end of the second larval instar. Another possibility is that residual signalling by other members of the TGFβ super-family of proteins, such as glass bottom boat (gbb) (Ray and Wharton, 2001). Eventually, mutant larvae do eclose as adults with very small wings, resembling those seen in vestigial (vg) mutant flies (Figure 3.3 G-H). Based on these observations, we conclude that \textit{dpp} expression is required in the pouch for normal growth.
Figure 3.3 – Growth of the prospective wing requires Dpp expression within the pouch
(A-D’) Inactivation of dpp<sup>FRT-CA</sup> in the pouch (with rn-gal4, UAS-FLP) leads to de-repression of brinker and reduced growth (shown here in discs fixed at 96 and 120 hours AEL). The edge of the pouch is marked by the weak inner ring of homothorax expression. However, since the outer ring is more readily visible, this is the marker we used to measure pouch size (thus overestimating). (E, F) Quantification of the area enclosed by the Hth outer ring at the two stages (each dot/square represents one imaginal disc). (G, H) Wings from control (G) and experimental (J) adults. The scale bar, which represents 50 microns, applies to panels (A-D’). In panels (E-F) statistical significance was assessed with a Student’s t-test, assuming equal variance and a Gaussian distribution (p<0.0001).
Chapter 3. Results

3.4 Wing growth requires the central stripe of Dpp expression

In 2015, an article was published that suggested that the central stripe of Dpp (located at the A/P boundary) was not required for wing disc growth during the second half of larval development (Akiyama and Gibson, 2015). In this study, the authors created a conditional allele of Dpp by inserting FRT sites surrounding the first coding exon (this allele is referred to as \textit{dpp}^{FRT-TA}). Surprisingly, inactivation of \textit{dpp}^{FRT-TA} with \textit{dpp-gal4} and \textit{UAS-FLP} was reported to have no adverse effect on growth. In this genetic background, pMad and Omb were disrupted, indicating that \textit{dpp} expression was indeed impaired. However, when they inactivated their allele with a pouch-specific driver (\textit{nub-gal4}) or a driver solely expressed in the anterior compartment (\textit{en-gal4}), growth defects were observed. It was therefore suggested by the authors that the stripe of Dpp expression may not be needed for growth because of the existence of another source of Dpp in the anterior compartment (Akiyama and Gibson, 2015).

We wanted to address this surprising claim before doing any further experiments pertaining to Dpp and growth. First, we decided to repeat the same experiment (inactivation of Dpp with \textit{dpp-gal4} and \textit{UAS-FLP}) with our conditional allele. This was done using the same \textit{dpp}^{BLK-gal4} transgene (Staehling-Hampton et al., 1995) to enable proper comparison with the results of Akiyama and Gibson (Akiyama and Gibson, 2015). The \textit{dpp}^{BLK-gal4} driver was used to drive the expression of FLP recombinase in \textit{dpp}^{FRT-CA} homozygotes. Following this, the efficiency of excision was assessed by staining wing imaginal discs with anti-HA, which marks functional, mature Dpp in unexcised alleles. At both 96 and 120 hours AEL, HA immunoreactivity was eliminated from the whole disc, except for a small area located outside of the pouch, in the posterior hinge (Figure 3.4 A-D). Such residual expression is reproducible and likely represents an area where \textit{dpp}^{BLK-gal4} does not recapitulate the endogenous Dpp expression domain. However, in the rest of the wing imaginal disc, including the entire pouch, the \textit{dpp}^{BLK-gal4}, \textit{UAS-FLP} combination seemed to trigger proper gene inactivation. This was associated with the de-repression of \textit{brinker} (Figure 3.4 A-D) and a marked reduction in pouch size (84%). This was quantified by staining both control and mutant wing imaginal discs with anti-Hth and measuring the area enclosed by the strong ring of Hth expression (Figure 3.4 E). This
result contradicts the report that \( dpp^{FRT-CA}; dpp^{BLK-gal4} > UAS-FLP \) wing imaginal discs attain a normal size and express Brk throughout the pouch at 120 hours AEL. Thus, our goal was to understand the discrepancy between these two results.

### 3.4.1 The expression of \( dpp \) is confined to the stripe along the A/P boundary

As mentioned above, Akiyama and Gibson hypothesized the existence of an alternate source of low-level Dpp in the anterior compartment of the wing imaginal disc (Akiyama and Gibson, 2015). This source of Dpp would provide sufficient signalling activity to permit growth in the pouch. Indeed, long-term lineage tracing using G-TRACE suggests that progenitors of cells located anterior to the stripe could express Dpp, at least at some point during development (Evans et al., 2009). To gain more information on the pattern of \( dpp \) expression in the wing disc, we generated a reporter line (\( dpp^{FRT-REP} \)) that expresses the readily detectable marker CD8-GFP from the \( dpp \) locus (Figure 3.5 A). An excisable cassette expressing Dpp was included upstream of the CD8-GFP coding sequences to allow expression of functional Dpp during embryogenesis (which requires both functional alleles). Thus, CD8-GFP is only expressed after FLP-mediated excision of this cassette. Excision was induced after embryogenesis with \( rn-gal4 \) and \( UAS-FLP \), making CD8-GFP a reporter of \( dpp \) transcription in the pouch. At 72, 96, and 120 hours AEL, GFP expression was only detected in a narrow stripe of cells along the A/P boundary (Figure 3.5 B-D). This result suggests that the activity of the \( dpp \) promotor anterior to the stripe must be very low or occur at a time before 72 hours AEL. Therefore, our results do not support the idea that an alternative source of Dpp exists outside of the central stripe during the third larval instar.
Figure 3.4 – Growth of the prospective wing requires the stripe of Dpp expression

(A-D) Inactivation of \(dpp^{FRT-CA}\) in the normal domain of Dpp expression (with \(dpp^{BLK-gal4, UAS-FLP}\)) leads to de-repression of \(brinker\) and severely reduced growth (shown here in discs fixed at 96 and 120 hours AEL). A zone of \(brinker\) repression can be seen in the prospective hinge around weak residual Dpp expression (arrowhead in C, D). (E) Quantification of pouch area (area enclosed by the outer ring of Hth) in control and mutant wing imaginal discs (each dot/square represents one disc). Statistical significance was calculated using a Student’s t-test, assuming equal variance and a Gaussian distribution (\(p<0.0001\)). Scale bar = 50 microns.
Figure 3.5 – *dpp*<sup>FRT-REP</sup> expression is confined to the stripe along the A/P boundary

(A) A schematic of the *dpp*<sup>FRT-REP</sup> allele, designed to act as a reporter following excision of the FRT-flanked HA-Dpp-containing cassette. (B-D) Expression of CD8-GFP from *dpp*<sup>FRT-REP</sup> within the pouch at 72, 96, and 120 hours AEL. Expression is only seen in the stripe. Scale bar = 50 microns.

Figure 3.6 – Excision with an untagged version of *dpp*<sup>FRT-CA</sup> also produce growth defects

(A) Control wing disc stained with an antibody against Brk. (B) Inactivation of *dpp*<sup>FRT-NoTag</sup> with *dpp-gal4* and *UAS-FLP* also results in growth defects, including a severe reduction in pouch size. De-repression of *brk* is seen throughout the wing imaginal disc (except for a region in the posterior hinge). Scale bar = 50 microns.
3.4.2 Growth defects are also associated with an untagged version of \(dpp^{FRT-CA}\)

Many scientists have struggled to tag Dpp downstream of its three furin cleavage sites because a big tag, such as GFP (238 amino acids) likely interferes with protein production or function and results in flies that are not homozygous viable. This is one of the reasons we selected an HA tag (11 amino acids) for \(dpp^{FRT-CA}\). However, we were still concerned that two HA tags could make our conditional allele act as a hypomorph and that this was the reason for the lack of growth seen in \(dpp^{FRT-CA}; dpp-gal4, UAS-FLP\) wing imaginal discs. In order to test this, we made an untagged version of \(dpp^{FRT-CA}\) (herein referred to as \(dpp^{FRT-NoTag}\)). Besides the absence of any tag, this allele is exactly the same as \(dpp^{FRT-CA}\). We used this conditional allele to inactive Dpp in its own domain of expression with \(dpp-gal4\) and \(UAS-FLP\). As seen with \(dpp^{FRT-CA}\), wing discs showed severe growth phenotypes (Figure 3.6 A-B). De-repression of \(brk\) was seen throughout the entirety of mutant wing discs, except for a small region in the posterior hinge (Figure 3.6 B). Thus, it is very unlikely that the growth defects seen in \(dpp^{FRT-CA}; dpp-gal4, UAS-FLP\) wing imaginal discs are due to the hypomorphic nature of our tagged, conditional allele.

3.4.3 Inactivation of \(dpp^{FRT-TA}\) with \(dpp-gal4\) leads to the de-repression of \(brk\) only at late stages of larval development

In the previous sections of this chapter, I have confirmed that inactivation of \(dpp^{FRT-CA}\) with \(dpp-gal4, UAS-FLP\) leads to the abrogation of growth. Furthermore, I have shown that during the third larval instar, \(dpp\) expression is most likely confined to a narrow stripe along the A/P boundary and that \(dpp^{FRT-CA}\) does not act as a hypomorph due to the presence of two HA tags. Since our experiments were done with the same \(dpp-gal4\) driver used by Akiyama and Gibson (Akiyama and Gibson, 2015), I began to think that perhaps there was a problem with the \(dpp^{FRT-TA}\) allele. For example, perhaps the excision of the first coding exon in \(dpp^{FRT-TA}\) could be inefficient. In order to test this, we wanted to re-examine \(dpp^{FRT-TA}; dpp-gal4, UAS-FLP\) wing imaginal discs, not only at 120 hours AEL, but also at much earlier stages in larval development. In their paper, Akiyama and Gibson do not detect immunoreactivity against Dpp and pMad in mutant wing discs at 72, 96, and 120 hours AEL (Akiyama and Gibson, 2015). However, it is conceivable that immunofluorescence images may not show low levels of protein, which could very well
be sufficient for growth in this case. Thus, we decided to look at the expression pattern of Brk at earlier time points (before 120 hours AEL), something that the authors had failed to do. We confirmed that $dpp^{FRT-TA}; \ dpp\text{-gal4}, \ UAS\text{-FLP}$ wing discs attain a normal size and express Brinker at 120 hours AEL (Figure 3.7 C). However, at 90 and 96 hours AEL, during the growth phase, Brinker was still repressed within the pouch, a clear indication that Dpp signalling is still active at these stages (Figure 3.7 A-B). These results suggest that in $dpp^{FRT-TA}; \ dpp\text{-gal4}, \ UAS\text{-FLP}$ wing imaginal discs, Dpp signalling is not fully eradicated until 120 hours AEL, when most growth has already taken place. This also implies that the $dpp^{FRT-TA}$ allele may not be as readily inactivated by $dpp\text{-gal4}$ and $UAS\text{-FLP}$ as the $dpp^{FRT-CA}$ allele.

**Figure 3.7 – Inactivation of $dpp^{FRT-TA}$ only leads to the de-repression of brk at late stages**

(A-C) Inactivation of $dpp^{FRT-TA}$ in the normal domain of Dpp expression (with $dpp\text{-gal4}$ and $UAS\text{-FLP}$) only leads to Brk derepression after most growth has taken place (120 hours AEL). At earlier stages (90 and 96 hours AEL), Brinker is still repressed in the pouch, indicating residual Dpp signalling activity. Scale bar = 50 microns (applies to all three panels).
3.4.4 Inactivation efficiency is addressed for both alleles with qRT-PCR

In order to test the efficiency of gene activation for both of the alleles, we used a hs-FLP transgene to express FLP recombinase under identical heat-shock conditions and measured brinker expression by qRT-PCR. This experiment was done by Pablo Sanchez-Bosch (a PhD student in the laboratory of Konrad Basler). Please note that Pablo Sanchez-Bosch and Konrad Basler were collaborators that we worked with to understand the role of the Dpp stripe in the growth of the wing disc during the third larval instar. Our results were published online in eLife in 2017 (Bosch et al., 2017). In this experiment, Pablo inactivated \( dpp^{FRT-TA} \) and \( dpp^{FRT-CA} \) with hs-FLP (induced at 102 hours AEL). Then, he dissected wing discs at 120 hours AEL and quantified brinker mRNA levels by performing qRT-PCR on mutant and control samples. The results of this experiment show that brinker expression was de-repressed in both cases, but less so with \( dpp^{FRT-TA} \) compared to \( dpp^{FRT-CA} \) (Figure 3.8 A). Altogether, these results provide further proof that \( dpp^{FRT-TA} \) is less readily excised than \( dpp^{FRT-CA} \).

![Graph showing normalized brinker mRNA levels](image)

**Figure 3.8** – Inactivation efficiency for \( dpp^{FRT-TA} \) and \( dpp^{FRT-CA} \) is addressed with qRT-PCR (A) Efficiency of inactivation for \( dpp^{FRT-TA} \) and \( dpp^{FRT-CA} \) is addressed by inducing the expression of hsp70-FLP at 102 hours AEL and performing qRT-PCR on wing discs dissected at 120 hours AEL. Graph shows the level of brinker mRNA normalized to that in non-heat-shocked controls. Each bar shows average mRNA level +/- SEM. A two-way ANOVA test showed statistically different brinker expression between \( dpp^{FRT-TA} \) and \( dpp^{FRT-CA} \) (p<0.0001).
3.5 Dpp expression is required in the wing imaginal disc throughout larval development for normal growth

I also wanted to understand precisely when (during larval stages) Dpp was required for growth. In order to investigate this, I inactivated $dpp^{FRT-CA}$ at different times using $dpp$-gal4, UAS-FLP and tub.gal80ts. Larvae were shifted from 18 to 29 degrees Celsius at 96, 144, and 192 hours AEL in order to inactivate GAL80 and permit GAL4 activity (Figure 3.9 A). Then, wandering L3 larvae were dissected, fixed, and stained with an antibody against Brk. In addition, $dpp^{FRT-REP}$ was incorporated into the genetic background ($dpp^{FRT-REP}/ dpp^{FRT-CA}; dpp$-gal4, UAS-FLP/tub.gal80ts), allowing us to visualize where Dpp was inactivated (please refer to section 3.4.1 for a description of $dpp^{FRT-REP}$). Finally, pouch surface area was measured in mutant wing discs and normalised to the average surface area of control discs (those with constitutively active GAL80).

The inactivation of Dpp at 96 and 144 hours AEL (with $dpp$-gal4 and UAS-FLP) resulted in severe growth defects and widespread de-repression of brk (Figure 3.9 D-E). By contrast, late excision of Dpp (192 hours AEL) had a relatively mild (although still significant) effect on pouch size (Figure 3.9 C, F). Interestingly, these wing discs appeared to have a wildtype pattern of Brk expression despite the mild reduction in pouch size (Figure 3.9 C, F). The weaker impact of Dpp inactivation at 192 hours AEL could be due to the perdurance of Dpp or downstream events. It is also important to note that inactivation of Dpp at 192 hours AEL is not instantaneous, as it will take time for GAL4 activity to build up. Furthermore, any effect on growth might be difficult to detect at this time point because the growth rate of imaginal discs decreases with age (Johnston and Sanders, 2003). Nevertheless, our experiments clearly show that Dpp must be continuously produced, at least up to mid-third larval instar (and probably beyond this time point), for the normal growth of the wing imaginal disc.
**Figure 3.9 – Dpp inactivation at different time-points in larval development**

(A) Timeline of larval development at 18°C (hours after egg laying). Flies were kept in a cage at 18°C. Larvae were picked from food plates and transferred to 29°C at three different time-points: 1st molt, 2nd molt, and 48 hours after the 2nd molt (~192 hours AEL). Crawling larvae were dissected and fixed. (B-E) Wing imaginal discs dissected from crawling larvae following the inactivation of \(dpp^{\text{FRT-CA}}\) at 96, 144, and 192 hours AEL. Discs were shifted from 18 to 29 degrees Celsius at the indicated time points in order to remove GAL80 suppression of GAL4 activity. Inactivation of Dpp at 96 and 144 hours AEL leads to the de-repression of \(brk\) and a significant decrease in pouch size. However, Dpp inactivation at 192 hours AEL has a milder effect on pouch size and does not seem to change the expression pattern of Brk. (F) The pouch area of each wing imaginal disc was measured (this was approximated using the tissue folds). The surface area of the pouch for each wing imaginal disc was normalized to the average pouch area of control discs (n=20 for control discs, n=15 for 192 and 144 hours AEL, and n=9 for 96 hours AEL). Measurements for each time point were compared to the control area with a Student’s t-test. The p-value was highly significant (<0.0001) for each comparison except for 192 hours AEL vs. 240 hours AEL (p-value = 0.0028). Scale bar = 50 microns.
3.6 Chapter Discussion

In Chapter 3, I have described the methodology used to generate two conditional alleles with CRISPR/Cas9 - dpp$^{FRT-CA}$ and brk$^{FRT}$. These alleles have been used to investigate the requirement of Dpp signalling for the growth of the wing imaginal disc. First, we showed that Dpp is required in the prospective wing blade (the pouch) for normal growth, as dpp$^{FRT-CA}$, rn-gal4, UAS-FLP wing discs have a significantly reduced pouch size. Our experiments also address a recent claim that the central stripe of Dpp, which is produced along the A/P compartment boundary, is not required for the normal growth of the wing imaginal disc during the third larval instar. Indeed, inactivation of dpp$^{FRT-TA}$ in the normal domain of Dpp expression (with FLP driven by a disc-specific dpp regulatory element) was reported to have a minimal impact on growth (Akiyama and Gibson, 2015). The authors suggest that Dpp expressed from another source in the anterior half of the pouch could suffice to sustain growth. Consistent with this hypothesis, inactivation of dpp$^{FRT-TA}$ throughout the pouch with nubbin-gal4 and UAS-FLP led to a strong growth reduction. This is an observation that we confirmed with our conditional allele and rn-gal4, UAS-FLP. However, inactivation of dpp$^{FRT-CA}$ with dpp-gal4 and UAS-FLP (the same source of FLP used by Akiyama and Gibson, 2015) led to a severe impairment of growth, in contrast to the findings with dpp$^{FRT-TA}$. Our analysis of Brk expression at 90, 96, and 120 hours AEL in dpp$^{FRT-TA}$; dpp-gal4, UAS-FLP wing discs shows that brinker is only de-repressed in the pouch at 120 hours AEL, during the final stages of larval development. This data suggests that dpp$^{FRT-CA}$ is more readily inactivated than dpp$^{FRT-TA}$, a hypothesis that is also supported by qRT-PCR results that show a significant difference in the levels of brinker mRNA between dpp$^{FRT-CA}$ and dpp$^{FRT-TA}$ wing imaginal discs exposed to hs-FLP at 102 hours AEL. It is likely that in dpp$^{FRT-TA}$; dpp-gal4, UAS-FLP wing discs, some dpp expressing cells would linger long enough to provide sufficient signalling activity to repress brinker and therefore promote growth and cell proliferation. Eventually, at some point after 96 hours AEL, these lingering cells would undergo proper excision so that by the end of the third larval instar, no Dpp signalling would remain and brk would be fully de-repressed in the pouch. The reason for this delayed inactivation is unclear, but a closer look at the sequence for dpp$^{FRT-TA}$ reveals that one of the FRTs is very close a LoxP site, which could impair recombination
(both form hairpin structures). In any case, my results show that removal of the central stripe of Dpp leads to the impairment of growth.

After assessing the spatial requirements of Dpp in the wing disc, I also wanted to understand if Dpp signalling was continuously required for growth during the third larval instar. By inactivating Dpp at different time-points with a *tub.GAL80ts* transgene, I was able to show that the central stripe of Dpp expression is indeed required up until the mid-third larval instar (and most likely beyond this time period). Wing imaginal discs dissected from larvae that were shifted to 29 degrees Celsius at 192 hours AEL (Figure 3.9 C) show a relatively normal pattern of Brk expression. Thus, it can be inferred that Dpp inactivation (in this experimental setup) is a long process. However, it should still be noted that wing discs are slightly smaller compared to their wildtype counterparts, perhaps due to low levels of Brk in the pouch that are not detected with our antibody. Ultimately, our results verify the importance of the central stripe of Dpp throughout the development and growth of wing imaginal disc.
Chapter 4. Results

Re-visiting old models of Dpp-dependent growth regulation with new tools

4.1 Chapter Introduction

It is well known that morphogens, such as Dpp, provide a growing tissue with positional information and thereby orchestrate the specification of different cell fates during development. In addition, many morphogens are closely linked to the regulation of growth and cell proliferation within a developing tissue. As shown in Chapter 3, loss of Dpp signalling in the wing imaginal disc gives rise to adult flies with small wings. It has also been shown that clones of cells over-expressing Dpp induce proliferation in surrounding cells (Zecca et al., 1995). Despite many years of research, a comprehensive understanding of growth regulation via Dpp signalling remains lacking.

In wildtype wing imaginal discs, it has been shown that proliferation is more or less uniform (Aegerter-Wilmsen et al., 2007, Aegerter-Wilmsen et al., 2012, Hufnagel et al., 2007, Shraiman, 2005). This phenomenon has puzzled developmental biologists for some time – how does a graded distribution of protein (i.e. a morphogen) trigger a uniform growth response? In 2005, a paper proposed that the gradient of Dpp was itself a trigger for uniform growth (Rogulja and Irvine, 2005). Rogulja and Irvine showed that clones expressing an activated form of Thickveins (Tkv) create abrupt differences in Dpp signalling and triggered non-autonomous proliferation in neighbouring cells. They also found that wing discs expressing uniform Dpp (UAS-Dpp) exhibited an uneven pattern of proliferation. Thus, they argued, this provided the necessary evidence to show that graded Dpp expression was required for uniform growth rates in the wing disc. In this chapter, we challenge this hypothesis by removing Dpp from the pouch, while simultaneously using a transgene to express uniform, low levels of Dpp in this region.

Another model, proposed by Schwank et al. claims that Dpp controls growth entirely via the repression of brinker and that Dpp/Brk are responsible for ironing out otherwise uneven growth rates in the wing disc (Schwank et al., 2008). In their paper,
Schwank et al. show that \( brk^{M68} \) and \( tkv^{Q235D} \) (a constitutively active form of Tkv) clones behave the same in medial or lateral regions of the wing imaginal disc. Furthermore, they show that \( brk \) mutant wing discs exhibit the same phenotype as \( brk/dpp \) double mutant discs – increased proliferation in the lateral regions of the wing disc and decreased proliferation in the medial regions. They do not provide an explanation for the regional proliferative differences in mutant wing discs, but they hypothesize the existence of a diffusible factor or a growth inhibitor that is repressed by Brk (Schwank et al., 2008). In this chapter, I use \( dpp^{FRT\text{-CA}} \) and \( brk^{FRT} \) to test whether Dpp does indeed mediate growth solely via the repression of Brinker. I also closely examine \( brk \) mutant wing discs at different times during larval development, carefully quantifying pouch size and cell proliferation. Thus, Chapter 4 attempts to resolve the following questions:

1. Is a gradient of Dpp needed to promote growth and uniform rates of cell proliferation in the pouch?
2. Does Dpp regulate growth solely by repressing and creating an inverse gradient of \( brk \) in the wing imaginal disc?
3. What is the spatial profile of cell proliferation in \( brk \) mutant wing discs? Do changes in cell proliferation have an impact on tissue shape?
4.2 Uniform levels of Dpp are sufficient to stimulate growth and cell proliferation

In order to rigorously test the slope model, we eliminated endogenous \textit{dpp} expression from the pouch and simultaneously induced uniform, low levels of Dpp from a transgene. The \textit{rn-gal4} driver was used in combination with \textit{UAS-FLP} to simultaneously excise the FRT cassettes of \textit{dpp}^{FRT-CA} and \textit{Tubal1}-FRT-f'-FRT-dpp. This transgene, which was kindly provided to us by Gary Struhl, has been shown to trigger intermediate signalling activity, sufficient to activate \textit{omb} but not \textit{salm} expression (Zecca et al., 1995). At first, we looked to see how patterning was affected by uniform levels of Dpp, fully expecting that vein positions would be mis-specified or not specified at all. In the resulting ‘rescued discs,’ Omb was expressed more uniformly, although at a reduced level (Figure 4.1 A-B). Brk was repressed and pMad immunoreactivity was barely detectable in the pouch, suggesting that the level of signalling achieved by \textit{Tubal1-dpp} is similar to that present far from the normal stripe of Dpp expression (Figure 4.1 C-F). Finally, ‘rescued’ discs were also stained for \textit{Drosophila serum response factor} (\textit{DSRF}), which is repressed in the prospective veins (Figure 4.1 G-H, yellow arrows). Since veins form at stereotypical positions in \textit{Drosophila} wing imaginal discs, they provide a convenient marker of patterning. Anti-\textit{DSRF} showed that the pattern of veins in ‘rescued’ discs was markedly disrupted, with only two zones of repressed DSRF remaining. One of these was located around the D/V boundary, where vein 1 normally forms under the control of Wingless (Couso et al., 1994, Rulifson and Blair, 1995). The other zone was around proveins 3 and 4, which are mainly specified by Hedgehog signalling in wildtype wing discs (Blair, 2007). The areas of DSRF repression corresponding to veins 2 and 5 were missing, consistent with the idea that a gradient of Dpp is necessary to specify these domains (Blair, 2007) (Figure 4.1 G-H).

Despite these patterning defects, approximately half of the discs of this genotype exhibited a normal pouch size at the end of the third larval instar, while the other half overgrew slightly in the posterior compartment (i.e. Figure 4.1 B). This variability is most likely caused by the \textit{rn-gal4} driver, which is expressed in a circular domain that encroaches on the hinge in the posterior compartment (Figure 4.2). Thus, the expression
Chapter 4. Results

of Tubal-FRT-f'-FRT-dpp is not perfectly confined to the pouch. Consequently, in some cases, the repression of brk in the posterior hinge causes slight overgrowth in this area. In this experiment, growth was quantified by dissecting wing discs from crawling L3 larvae and counting the number of mitotic cells (identified by staining for Phospho-Histone H3) in a given area. Control and ‘rescued’ discs had similar levels of PH-3 density, while disks lacking Dpp signalling in the pouch proliferated at a much lower rate (Figure 4.3 A-D). ‘Rescued’ larvae did survive to adulthood and thus, we were able to analyze adult wings for patterning elements and growth (Figure 4.3 E-G). Many of these adult wings appeared to be made entirely of crumpled vein material, which made it difficult for us to assess size (Figure 4.3 G). This phenotype could arise because Dpp promotes vein differentiation throughout pupal development (its expression is heightened along all of the veins). Thus, the Tubal-FRT-f'-FRT-dpp transgene may, in some cases, produce levels of Dpp that are high enough to induce vein specification throughout the majority of the pupal wing. Nevertheless, some adult wings were well formed, perhaps because they experienced lower levels of Dpp signalling than those needed for vein differentiation. In these adult wings, vein patterning was disrupted, but in a reproducible manner. Each adult wing contained a broad swath of vein tissue near the A/P boundary (reminiscent of the phenotype seen in wing imaginal discs). More-so, these adult wings reached an appreciable size (Figure 4.3 F). However, it should be noted that they were still significantly smaller than wildtype counterparts. The reasons for this are unclear, especially since dissected wing discs did not exhibit a smaller pouch size. At the moment, we attribute the size differences between control and mutant adult wings to events that take place (or fail to take place) during pupal development. Although this is pure speculation, it is conceivable that the rounds of cell division that normally occur in pupal wings do not take place, thus accounting for the smaller wing size.

It is important to note that there is already some data refuting the slope model for Dpp. For example, salE-GAL4>tkvQ235D wing imaginal discs exhibit a normal profile of cell proliferation (Schwank et al., 2008). However, our group is the first to look carefully at patterning and observe adult wings. Our results nicely show that uniform levels of Dpp in the pouch are sufficient for normal growth and cell proliferation, but not for patterning.
In addition, our results suggest that very low levels of Dpp are sufficient to suppress *brk* and promote growth.

**Figure 4.1 – Low level, uniform Dpp disrupts patterning in wing imaginal discs**

(A-H) Comparison of wild-type discs (A, C, E, G) to discs lacking endogenous Dpp in the pouch and expressing low level, uniform Dpp from the *Tuba1-FRT-f'-FRT-dpp* transgene instead (B, D, F, H). Omb is expressed uniformly in mutant wing discs and also at a relatively lower level than in control discs (samples shown in A and B were stained and imaged under identical conditions on the same day). Please also note the repression of Brk and the undetectable levels of pMad in the medial regions of mutant wing discs. Despite exhibiting a normal size, mutant wing discs show abnormal vein patterning, with only two recognizable vein territories (marked by the loss of DSRF). The scale bar applies to all panels and is equal to 50 microns.
Figure 4.2 – The domain of \( rn \) expression is larger than the pouch territory

(A) Please note that the \( rn\)-gal4 driver is expressed in a wider domain than the pouch (compare the shaded light blue area to the dark blue area) in the posterior compartment. As a result, \( Tub\alpha1\cdot FT^T\cdot FRT\cdot dpp \) is expressed in the posterior hinge, sometimes leading to the de-repression of \( brk \) in this region and thereby causing some overgrowth.

Figure 4.3 – Uniform levels of Dpp are sufficient for normal growth and cell proliferation

(A-B) \( dpp^{FRT\cdot CA} \) and \( dpp^{FRT\cdot CA} \cdot rn\cdot gal4\cdot UAS\cdot FLP, tub\alpha1\cdot FRT\cdot f^T\cdot FRT\cdot dpp \) wing imaginal discs stained for PH-3 show a relatively uniform proliferation throughout the entire tissue. (C) \( dpp^{FRT\cdot CA} \cdot rn\cdot gal4\cdot UAS\cdot FLP \) wing imaginal discs show low levels of PH-3 in the pouch region. (D) Quantification of PH-3 density is based on 14 rescued discs, 9 control discs, and 11 non-rescued discs. Statistical significance was assessed with a Student’s t-test, assuming equal variance and a Gaussian distribution. Mitotic density (PH-3 spots/area) was determined for each individual disc using Fiji/ImageJ software (see Materials & Methods). (E) Female \( dpp^{FRT\cdot CA} \) adult wing. (F-G) Two variants of adult wings from \( rn\cdot gal4\cdot UAS\cdot FLP, tub\alpha1\cdot FRT\cdot f^T\cdot FRT\cdot dpp \) flies. A majority of wings (Type B, 15/20) had excess vein tissue, while the remainder (Type A, 5/20) had one central vein around the position of the A/P boundary. Scale bar = 50 microns.
4.3 Dpp controls growth primarily via the repression of *brinker*

To understand the extent that Dpp controls growth through *brk* repression, we simultaneously inactivated both Dpp and Brk in the pouch. Normally, wing imaginal discs lacking Dpp in the prospective wing blade exhibit growth defects (Figure 4.4 B). However, if Dpp does indeed regulate growth solely via the repression of *brk*, then the inactivation of both genes in the pouch should fully restore the abrogation of growth. The *rn*-gal4, *UAS-FLP* combination was used to inactivate both *dpp*\(^{FRT,CA}\) and *brk*\(^{FRT}\). Indeed, double mutant wing imaginal discs (dissected at 120 hours AEL and stained for Brk or V5) exhibited a normal-sized pouch when compared to *dpp*\(^{FRT,CA}\); *rn*-gal4>*UAS-FLP* wing discs (Figure 4.4 B-C). The rescue of growth was even more apparent when we looked at adult wings (Figure 4.4 D-F). *dpp*\(^{FRT,CA}\); *rn*-gal4>*UAS-FLP* pupae eclose as adults with extremely small wings (Figure 4.4 E). However, simultaneous removal of Brk in this genetic background rescued a large portion of the growth defects. Double mutant adult wings were similar in size to wildtype, but they did exhibit a shape change. These rounded wings also contained no veins, most likely due to the complete absence of Dpp signalling in the pouch. These results support the growth equalisation model (Schwank et al., 2008) and provide evidence to support the claim that Dpp mainly controls growth via the inactivation of the transcriptional repressor, *brinker*.
Figure 4.4 – Dpp primarily regulates growth indirectly via the repression of brk

(A) Control wing disc stained for Brk. (B) Dpp inactivation in the pouch with rn-gal4 and UAS-FLP leads to stunted growth and the de-repression of brk in the prospective wing blade. (C) The growth defects seen in panel B are restored if Brk is removed concomitantly with Dpp. DAPI is shown in this panel to give a better sense of the contour of the wing disc. Dotted white lines show the boundary of the pouch (determined by stereotypical tissue folds). Scale bars = 50 microns. (D-F) Female adult wings for the corresponding genotypes. Double mutant adult wings exhibit a substantial increase in size when compared to dpp<sup>FRT-CA</sup>; rn-gal4>UAS-FLP wings.
4.3.1 The removal of Brk in the pouch does not have a large effect on growth

I also wanted to confirm that the removal of Brk itself in the pouch had no significant effect on growth. In order to do this, we inactivated \( brk^{FRT} \) with \( rn\text{-}\text{gal4}, UAS-FLP \) and dissected crawling L3 larvae. Immunostainings against V5 (Brk) show proper excision of \( brk \) in the domain of \( rotund (rn) \) expression (Figure 4.5 A-B). Mutant wing discs exhibit a normal pouch size, although some overgrowth was observed in the P compartment. As mentioned previously, the expression of \( rn \) overlaps with certain regions of the posterior hinge. Thus, the inactivation of \( brk \) with the \( rn\text{-gal4} \) driver will also result in some excision in the posterior hinge, causing an increase in proliferation. Emerging adults of this genotype had round wings, missing L2 and L5 completely (Figure 4.5 C-D). Using Fiji/ImageJ software, I was able to measure the total surface area of the wing blade in mutant samples. In addition, the surface area of the anterior half of the wing blade was measured, as the domain of \( rn \) expression does not seem to encroach on hinge territories in the A compartment of wing discs (Figure 4.5 E-F). All measurements were normalized to the average area of control adult wing blades. In both cases, there was a significant difference between control and mutant samples. Once again, this raises the question – why are adult wings smaller compared to wildtype despite no obvious reduction in pouch size? As stated previously, this could be a result of certain processes during metamorphosis. It is also interesting to note that the shape of the pouch does seem to change slightly upon the inactivation of \( brk \) and this clearly has an effect on the final shape of the adult wing. Altogether, this data suggests that removal of Brk with \( rn\text{-gal4} \) and \( UAS-FLP \) does not drastically affect the size of the pouch, but it does seem to cause a shape change that is more clearly seen in adult wings.
Figure 4.5 – The removal of Brk in the pouch does not substantially affect growth

(A) Control wing disc stained for V5 (Brk). (B) The removal of Brk in the pouch does not have a large effect on growth. Some overgrowth is observed in the P compartment (yellow arrow) as \( \textit{rn} \) expression extends into parts of the hinge. (C-D) Female adult wings from corresponding genotypes. Mutant adult wings are rounded and seem to be missing a region in the P compartment. (E-F) In \( \textit{brk}^{\text{FRT}} \); \( \textit{rn-gal4>FLP} \) adult wings, the area of the total wing blade and the anterior half of the wing blade is significantly smaller than wildtype. However, the phenotype is quite variable and some mutant adult wings do reach wildtype size. In each case, statistical significance was determined using a Student’s t-test, assuming equal variance and a Gaussian distribution (p<0.0001 and p = 0.0020).
4.4 Wing imaginal discs mutant for brk or brk/dpp show the same phenotype: a significant decrease in proliferation in medial areas

Schwank et al. have used hypomorphic alleles to show that wing imaginal discs deficient in brk activity (or both brk and dpp activity) overgrow in the “lateral” regions of the wing imaginal disc, while medial regions exhibit a severe decrease in proliferation (Schwank et al., 2008). I wanted to repeat these experiments with our newly-generated conditional alleles to see if they would produce the same results. Furthermore, I wanted to closely analyze PH-3 density and overall shape changes in wing discs after the inactivation of brk at different stages of larval development. The esg-gal4 driver was used in combination with UAS-FLP to inactivate brk from the entire wing imaginal disc. This driver is expressed very early, thus mutant wing discs should never be exposed to brk expression. In this experiment, wing discs were dissected and fixed at 72, 96, and 120 hours AEL. Discs were stained for V5 (Brk) and PH-3, a marker of cells undergoing mitosis. At 72 hours AEL, there were no obvious differences in PH-3 density throughout the tissue (Figure 4.6 A, D). However, at 96 hours AEL, mutant wing discs already exhibited slight changes in shape, although it was not apparent that there was an increase in the number of dividing cells in the lateral regions of the wing disc (Figure 4.6 B, E). This shape change, also seen in mutant discs dissected at 120 hours AEL, seems to be characterized by an extension of the D/V axis (Figure 4.6 C, F). By the end of larval development (120 hours AEL), it was also apparent that the pattern of proliferation in mutant wing discs had completely changed (Figure 4.6 C, F). Please note that the same phenotype was seen in brk/dpp mutant wing discs at 120 hours AEL as well (not shown). In the following section, I describe the methodology used to quantify cell proliferation in these wing discs at 96 and 120 hours AEL.
Figure 4.6 – Inactivation of brk throughout the entire wing disc with esg-gal4, UAS-FLP

(A-C) Control wing discs dissected and fixed at three major time points during larval development (72, 96, and 120 hours AEL). Wing discs were stained with antibodies against Brk and PH-3 and show a more-or-less uniform profile of proliferation throughout the tissue. (D-F) After removing Brk throughout the entire tissue with esg-gal4, UAS-FLP, wing discs exhibited a shape change (elongation along the D/V axis) and a heterogeneous profile of PH-3 (dividing cells). Lateral regions appear to proliferate more than medial regions. Scale bar = 50 microns (applies to all panels in the figure).
4.4.1 Quantifying proliferation (PH-3 density) in brk mutant wing discs

To quantify PH-3 density in wing imaginal discs, I used a program (referred to as R_Cell) that was written with Python and OpenCV (Open Source Computer Vision Library), a library of programming functions geared towards computer vision. In essence, R_Cell processes and segments each image (see Materials & Methods for a detailed description). Then, it uses a series of algorithms to split the wing disc into three different regions: the left (lateral) region, the medial region, and the right (lateral) region. This is done by approximating an ellipse and cutting it at pre-defined positions. First, the program finds the widest part of the wing disc (D1) and divides it in half to find the center of the ellipse (xc, yc). Then, it identifies the minor axis by finding the distance from the center of the ellipse to the lowest point of the wing disc (D2). The top of the ellipse is cut at 0.8*D2, which roughly identifies the lower limit of the notum and this region is excluded from our calculations. (Figure 4.7 A). Finally, the left and the right regions are determined by vertical lines that are placed at 0.25*D1 (Figure 4.7 B). In an ideal scenario, the lateral regions would be determined by the expression of brk. However, since we do not have a marker for excision events in brk mutant wing imaginal discs (like dppFRT-REP), we need to approximate. In wildtype wing discs the extent of brk expression reaches into about a quarter of the wing disc, thus the two vertical lines that separate the left, medial, and right regions are placed at 0.25*D1.

R_Cell was used to quantify PH-3 density in the three different regions (number of spots/area). Although a slight shape change (elongation of the D/V axis) was seen in brk mutant wing discs at 96 hours AEL, there were no significant differences in PH-3 density between the various regions (Figure 4.8 A, C, E). Proliferation was more-or-less uniform throughout both wildtype and mutant wing discs. On the other hand, at 120 hours AEL, a significant decrease in PH-3 density was detected in the medial regions between mutant and wildtype samples (Figure 4.8 B, D, F). Surprisingly, our quantifications suggest that the ‘left’ and ‘right’ lateral regions do not overproliferate compared to wildtype. However, it should be noted that clusters of cells within the ‘left’ and ‘right’ lateral regions, which normally express brk, exhibit an increase in PH-3 density after the inactivation of brk (this observation is not based on quantifications, only on visual observations). Conversely, cells that do not normally express brk exhibit a non-
autonomous decrease in PH-3 density. Consequently, proliferation is heterogeneous within the specified ‘left’ or ‘right’ domain and this may be the reason why a change in proliferation is not detected by our program in these lateral regions. Overall, our data shows that, in brk mutant wing discs, medial regions suffer a significant loss in PH-3 density, but this only happens at late stages of larval development (beyond 96 hours AEL).

**Figure 4.7 – Approximating an ellipse to quantify PH-3 density in wing discs**

(A) Our program, R_Cell, finds the widest part of the wing imaginal disc (D1) and divides it in half to find the center of our ellipse (xc, yc). Then, it finds the distance from the center of the ellipse to the lowest point in the wing disc, which we refer to as the minor axis (D2). The top of the ellipse is cut at 0.8*D2 to separate the notum from the rest of the wing disc. The notum is excluded from our calculations. (B) The left, medial, and right regions are established by two vertical lines that are placed at 0.25*D1.
Figure 4.8 – Using R_Cell to quantify PH-3 density in wildtype and brk mutant wing discs

(A-D) R_Cell was used to segment each image and quantify the number of PH-3 spots in a given area (left, medial, or right) at both 96 and 120 hours AEL. (E-F) Quantification of PH-3 density at 96 hours AEL is based on 13 control discs and 10 mutant discs and on 11 control discs and 11 mutant discs at 120 hours AEL. For each region, significance was determined using a Student’s t-test, assuming equal variance and a Gaussian distribution. The only significant difference between control and mutant samples was seen in medial regions at 120 hours AEL (p=0.0018). Scale bar = 50 microns.
4.4.2 Quantifying pouch/wing blade size in brk mutant wing discs

Although brk mutant wing discs do exhibit a shape change, it was not obvious that they were overgrown at either 96 or 120 hours AEL (in comparison to wildtype samples). Thus, I also decided to quantify pouch size to determine if there was some overgrowth or if the shape of the pouch had just changed (extending along the D/V axis and shrinking along the A/P axis). In order to do this, brk\textsuperscript{FRT} and brk\textsuperscript{FRT}; esg-gal4>FLP wing discs were dissected at 120 hours AEL and fixed. Then, they were stained with an antibody against Zinc finger homeodomain 2 (Zfh2), a gene that has been shown to be involved in the specification of proximal and distal structures in the wing disc. More specifically, it has been suggested that the repression of zfh2 permits the specification of the pouch, the distal-most fate (Terriente et al., 2008). Thus, we used the ring of Zfh2 staining as an estimate for pouch size. In wing discs dissected at 120 hours AEL, there was no difference between pouch surface area in control and mutant samples (Figure 4.9 A-C). However, it is important to note that eventually, these wing discs do overgrow because they do not pupariate at 120 hours AEL like their wildtype counterparts. Instead, they continue to grow and their lateral regions continue to proliferate (the delay has not been quantified in this study). Thus, brk mutant wing discs that are dissected at the wandering L3 stage show even more serious morphological changes than those seen in Figure 4.9 (not shown). Ultimately, I believe this data shows that brk mutant discs do not necessarily ‘overgrow’ up until 120 hours AEL. Instead, it seems like the normally homogenous distribution of proliferating cells is disrupted and this gives rise to shape changes that alter the final size of the wing blade.

Inactivation of brk with esg-gal4 and UAS-FLP caused lethality during the pupal stages, most likely because the driver is expressed in several other imaginal discs. Thus, in order to observe adult wings, the vg-gal4 driver was used instead of esg-gal4. The same phenotype was observed in wing imaginal discs upon inactivation of Brk with vg-gal4>FLP or esg-gal4>FLP. However, brk\textsuperscript{FRT}; vg-gal4>FLP pupae survived during metamorphosis and eclosed as adult flies. Surprisingly, adult wings were not overgrown, but much smaller compared to wildtype (Figure 4.10 A-C). Mutant adult wings were also completely rounded, suggesting that the more Brk that is removed from the wing disc, the more rounded the adult wing becomes (compare Figure 4.5 D to Figure 4.10 B). In
order to make sure that this phenotype was not associated with cell death, \( \text{brk}^{FRT}; \text{esg-gal4}>\text{FLP} \) wing discs were stained with an antibody against Death caspase-1 (Dcp-1). No major differences in Dcp-1 staining were seen between wildtype and mutant samples (not shown). This data supports our hypothesis that wing imaginal discs mutant for \( \text{brk} \) do not necessarily overgrow, but exhibit a dramatic shape change (as a result of a heterogeneous profile of dividing cells) that also affects the final shape of the adult wing.
Figure 4.9 – Measuring pouch surface area in brk mutant wing discs using anti-Zfh2

(A-B) Control and brk mutant wing discs dissected at 120 hours AEL and stained with an antibody against Zfh2 (a gene involved in specifying proximal and distal fates). This staining was used to estimate pouch surface area in both sets of samples. (C) Quantification of pouch surface area at 120 hours AEL. Significance was determined by performing a Student’s t-test, assuming equal variance and a Gaussian distribution. There is no significant difference between control and mutant samples. Scale bar = 50 microns.

Figure 4.10 – Inactivation of brk with vg-gal4 results in adults with small, rounded wings

(A-B) Female adult wings from the corresponding genotypes. Removal of Brk in the entire wing disc (with vg-gal4 and UAS-FLP) results in adult flies with small, rounded wings. (C) The surface area of adult wings was normalized to the average of control samples. Statistical significance was determined using a Student’s t-test, assuming equal variance and a Gaussian distribution (p<0.0001).
4.5 Chapter Discussion

In this chapter, our newly-generated conditional alleles are used to investigate the slope model (Rogulja and Irvine, 2005) and the growth equalisation model (Schwank et al., 2008). Inactivation of Dpp in the pouch and simultaneous expression of Tubal-dpp (uniform, low levels of Dpp) in this region resulted in wing discs with no apparent growth defects. However, discs exhibited severe patterning errors, including the loss of many proveins. Despite attaining a normal pouch size, ‘rescued’ adult wings were slightly smaller compared to their wildtype counterparts, a phenotype that we still do not fully understand, but attribute to events during metamorphosis. Nevertheless, our results do suggest that a gradient of Dpp is not needed in the pouch to promote growth and uniform cell proliferation. They also confirm that the low levels of Dpp produced by the Tubal-FRT-f’-FRT-dpp transgene, which are not sufficient to activate the expression of salm (Nellen et al., 1996), are indeed sufficient to repress the activity of brk. In conclusion, it appears that if brk is repressed in the pouch, then growth is permitted. Conversely, if brk is de-repressed in this region, proliferation is stunted, but not completely abolished (Figure 4.3 C). It is important to note that this principle does not apply to regions outside of the pouch, where any loss of brk expression triggers a sudden increase in proliferation.

To understand whether or not Dpp solely regulates growth via the repression of brk, dppFRT-CA and brkFRT were simultaneously inactivated in the pouch with rn-gal4, UAS-FLP. Normally, the inactivation of dpp in the pouch leads to the de-repression of brk and to the abrogation of growth (Figure 4.4 B). However, by simultaneously excising brkFRT in this context, growth is almost fully restored in both wing discs and adult wings. In contrast, the inactivation of brk or dpp/brk throughout the entire wing disc (with esg-gal4 or vg-gal4) leads to very different results, namely a dramatic change in the homogenous profile of dividing cells that is normally seen in wildtype discs. Groups of cells that lose brk expression seem to proliferate more. Conversely, areas that do not normally express brk, such as the medial regions of the wing disc, exhibit a significant loss in proliferation by 120 hours AEL (but not at 96 hours AEL). Interestingly, this late phenomenon suggests that brk might have a late contribution to growth regulation. Furthermore, it is entirely unknown how these differences in proliferation arise. brkFRT,
esg-gal4>FLP and brk<sup>FRT</sup>; dpp<sup>FRT-CA</sup>; esg-gal4>FLP wing discs are theoretically never exposed to Brk (because of the early activity of the esg-gal4 driver). Thus, differences in proliferation must arise from another signalling pathway or perhaps even from mechanical tension generated throughout development. In the next chapter, we focus our attention on trying to identify which pro-growth genes are regulated by the Dpp signalling pathway. In addition, we begin to correlate different levels of Brk with cell proliferation.
Chapter 5. Results

Uncovering the mechanisms that Brk utilizes to suppress growth

5.1 Chapter Introduction

Clearly, the expression of brk represses growth in some way. As shown in Chapter 4, the de-repression of brk in the pouch significantly decreases the number of dividing cells in the region. Conversely, the removal of Brk outside of the prospective wing blade increases the rate of division in the manipulated area. Thus, it seems that brk expression is required in the lateral regions of the wing disc to curb proliferation rates. However, very little is known about the actual mechanisms that Brk employs to suppress growth. In 2013, ChIP-Seq. was performed to identify potential target genes of Brk. This study identified 1,671 genes with peaks that were enriched compared with normalized input control. Out of this list, the authors chose to focus on Drosophila myc (d-myc), a gene that contains several Brk binding sites. Doumpas et al. show that brk mutant clones seem to upregulate their expression of d-myc. Somewhat disappointingly though, nubbin-gal4>UAS-Brk, UAS-dMyc adult wings were only slightly larger compared to nubbin-gal4>UAS-Brk counterparts (Doumpas et al., 2013). Thus, we decided to follow up on this data by performing several different experiments. First, RNAi against d-myc was used to try and rescue the increased proliferation seen in the lateral regions of brk mutant wing discs. In addition, I generated a transgene that expresses d-myc at more physiological levels (compared to UAS-dMyc). This construct will be used to try and rescue pouch growth in dpp mutant wing discs.

Another possible mediator of growth downstream of Dpp signalling is vestigial (vg). In 1997, it was suggested that Mad binds to and is required for the activation of an enhancer within vestigial (vg), a gene that is absolutely essential for wing and haltere formation (Kim et al., 1997). Interestingly, the ChIP-Seq. data set generated by Doumpas et al. also indicates that Brk binds to regions in the vg locus (Doumpas et al., 2013). Thus, we also wanted to understand how Dpp signalling regulates the expression of vg in the wing disc. In order to test this, we observed Vg expression in dpp mutant wing discs. In
addition, a transgene (Tubα1-FRT-STOP-FRT-vg) was used to try and rescue the growth defects seen in dpp\textsuperscript{FRT-CA}; rn-gal4>FLP wing imaginal discs. Thus, in this chapter, we use dpp\textsuperscript{FRT-CA}, brk\textsuperscript{FRT}, and our newly-generated constructs to test if Dpp signalling regulates growth in the wing disc by regulating the expression of either \textit{d-myc} or vg.

In addition to identifying target genes, we felt it was equally important to understand how different levels of Brk affect growth and cell proliferation. Based on our stainings for V5 in brk\textsuperscript{FRT} wing discs, it seems that Brk is indeed expressed in an inverse gradient to Dpp, with the highest levels seen in the hinge and the lowest levels found in the periphery of the pouch. However, it is not known if these low levels of Brk contribute to the repression of growth. In addition, it is not known whether the relationship between Brk and growth is linear (i.e. if increasing levels of Brk lead to decreasing levels of cell proliferation)\? In this chapter, the following questions are addressed:

1. Does Brk regulate the expression of \textit{d-myc}? Can the expression of d-Myc rescue \textit{dpp} mutant phenotypes in the wing disc?
2. Does Dpp signalling control growth through the regulation of \textit{vg}? Can the expression of Vg rescue \textit{dpp} mutant phenotypes in the wing disc?
3. Does Brk “invade” the pouch over developmental time? Do low levels of Brk repress growth in the pouch?
5.2 Knockdown of d-Myc with RNAi does not rescue brk mutant phenotypes

In order to better characterize the relationship between Brk and d-Myc in the wing imaginal disc, three different RNAi lines against d-myc (BL51454, BL36123, BL25784) were used to try and rescue the brk mutant phenotype. First, each RNAi line was validated by crossing it to the vg-gal4 driver. It is well-known that the removal of d-myc expression in the wing disc results in flies with normally shaped, but smaller wings (Pierce et al., 2008). This phenotype was also observed in vg-gal4>UAS-dMycRNAi-25784 adult wings, whose surface area was significantly smaller compared to w1118 (wildtype) counterparts (Figure 5.1 A). Since the expression of dMycRNAi-51454 and dMycRNAi-36123 was lethal during pupal stages, I was not able to observe adult wings from these genotypes. Overall, this data shows that these particular d-Myc RNAi lines do have an effect on growth when expressed in wing imaginal discs.

In this experiment setup, I wanted to express the RNAi lines solely in the domain of brk excision (brkFRT/brkGAL4; UAS-FLP/UAS-dMycRNAi). Thus, I decided to knock in the sequence of GAL4 directly into the brk locus to generate brkGAL4, a null allele. Please note that a brk-gal4 line does already exist (McKay and Lieb, 2013), but this is a synthetic construct that was inserted into the genome at random (P-element insertion). It is not a null allele and thus, it cannot be used in our experiment. The brkGAL4 allele was generated by injecting RIV-GAL4 (Figure 5.1 B) into brk KO embryos containing an attP site. Following attP/B-mediated recombination, larvae were screened for proper insertion by observing pax-GFP expression. Once candidates were identified, they were properly balanced with FM7-GFP. The brkGAL4 allele was used to drive the expression of FLP recombinase and RNAi against d-Myc (brkFRT/brkGAL4; UAS-FLP/UAS-dMycRNAi). Wing imaginal discs were dissected from L3 crawling larvae and stained with an antibody against PH-3 to observe dividing cells. Surprisingly, none of the RNAi lines had any effect on cell proliferation in brk mutant wing discs. In fact, brkFRT/brkGAL4; UAS-FLP/UAS-dMycRNAi wing discs did not appear any different from their brkFRT; esg-gal4>FLP counterparts (Figure 5.1 C-D). Discs still exhibited increased proliferation in the domain of brk excision and a significant decrease in proliferation in medial regions.
Furthermore, \( brk^{FRT/brk^GAL4}; UAS-FLP/UAS-dMyc^{RNAi} \) discs also displayed a shape change, consistent with an elongation of the D/V axis. Overall, this data shows that the expression of d-Myc RNAi does not rescue the growth defects seen in \( brk \) mutant wings discs, suggesting that Brk and d-Myc regulate growth independently of one another. It is important to note that the levels of expression achieved with \( brk^GAL4 \) may not be as high as the levels of d-Myc RNAi that are produced with \( vg^gal4 \). Thus, further experiments are required to completely rule out an association between Brk and d-Myc.

5.2.1 Visualizing the expression of Myc in \( brk \) mutant wing discs

Immunostainings show that d-Myc and Brk are normally expressed in complementary domains of the wing disc at 120 hours AEL (Doumpas et al., 2013). Furthermore, in their study, Doumpas et al. show that clones expressing activated Thickveins (Tk\(^{V235D}\)) upregulate their expression of \( d\text{-}myc \). In light of this, the idea that Brk represses the expression of \( d\text{-}myc \) seems quite compelling. However, nobody has actually looked at the expression of d-Myc in \( brk \) or \( dpp \) mutant wing discs. Since the antibody used to visualize d-Myc is not available anymore, we decided to make Myc-GFP, a C-terminal insertion of GFP into the \( d\text{-}myc \) locus. This was done by using a vector (929CHE) obtained from Yohanns Bellaïche (group leader at the Institut Curie, Paris). This vector contains the sequences for GFP and \( pax\text{-}Cherry \), which is used as a selection marker in this case and is flanked by LoxP sites to enable removal upon successful identification of candidates. There are also multiple cloning sites, which allow for the insertion of 5’ and 3’ homology arms (approximately 1-1.5 kb each). After successful cloning of the 929CHE vector, it was injected into \( nos\text{-}cas9 \) embryos together with the CFD4 vector (containing the sequences for our gRNAs). After three rounds of injections, we obtained 8 candidates, which are all homozygous viable. Although homozygous \( c\text{-}Myc\text{-}eGFP \) mice are viable and fertile (and the fusion protein seems to retain endogenous functionality), we could not observe GFP expression in wing discs homozygous for our insertion. This could be due to suppressed expression by the \( pax\text{-}Cherry \) cassette. Thus, at the moment, \( hs\text{-}cre \) is being used to remove the \( pax\text{-}Cherry \) cassette from the locus of all 8 candidates. After this, wing imaginal discs will be stained for GFP again. We hope this adjustment will enable us to visualize the expression of d-Myc in wing imaginal discs.
Figure 5.1 – dMyc RNAi does not rescue defects associated with brk mutant discs

(A) Validation of three different RNAi lines against d-Myc. $vg\text{-gal4}>UAS-dMyc^{RNAi-25784}$ wings are smaller than their $w^{1118}$ counterparts (the surface area of adult wings was measured and normalized to the average of control samples). Statistical significance was determined using a Student’s t-test, assuming equal variance and a Gaussian distribution ($p<0.0001$). $vg\text{-gal4}>UAS-dMyc^{RNAi-36123}$ and $vg\text{-gal4}>UAS-dMyc^{RNAi-51454}$ flies died in the pupal stages. (B) Schematic showing the RIV-GAL4 construct, which contains an attB site, the GAL4 coding sequence followed by a poly(A) tail, a LoxP site, and a $pax$-GFP selection marker. RIV-GAL4 was injected into $brk$ KO embryos (where the only coding exon has been replaced with an attP site). Proper attP/B-mediated homologous recombination generates $brk^{GAL4}$. (C–D) Expression of d-Myc RNAi with $brk^{GAL4}$ does not rescue the phenotype seen in $brk$ mutant wing discs – increased proliferation in lateral regions and decreased proliferation in medial regions. Scale bar = 50 microns.
5.2.2 Generation of Tubα1-FRT-STOP-FRT-dmyc

The expression of UAS-Brk with nub-gal4 results in adults with very small wings. However, co-expression of UAS-dMyc does not impressively rescue wing blade size (Doumpas et al., 2013). As previously discussed, we wanted to repeat these experiments with a source of d-Myc that produces more physiological levels of the protein and does not overload cells with a potent growth factor. Thus, I decided to generate a vector that could express relatively low, uniform levels of d-Myc in a region of interest. In order to do this, we cloned the cDNA for d-myc into the FTS vector, which was generated by a senior investigator in our laboratory named Cyrille Alexandre. The FTS vector contains the sequence for the Tubα1 promoter, a poly(A) tail (STOP cassette) flanked by FRT sites, a MCS, and P elements. In this case, we used the MCS to insert the cDNA for d-myc and the 3’ UTR of the gene. Afterwards, this vector was injected into w1118 embryos with a source of transposase. Following the development of injected embryos, surviving adults were crossed to w flies and the progeny of this cross were scored for red eye color. Detection of w+ indicated successful integration of the construct into the genome. In future experiments, Tubα1-FRT-STOP-FRT-dmyc flies will be used to try and rescue the growth defects seen in dpp mutant wing discs. In order to do this, I will use rn-gal4, UAS-FLP to simultaneously excise the FRT cassettes in both dppFRT-CA and Tubα1-FRT-STOP-FRT-dmyc. We hope that the results of this experiment will provide further insight into the relationship between Brk and d-Myc.
5.3 Vg expression does not rescue dpp mutant phenotypes

Experiments investigating the relationship between Dpp signalling and vestigial were conducted by Sean B. Carroll’s group and published in 1996 and 1997. Most of these were clonal studies, showing that homozygous clones for Tkv or the strong Mad1,2 allele seem to down-regulate their expression of Vg. However, it should be noted that some of these stainings are difficult to interpret, only showing a slight reduction of Vg expression in a small part of the actual clone (see Figure 5 E, F in (Kim et al., 1996). Despite this, Sean B. Carroll’s group did indeed show that MAD homology region 1 (MH1) can bind to the vg quadrant enhancer (QE) and if the 12-bp binding site is mutated, it severely diminishes the expression of a QE-lacZ reporter gene (Kim et al., 1997).

Altogether, these results suggest that Dpp signalling regulates the expression of vestigial in some way.

To follow up on this data, the expression of Vg was assayed in dpp mutant wing imaginal discs using an antibody that was kindly provided to us by Sean B. Carroll (Figure 5.2 A-B). dpp\textsuperscript{FRT-CA}; \textit{rn-gal4}>FLP wing discs exhibited a loss of Vg expression in a somewhat narrow stripe of cells located near the A/P boundary (Figure 5.2 B). However, Vg expression along the D/V boundary was unaffected, consistent with the finding that the vg boundary enhancer (BE) is controlled by Notch signalling (Kim et al., 1996, Neumann and Cohen, 1996). It is interesting to note that the cells that lose Vg expression seem to be located within the domain of \textit{dpp} expression (the central stripe of Dpp). These results suggest that Dpp signalling does indeed activate the \textit{vgQE}, albeit in a very specific domain. Alternatively, Dpp signalling in this region could also regulate the expression of another factor, which in turn influences the expression of \textit{vestigial}. It is important to note that the QE is also regulated by other inputs since the expression of Vg in the posterior compartment of \textit{dpp}-deficient discs looks completely normal.

Because Vg expression is lost in a stripe of cells near the A/P boundary in \textit{dpp} mutant discs, we thought that perhaps relatively high levels of Dpp/Mad could be required to activate the \textit{vgQE}. In order to test this theory, we also looked at the expression of Vg in \textit{dpp}^\text{FRT-CA}; \textit{rn-gal4}>FLP, \textit{Tub\alpha1-FRT-f-FRT-dpp} wing discs (where endogenous
levels of Dpp are replaced by uniform, low levels of Dpp in the pouch). Interestingly, these wing discs also had an altered pattern of Vg expression (Figure 5.2 C). Immunostainings show a loss of Vg in the peripheral regions of the pouch, but solely in the A compartment (Figure 5.2 C, yellow arrows). This result suggests that the activation of *vestigial* in this precise domain requires high levels of Dpp. Furthermore, it indicates that pMad not only represses *brk*, but may also directly activate *vg*. Further experiments need to be performed to better understand the relationship between Dpp signalling and Vg. However, this data shows that Dpp does regulate the expression of *vestigial* (*vg*), though only in a specific domain in the A compartment. Ultimately, our results also imply that Dpp can regulate growth in two different ways – first, via the inactivation of *brk* and secondly, via the activation of *vg* (this is discussed in further detail in the chapter discussion).
Figure 5.2 – The expression of Vg is altered in dpp mutant wing imaginal discs

(A-C) Wing imaginal discs of the corresponding genotype were dissected and stained with an antibody for Vestigial (Vg). The inactivation of Dpp in the pouch results in a loss of Vg expression in a somewhat narrow stripe of cells near the A/P boundary (but not at the D/V boundary). It is possible to rescue a significant portion of the growth defects seen in \( dpp^{FRT-CA} \); \( m\text{-gal4>FLP} \) wing discs by simultaneously expressing \( Tub\alpha -FRT^-{FRT-dpp} \). Despite this, these wings discs also have an altered pattern of Vg expression. Each scale bar = 50 microns.
5.3.1 Vg does not rescue growth defects seen in dpp mutant wing discs

In order to understand how much growth can be rescued by the expression of Vg in dpp mutant wing discs, $rn\text{-}gal4$ and $UAS\text{-}FLP$ were used to simultaneously remove the FRT cassettes from $dpp^{FRT\text{-}CA}$ and $Tuba1-FRT\text{-}STOP\text{-}FRT\text{-}vg$ (a construct made by Cyrille Alexandre). However, before performing this experiment, we wanted to show that $Tuba1-FRT\text{-}STOP\text{-}FRT\text{-}vg$ can rescue the lack of growth seen in $vg$ mutant adult wings. To do this, we made use of a conditional allele ($vg^{FRT}$) that was generated by Cyrille Alexandre using CRISPR/Cas9. This conditional allele was used to inactivate Vg in the pouch domain ($vg^{FRT}; \text{rn-gal4, UAS\text{-}FLP}$), resulting in adult flies with very small wings (Figure 5.3 A-B). However, by expressing $Tuba1-FRT\text{-}STOP\text{-}FRT\text{-}vg$ at the same time, a large portion of the growth was restored. Although $vg^{FRT}; \text{rn-gal4, UAS\text{-}FLP, Tuba1-FRT\text{-}STOP\text{-}FRT\text{-}vg}$ adult wings were still smaller compared to wildtype, they were substantially bigger compared to $vg$ mutant wings (Figure 5.3 A-C). This shows that the transgene can rescue growth in the absence of endogenous Vestigial protein. Thus, it was also used in combination with $dpp^{FRT\text{-}CA}$ and $rn\text{-}gal4, UAS\text{-}FLP$ to see if the size of the pouch or the wing blade could be restored in dpp mutant wing discs. Wing imaginal discs ($dpp^{FRT\text{-}CA}$ and $dpp^{FRT\text{-}CA}; \text{rn-gal4, UAS\text{-}FLP, Tuba1-FRT\text{-}STOP\text{-}FRT\text{-}vg}$) were dissected and stained with an antibody for Zfh2, which allowed us to visualize and estimate pouch size. Mutant wing discs still exhibited a small pouch size compared to wildtype discs (Figure 5.3 D-E). Furthermore, mutant adult wings were not any bigger than $dpp^{FRT\text{-}CA}; \text{rn-gal4, UAS\text{-}FLP}$ adult wings, indicating that this transgene has no effect on either pouch (or wing blade) size (Figure 5.3 F-G). These results suggest that although Dpp may regulate the expression of vestigial, this does not have a profound impact on the overall size of the pouch. However, it should be noted that $Tuba1-FRT\text{-}STOP\text{-}FRT\text{-}vg$ does not restore all growth in a $vg$ mutant background, most likely because the levels of Vg produced by this transgene are relatively low. Thus, in the future, these experiments should be repeated with $UAS-Vg$, which does rescue 100% of growth in $vg$ mutant wing discs.
Figure 5.3 – Expression of Vg in dpp mutant wing discs does not rescue growth defects

(A-C) Female adult wings from the corresponding genotypes were mounted on slides and imaged with a 2.5x objective. Inactivation of Vg in the pouch gives rise to adults with no wings. However, expression of Tubα1-FRT-STOP-FRT-vg in this genetic background rescues a significant portion, albeit not all, of the growth defects. (D-E) Tubα1-FRT-STOP-FRT-vg was used to try and rescue the abrogation of growth seen in dppFRT-CA; rn-gal4>FLP wing discs. Pouch size is estimated by staining for Zfh2 (a gene involved in specifying proximal and distal regions in the wing disc). White dotted lines show the outline of the pouch (based on the staining for Zfh2). Scale bars = 50 microns. (F-G) Inactivation of Dpp in the pouch results in adult flies with very small wings. Expression of Tubα1-FRT-STOP-FRT-vg does not rescue any of these growth defects. In fact, dppFRT-CA; rn-gal4>FLP, Tubα1-FRT-STOP-FRT-vg adult wings are comparable to dppFRT-CA; rn-gal4>FLP, Tubα1-FRT-STOP-FRT-vg wings.
5.4 Low levels of Brk in the pouch still suppress growth

Even though Dpp signalling seems to regulate the expression of vestigial, our experiments show that this does not seem to have a large effect on the growth of the wing imaginal disc. In fact, most of our data suggests that Dpp primarily controls growth via the inactivation of brk (in agreement with the growth equalization model). Thus, by identifying the pro-growth genes that Brk regulates, we will gain a better understanding of the relationship between Dpp signalling and cell proliferation. In addition, it is also important to devise experiments that can correlate the level of brk expression with growth. At 120 hours AEL, a significant portion of cells in the lateral regions of the pouch express low to relatively moderate levels of Brk (Figure 5.4 A, yellow arrow). In order to better understand the precise mechanisms that Brk utilizes to repress growth, we wanted to test whether low levels of Brk could also suppress cell proliferation. Thus, we used the FTS vector (described in 5.2.2) to generate Tubα1-FRT-STOP-FRT-brk:V5. Transposable P elements facilitated the insertion of this transgene into random locations in the genome and the insertion site for each candidate was mapped. After the generation of transgenic flies, Tubα1-FRT-STOP-FRT-brk:V5 was expressed in the pouch with rm-gal4 and UAS-FLP. Control and mutant wing discs were dissected at 120 hours AEL, stained in the same antibody solution, and imaged on the same day using identical microscope settings. As expected from the low activity of the Tubα1 promoter, the levels of Brk produced by this transgene are hardly detectable (Figure 5.4 B). However, even the expression of these low levels of Brk generated a significant effect on growth (assayed by looking at the size of female adult wings). Mutant adult wings were substantially smaller than their wildtype counterparts (Figure 5.4 C-D). Although these experiments suggest that even low levels of Brk can repress growth, more sophisticated experiments are needed to ascertain how different levels of Brk affect cell proliferation. In the future, we hope to find a way to express varying levels of a protein. We also plan to devise a program that can measure the concentration of Brk in a small region of the wing disc and compare this to the number of dividing cells in the same area.
Figure 5.4 – The expression of low levels of Brk can repress growth

(A-B) Wing imaginal discs from the corresponding genotypes were stained with an antibody against V5 (Brk). Discs were imaged on the same day using identical microscope settings. The level of Brk produced by Tub1-FRT-STOP-FRT-brk:V5 is barely detectable and seems to be much lower than the endogenous levels of Brk found in the lateral regions of the pouch (yellow arrow). Scale bar = 50 microns. (C-D) Female adult wings from either control or mutant flies. The expression of Tub1-FRT-STOP-FRT-brk:V5 in the pouch with rn-gal4, UAS-FLP results in flies with significantly smaller wings compared to wildtype counterparts.
5.5 Chapter Discussion

At the beginning of this chapter, our main objective was to determine whether \(d\)-myc or \(v\)g are regulated by the Dpp signalling pathway. Our results show that the expression of RNAi against \(d\)-Myc cannot rescue the uneven pattern of proliferation (or aberrant shape change) seen in \(brk\) mutant wing discs. Although the \(d\)-myc locus contains several Brk binding sites and the two genes are expressed in complementary domains, our results suggest that Brk does not regulate growth via \(d\)-myc signalling. However, this does not mean that in a different context (i.e. cell competition), Brk cannot regulate its expression. At the moment, the literature suggests that \(d\)-Myc controls growth primarily by modulating the overall size of the wing imaginal disc in response to extrinsic signals, such as mTOR (Teleman et al., 2008, Parisi et al., 2011). Furthermore, wing discs lacking \(d\)-myc expression grow slower and are significantly smaller than wildtype counterparts. However, they do not exhibit any morphological defects (Schwinkendorf and Gallant, 2009, Wu and Johnston, 2010). On the other hand, the removal of Dpp has a severe effect on the morphology of the wing disc and on the overall size and shape of the adult wing blade. Keeping this in mind, it is most likely that Brk and \(d\)-Myc regulate cell proliferation in different ways. However, it will still be interesting to observe the expression of Myc-GFP in both \(brk\) and \(dpp\) mutant wing discs.

As shown above, the expression of Vg is altered in \(dpp^{FRT-CA};\ rn-gal4>FLP\) and \(dpp^{FRT-CA};\ rn-gal4>FLP,\ Tub\alpha1-FRT-f^\prime-FRT-dpp\) wing discs. It is interesting to note that the expression pattern is only affected in the A compartment, in the proximal regions of the pouch. This finding, along with the fact that Mad binds to the \(vgQE\) (Kim et al., 1997), suggests that Dpp signalling does regulate the expression of Vg. However, as discussed previously, Dpp is probably not the sole activator of the QE, since Vg expression in the P compartment of mutant wing discs is normal. Naturally, because Vg is essential for wing formation, it seems likely that Dpp signalling would regulate growth by controlling its expression. However, a transgene that expresses uniform levels of Vg in the pouch, was unable to rescue the abrogation of growth seen in \(dpp\) mutant wing discs. In fact, this transgene seemed to have no effect on cell proliferation, as \(dpp^{FRT-CA};\ rn-gal4>FLP\) and \(dpp^{FRT-CA};\ rn-gal4>FLP,\ Tub\alpha1-FRT-STOP-FRT-vg\) female adult wings were the same.
size. This is surprising considering that the removal of vg expression from the pouch results in adult flies with no wings. Thus, in order to confirm that the expression of Vg cannot rescue the growth defects associated with dpp mutant wings, the experiment should ideally be repeated with another source of Vg, which produces much higher levels of the protein compared to Tubal-FRT-STOP-FRT-vg. The altered pattern of Vg expression in dpp\textsuperscript{FRT-CA}; rn-gal4>FLP, Tubal-FRT-f'-FRT-dpp wing discs might also explain the adult wing phenotype seen in Figure 4.2 F. Although pouch size (assessed by looking at the stereotypical folds in the tissue) did not seem to be affected in dpp\textsuperscript{FRT-CA}; rn-gal4>FLP, Tubal-FRT-f'-FRT-dpp wing discs, the final size of adult wings was smaller compared to control samples. This was something that we did not fully understand at the time. However, it is certainly possible that the loss of Vg expression, which specifies wing identity, causes this phenotype. Altogether, these results suggest that Dpp does regulate the expression of vg. However, it is still unclear how and to what extent this regulation affects the growth of the wing imaginal disc.

Finally, this chapter ends by looking at how low levels of Brk affect cell proliferation in the pouch. In order to do this, Tubal-FRT-STOP-FRT-brk:V5 is expressed in the pouch with rn-gal4, UAS-FLP. Our results show that this manipulation still has a significant effect on the growth of the wing disc and the wing blade. Although much more sophisticated experiments need to be carried out that precisely link different levels of Brk with growth, this data implies that even small amounts of Brk can have a substantial effect on cell proliferation. In my opinion, this suggests that in a wildtype scenario, even low to moderate levels of Brk (found in the lateral regions of the pouch) should play a role in the regulation of growth. In the future, we plan to measure the concentration of Brk in different regions of the wing disc and link low, intermediate, and high levels of the protein with the rate of cell proliferation.
Chapter 6. Discussion

6.1 Summary of Results and General Discussion

When I began my PhD, I was interested in studying morphogens and how they regulate growth during development. More specifically, my goal was to understand the mechanisms that Dpp utilizes to control growth and cell proliferation in the wing imaginal disc. However, at the same time that I joined the group, a new study was published that generated some controversy – Akiyama and Gibson mystified the field when they claimed that the characteristic stripe of dpp expression, running along the A/P boundary, was not required for the growth of the wing imaginal disc during the third larval instar. Considering that previous studies have demonstrated the importance of Dpp signalling in wing disc growth, this was a surprising claim. It has been shown that if dpp is deficient or brk is over-expressed in the pouch, then cell proliferation is abrogated in this region (Zecca et al., 1995, Martin et al., 2004). Therefore, it is hard to imagine how dppFRT-TA, dpp-gal4, UAS-FLP wing discs, which express high levels of Brk in the pouch, attain a normal size. The authors suggest that an alternative source of Dpp signalling must exist in the anterior region of the pouch. This is because they observe a negative effect on growth when dppFRT-TA is inactivated with drivers expressed in either the anterior compartment (ci-gal4) or the pouch region (nub-gal4) (Akiyama and Gibson, 2015). However, there is no evidence that such a source exists. In their paper, Akiyama and Gibson claim that it is not detectable using available antibodies. If this is true, then presumably, Dpp levels at this new source must be low. In addition, Dpp ligand must be able to spread incredibly far to promote growth in the peripheral regions of the posterior pouch. Taking these points into consideration, it seems more likely that the continued growth seen in dppFRT-TA, dpp-gal4, UAS-FLP wing discs can be attributed to a late inactivation caused either by the dpp-gal4 driver or dppFRT-TA. Either way, it became important to address the conclusions made by Akiyama and Gibson in their paper. Indeed, before testing whether graded levels of Dpp are required for growth or searching for downstream targets, we first had to understand whether or not the canonical stripe of dpp expression is important for the normal growth of the wing imaginal disc.
First, I inactivated $\text{dpp}^{\text{FRT-CA}}$ with $\text{rn-gal4}$, $\text{UAS-FLP}$ to confirm that Dpp signalling is required in the prospective wing blade for normal growth (both $\text{dpp}^{\text{FRT-TA}}$; $\text{nub-gal4}$, $\text{UAS-FLP}$ and $\text{dpp}^{\text{FRT-CA}}$; $\text{rn-gal4}$, $\text{UAS-FLP}$ wing discs showed growth defects). Following this, I used the same $\text{dpp-gal4}$ driver (from Akiyama et al., 2015) to inactivate $\text{dpp}^{\text{FRT-CA}}$. In our case, striking growth defects were observed throughout the entire wing imaginal disc. Why, in the same experimental setup, is growth affected with $\text{dpp}^{\text{FRT-CA}}$, but not with the $\text{dpp}^{\text{FRT-TA}}$ allele? This question raises a few possibilities: first, our allele could be a hypomorph. Indeed, it has not been possible to tag mature Dpp (downstream of all three furin cleavage sites) with a large fluorophore, such as GFP. Thus, even a relatively small tag (2xHA) could affect function. A second possibility is that the excision of $\text{dpp}^{\text{FRT-TA}}$ by $\text{dpp-gal4}$ might not be efficient, leading to a late inactivation. I tested both of these possibilities by generating an untagged version of $\text{dpp}^{\text{FRT-CA}}$, which is referred to as $\text{dpp}^{\text{FRT-NoTag}}$. Inactivation of $\text{dpp}^{\text{FRT-NoTag}}$ with $\text{dpp-gal4}$, $\text{UAS-FLP}$ also resulted in severe growth defects throughout the wing disc. At this point, I decided to re-examine the expression of Brk in $\text{dpp}^{\text{FRT-TA}}$; $\text{dpp-gal4}$, $\text{UAS-FLP}$ wing discs. I confirmed that at 120 hours AEL, $\text{brk}$ was de-repressed in the pouch region. However, I found expression of Brk at earlier stages of wing disc development, something that Akiyama and Gibson failed to assess in their study. At 90 and 96 hours AEL, Brk expression was absent from the prospective wing blade, suggesting that $\text{dpp}^{\text{FRT-TA}}$ may not be efficiently excised. These results are supported by qPCR experiments, which reveal that significantly lower levels of $\text{brk}$ mRNA are present in $\text{dpp}^{\text{FRT-TA}}$ wing discs (compared to $\text{dpp}^{\text{FRT-CA}}$ discs) after induction of $\text{hs-flp}$ at 102 hours AEL. At conferences, many scientists have asked me why the excision of $\text{dpp}^{\text{FRT-TA}}$ with $\text{dpp-gal4}$ and $\text{UAS-FLP}$ is inefficient. This is not clear and would most likely require the construction of new $\text{dpp}$ conditional alleles. However, a look at the sequence of $\text{dpp}^{\text{FRT-TA}}$ reveals the presence of a LoxP site in close proximity to a FRT site. Since both have been shown to form hairpin structures, it is possible that their proximity to one another could impede recombination.

It is important to recognize that Akiyama and Gibson do see an effect on growth when they use $\text{nub-gal4}$, a relatively late driver, to inactivate their allele. Thus, it is most likely the combination of driver and allele that results in a late inactivation of $\text{dpp}$
expression. For example, it is entirely possible that \textit{nub-gal4} is a much stronger driver compared to \textit{dpp-gal4} in the wing disc. It should also be noted that several studies have been published in recent years, supporting our conclusion that the characteristic stripe of \textit{dpp} expression is required for wing disc growth throughout the third larval instar. In 2015, Harmansa et al. blocked the spread of Dpp from the ‘central stripe’ region by using \textit{dpp-gal4} to express a membrane-tethered anti-GFP nanobody and eGFP:Dpp (in a \textit{dpp}^{d8d12} background). As a result, patterning was disrupted, but the size of the pouch was also reduced (Harmansa et al., 2015). This result indicates that if Dpp cannot spread from its domain of expression at the A/P boundary, growth is disrupted and any alternative sources of Dpp (if they exist) in the anterior pouch do not suffice to rescue the phenotype. Our conclusions are also supported by results from the group of Marco Milán. Barrio and Milán use a series of different \textit{dpp}-RNAi lines in combination with the \textit{dpp-gal4} driver and the temperature-sensitive GAL80 molecule to remove the expression of \textit{dpp} from the A/P boundary. As a result, pouch size is significantly reduced (Barrio and Milan, 2017).

In addition to our work, these studies provide compelling evidence to show that Dpp emanating from the ‘central stripe’ region is indeed required throughout larval development to promote growth and cell proliferation.

After clarifying the role of the Dpp stripe, I used our conditional alleles to test other models of Dpp-depandant growth regulation, in particular the slope model and the growth equalisation model. Using a conditional allele, which allows complete removal of gene function in an area of interest, gave us the opportunity to rigorously investigate these models without using hypomorphs or clones. In fact, for the first time, it was possible to completely remove \textit{dpp} expression solely from the pouch and to replace it with a source of uniform Dpp in the same region (\textit{dpp}^{FRT-CA}; \textit{rn-gal4}>UAS-FLP, \textit{tubα1-FRT-f^'-FRT-dpp}). By doing this, I was able to show that uniform levels of Dpp in the pouch can promote normal rates of cell proliferation (assessed by staining for PH-3). However, \textit{dpp}^{FRT-CA}; \textit{rn-gal4}>UAS-FLP, \textit{tubα1-FRT-f^'-FRT-dpp} adult wings are smaller compared to wildtype. I believe there are two main reasons for this incomplete rescue: in this genotype, some \textit{vg} expression is lost in the anterior compartment of the wing disc. At later stages, these mis-specified cells might undergo cell death and apoptosis. Furthermore, it is possible that our manipulation affects developmental processes in the
pupal wing, such as cell proliferation. For example, it is conceivable that the rounds of cell division that normally occur in pupal wings do not take place, thus accounting for the smaller wing size.

To address the growth equalisation model, we decided to repeat the experiments from Schwank et al., 2008 with our conditional alleles. In this paper, the authors show that \textit{brk} and \textit{dpp/brk} hypomorphs produce overgrown wing discs, with highly elevated rates of proliferation in the lateral regions of the wing disc and lower rates of proliferation in the medial areas. The authors argue that Dpp controls growth exclusively via the repression of \textit{brk} and that Dpp/Brk act in a growth modulatory system, evening out proliferative differences in the wing disc. In my thesis, I show that the simultaneous inactivation of \textit{brk} and \textit{dpp} in the pouch rescues the majority of growth defects seen in \textit{dpp\textsuperscript{FRT-CA}, rn-gal4, UAS-FLP} wing discs. Furthermore, the complete inactivation of either \textit{brk} or \textit{dpp/brk} throughout the entire wing disc gives rise to the same phenotype – mutant wing discs proliferate more in peripheral regions and less in medial regions. These results confirm that Dpp primarily controls growth through the repression of \textit{brinker}. However, in contrast to \textit{brk} hypomorphs, \textit{brk\textsuperscript{FRT}, esg-gal4>FLP} wing discs, dissected at 120 hours AEL, were not necessarily overgrown. In fact, at this time point, the pouch size of \textit{brk\textsuperscript{FRT}, esg-gal4>FLP} wing discs was comparable to wildtype. Although overgrowth was not seen at 120 hours AEL, a considerable shape change was noted in mutant discs, characterized by an extension of the A/P axis and a reduction of the D/V axis. This was also evident when mutant wing discs were dissected at 96 hours AEL, despite no apparent changes to cell proliferation in the wing disc at this time point. The origin of these shape changes is of major interest and will be the focus of future work. It will also be important to understand why proliferative differences arise in the absence of \textit{brk} during late stages of larval development, and whether they are correlated with the shape changes occurring throughout the tissue at earlier time points. In order to address these questions, first, we need to identify the target genes downstream of Brk that regulate cell proliferation.

Clonal studies and ChIP-Seq. data suggest that Brk regulates growth by repressing the expression of \textit{d-myc} in the peripheral regions of the wing disc (Doumpas et al., 2013). Indeed, the \textit{d-myc} locus contains four putative binding sites for Brk and \textit{brk} mutant clones
express d-Myc in lateral regions of the wing disc. However, the over-expression of d-Myc in the pouch only slightly rescues the size of nub-gal4>UAS-Brk adult wings (which are more than 80% smaller compared to wildtype wings). This unimpressive rescue prompted us to further investigate the relationship between brk and d-myc. First, I used brk-gal4 to simultaneously inactivate brk and to express three different d-myc RNAi lines in the domain of brk expression. If the increased rates of proliferation in the lateral regions of brkFRT; brk-gal4>FLP wing discs are caused by the de-repression of d-myc, then the RNAi lines should counteract this effect. Surprisingly, the expression of d-myc RNAi (previously validated with vg-gal4) did not alter the phenotype of brkFRT; brk-gal4>FLP wing discs. brkFRT/brkGAL4; UAS-FLP/UAS-dMycRNAi discs still exhibited tissue-wide shape changes and a heterogenous profile of dividing cells. Overall, these results do not provide evidence to show that Brk regulates the expression of d-myc. However, they also do not rule out such a possibility. Thus, I decided to generate a transgene, which would be able to express relatively low, uniform levels of d-myc in a region of interest. Tubα1-FRT-STOP-FRT-dmyc flies will be used in an attempt to rescue the reduced pouch size seen in dppFRT-CA; rn-gal4>FLP wing discs. As previously described, a similar experiment has been done with nub-gal4>UAS-Brk, UAS-dMyc. However, I believe our new approach brings an advantage – our transgene expresses more physiological levels of d-myc and does not overload cells with a potent growth factor.

Finally, I wanted to understand how, if at all, Dpp signalling affects the expression of vestigial, a nuclear protein with no known homologs. As described previously, the expression of vestigial specifies the wing primordia in the embryo (Cohen et al., 1993, Goto and Hayashi, 1997, Williams et al., 1991) and later drives wing blade development throughout the larval stages (Kim et al., 1996). Interestingly, it has also been suggested that Mad binds to, and is required for, the activation of the vg quadrant enhancer in the wing disc (Kim et al., 1997). Thus, the relationship between Dpp signalling, vg expression, and growth has been the subject of many conversations in our field. At the moment, there is little evidence to suggest that Dpp signalling regulates growth and cell proliferation by activating the expression of vg. I decided to test this hypothesis by 1) assessing Vg expression in dpp mutant wing discs, and 2) using tubα1-FRT-f+ -FRT-vg to try and rescue the growth defects associated with dpp mutant discs. I found that Vg
expression is disrupted in both \(dpp^{FRT-CA}; \, rn\text{-}gal4>UAS\text{-}FLP\) and \(dpp^{FRT-CA}; \, rn\text{-}gal4>UAS\text{-}FLP; \, tub\alpha1\text{-}FRT\text{-}f^*-FRT\text{-}dpp\) wing discs. More specifically, expression is lost in a stripe of cells near the A/P boundary, but not at the D/V boundary, suggesting that the activity of the \(vg\) quadrant enhancer is affected. Moreover, the fact that \(tub\alpha1\text{-}FRT\text{-}f^*-FRT\text{-}dpp\) cannot rescue the phenotype suggests that only the highest levels of Dpp signalling activate \(vg\) expression. It is interesting to note that this regulation seems to be specific to the anterior compartment, as no obvious changes in \(vg\) expression were detected in the P compartment of \(dpp\) mutant wing discs (confirmed by co-staining discs with an antibody against Patched, a marker of the A/P boundary). Overall, this shows that Dpp signalling regulates the expression of \(vg\) in a specific domain in the A compartment. However, it is still unclear how this contributes to growth. In order to answer this question, I compared the size of \(dpp^{FRT-CA}; \, rn\text{-}gal4>UAS\text{-}FLP\) adult wings to \(dpp^{FRT-CA}; \, rn\text{-}gal4>UAS\text{-}FLP; \, tub\alpha1\text{-}FRT\text{-}f^*-FRT\text{-}vg\) adult wings. No size differences were detected between adult wings, even though \(tub\alpha1\text{-}FRT\text{-}f^*-FRT\text{-}vg\) can rescue a significant portion of growth when \(vg\) is inactivated in the prospective wing blade. These results suggest that even though Dpp signalling does regulate \(vg\) expression, it seems unlikely that this regulation also promotes cell proliferation in the medial regions of the wing disc. Indeed, \(vg\) expression is altered in \(dpp^{FRT-CA}; \, rn\text{-}gal4>UAS\text{-}FLP; \, tub\alpha1\text{-}FRT\text{-}f^*-FRT\text{-}dpp\) wing discs, but this does not seem to have an effect on cell proliferation \((dpp^{FRT-CA}; \, rn\text{-}gal4>UAS\text{-}FLP; \, tub\alpha1\text{-}FRT\text{-}f^*-FRT\text{-}dpp\) discs do not show a loss of PH-3 in medial regions of the wing disc when compared to wildtype).
6.1.1 What is our model of growth regulation?

Altogether, our results support the notion that the characteristic stripe of \textit{dpp}, produced along the A/P boundary, is required for wing disc growth throughout larval development. Furthermore, our experiments demonstrate that a gradient of Dpp is not required to drive cell proliferation (although it is necessary for correct patterning). This conclusion is based on the following observations: cell proliferation is not affected when 1) \textit{dpp} expression is inactivated in the pouch and replaced with low, uniform levels of Dpp and 2) \textit{dpp} and \textit{brk} are completely inactivated in the prospective wing blade. Some may argue that the activity of the \textit{tubulin} promoter is not entirely uniform throughout the disc. However, the fact that cell proliferation can proceed normally in the absence of \textit{dpp}/\textit{brk} in the pouch is strong evidence that cells do not ‘read’ the gradient of Dpp when undergoing mitosis (see Figure 6.1). In light of this, I do not believe that Dpp directly regulates cell proliferation in the wing disc. In fact, most of my results agree with the growth equalisation model, which states that Dpp indirectly promotes growth through the repression of \textit{brk}. Indeed, reduced cell proliferation in \textit{dpp}^{FRT-CA}; \textit{rn-gal4>UAS-FLP} can be rescued by the concomitant inactivation of \textit{brk} in the pouch. Thus, it is my opinion that in the prospective wing blade, the gradient of Dpp is critical for specifying cell fates, but is dispensable for growth. Cell proliferation in the pouch occurs at normal rates as long as Dpp signalling remains above a pro-growth threshold. If Dpp signalling drops below this threshold, cell proliferation is affected negatively. On the other hand, I hypothesize that high levels of Dpp signalling, which exceed this threshold, should not have any effect on growth in the pouch because \textit{brk} is already fully repressed (or it is repressed enough so as not to hinder growth).

It is important to note that this model does not apply to the hinge territories, where the expression of \textit{brk} is necessary to equalize proliferation rates throughout the entirety of the wing disc. It is not known why wing imaginal discs devoid of \textit{brk} expression exhibit significantly decreased rates of cell proliferation in medial regions. Perhaps, the Dpp/Brk system is responsible for counteracting the proliferative differences generated by another diffusible factor (i.e. a growth factor with high activity in the lateral regions of the disc). However, this is pure speculation because there is no evidence for such a hypothetical factor. It is also possible that the Dpp-Brk system irons out differences in proliferation
rates that arise as a result of mechanical tension. It has been proposed that during the
growth of the wing imaginal disc, cells in the medial regions become compressed over
time. Conversely, cells in the peripheral regions become stretched and gain a growth
advantage compared to medial cells (assuming a growth-stimulatory role for stretching)
(Aegerter-Wilmsen et al., 2007). Brk would counter this advantage and promote uniform
growth. In the future, it will be interesting to explore the relationship between mechanical
forces, brk expression, and cell division.

The removal of brk expression in wing imaginal discs also has a significant effect
on the size and shape of adult wings, as seen in Figure 4.10. Mutant adult wings exhibit
a rounded phenotype. Although not as severe, a rounded phenotype is also seen in adult
wings with defects in planar cell polarity (i.e. dachsous mutants). I hypothesize that brk
expression is involved in determining the final shape of the adult wing, perhaps in part
by regulating planar cell polarity. In the future, I plan to look at the orientation of cell
divisions and the localisation of cytoskeleton-associated proteins in brk mutant wing
discs.
Chapter 6. Discussion

Figure 6.1 – Dpp regulates growth in the pouch solely via the repression of brk

(A-C) Each graph shows the expression of Dpp (green) and Brk (red) along the A/P boundary in the pouch region of the wing imaginal disc. Growth is also represented along the A/P boundary in blue. (A) In a wildtype scenario, Dpp signalling represses brk in the medial regions of the pouch. It is thought that the Dpp gradient is converted into an inverse gradient of Brk activity. (B) Uniform, low-level Dpp expression in the pouch stimulates normal rates of cell proliferation (brk is still silenced in medial regions). (C) The concomitant removal of Dpp and Brk from the pouch does not affect growth, showing that Dpp controls growth in the pouch region solely via the repression of brk.
6.2 Outstanding Questions and Future Experiments

We are merely partway to attaining a complete understanding of how Dpp and Brk affect cell proliferation in the wing imaginal disc. In the future, I believe the following questions must be rigorously addressed:

1. What are the downstream targets relevant for growth and cell proliferation?

It is important to identify the pool of pro-growth, anti-apoptotic genes that are regulated by brk in order to achieve a satisfactory understanding of growth control. This can be achieved by re-examining the ChIP-Seq. data published by Doumpas et al. and testing different candidates from their list of predicted target genes. However, it would also be useful to conduct RNA-Seq. on brk mutant wing discs to see what genes are mis-regulated in this genetic background. A comparison of RNA-Seq. and ChIP-Seq. data may allow us to more easily identify the target genes that are relevant for growth. Although it has been suggested that brk regulates cell proliferation by repressing d-myc and bantam, rescue experiments indicate that these are not the only target genes responsible for growth control.

2. What stages of the cell cycle are regulated by Brk?

It is not known what stage of the cell cycle is affected when brk is inactivated in the wing disc. EdU labelling and PH-3 stainings suggest that the expression of brk affects both the G1/S and G2/M transitions. Nevertheless, it may be useful to use a FUCCI sensor to closely monitor and readout cell cycle phases in mutant discs at different developmental time points.

3. What causes the shape changes seen in brk mutant discs?

In addition to identifying the origin of proliferative differences, it is equally important to consider the shape changes that arise throughout the development of brk mutant wing discs. Before this study, I assumed that the morphological changes (extension of the D/V axis, reduction of the A/P axis) seen in brk mutant discs at 120 hours AEL were a direct consequence of altered (heterogeneous) proliferation rates. However, my results suggest that the shape changes seem to occur before proliferation is
even affected. In the future, we hope to better characterize the cell shape changes and the orientation of cell divisions in brk mutant discs throughout larval development.

4. How do different levels of Brk affect growth and cell proliferation?

As mentioned previously, Brinker is thought to form an inverse gradient to pMad in the wing disc. Although it has been shown that high levels of Brk in the hinge repress growth, it is not known if lower levels of Brk in the peripheral regions of the pouch also function to suppress cell proliferation. Indeed, Brk seems to ‘invade’ the pouch over developmental time, so that at 120 hours AEL, its expression is clearly seen in the lateral regions of the prospective wing blade. Although experiments with Tubα1-FRT-STOP-FRT-brk:V5 suggest that low levels of Brk can still repress growth, it will be interesting to precisely correlate different concentrations of Brk with cell proliferation rates.

5. Does the inactivation of brk alter mechanical forces in the wing disc?

In addition to identifying target genes and characterizing cell cycle changes, it is important to consider mechanical tension in the context of growth control. Are changes in mechanical tension associated with the phenotype seen in brk and dpp/brk mutant wing discs? This is certainly a possibility that must be addressed. In the past, theoretical models have suggested that cells in the center of the wing disc undergo increased compression. Conversely, cells in the periphery become stretched and gain a growth advantage (they can divide more easily). This growth advantage would need to be counteracted by a growth repressor, such as brk, to maintain a uniform profile of cell division in the wing disc (Aegerter-Wilmsen et al., 2007). To better understand the relationship between mechanical tension and brk expression, new methods must be developed that allow scientists to alter mechanical forces in specific regions of the wing disc.
6.3 Final Remarks

Looking back on the work that I have done, I realize now that there are certain limitations to our study. Firstly, as this study only measures changes in surface area as a proxy for growth, much of the feedback that I have received revolves around characterizing growth in 3D, not 2D. In an ideal world, I would also evaluate the changes in volume that occur after certain genetic manipulations. Indeed, there are programs that can rapidly calculate the volume of a certain region from a stack of images. In the future, it will also be important to look at the density of nuclei or cells, something that I have not quantified in any of my experiments. In *Drosophila melanogaster*, this can easily be done by calculating the density of trichomes (denticles or hairs that arise from the cytoplasm on individual cells) in a particular region of the adult wing. Going forward, many different parameters of growth must be measured in order to gain a full understanding of how the Dpp/Brk system regulates growth and cell proliferation.

As stated previously, there are currently no known proteins that share strong homology with *brinker*. Moreover, in my literature review, I was surprised to read that TGFβ/BMP signalling in mice has been strongly associated with the inhibition of cell proliferation (the opposite of what occurs in our model system). For example, TGFβ has been shown to down-regulate the expression of *c-myc* in epithelial tissues, leading to the activation of Cdk inhibitors (Massague et al., 2000). Notwithstanding, studying the function of *dpp* and *brk* in wing imaginal discs can help us shed light on some basic questions in science; here, we sought to elucidate the relationship between patterning and growth. Our data confirms that Dpp primarily regulates patterning and cell proliferation by establishing an inverse gradient of Brk in the wing disc. Although the Dpp morphogen gradient does seem to play a direct role in patterning this tissue, our results suggest that its role in growth control is permissive (in agreement with the growth equalisation model). Thus - in contrast with some views - I do not believe that the Dpp gradient or changes in Dpp signalling activity can influence growth in the wing imaginal disc. Overall, it remains to be determined how different levels of Brk affect cell proliferation and what the origin of proliferative differences is in the absence of *brk* function.
Reference List


CUI, Y., JEAN, F., THOMAS, G. & CHRISTIAN, J. L. 1998. BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development. EMBO J, 17, 4735-43.


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Appendix

DNA sequence re-inserted in the attP site of dpp KO mutants to generate dpp^{FRT-CA}

AGCTCGACTTCCGGCCCCACACTGAGAGCTAGCTAAGCTAAGTTCCTATGATTTCTTTGCTCCAAGCTCACCGGCCGCCCGCGCACAGTGGTGGAGGTGCGGACGGTCCGCTCCCTGAAGCCGGCCCCACACCACCATGTACGCCTGCGCCGCAGCGCGGACGAGGCGCACGAGCGGTGGCAGCACAAGCAGCCGCTCCTGTTCACCTACACGGACGACGGGCGGCACAAGGCGCGCTCCATTCGGGACGTGTCTGGCGAGAGGGCGGTGGCAAGGGCGGCCGGAACAAGCGGCAGCCGAGACGGCCTACGAGGCGCAAGAACCACGACGACTACCCATACGACGTCCCTGACTATGCGGGCGGATATCCCTATGATGTTCCAGATTACGCTACCTGCCGGCGGCACTCGCTGTACGTGGACTTCTCGGACGTGGGCCTGGGACGACTGGATTGTGGCGCCTCTGGGCTACGATGCATATTACTGCCACGGGAAAGTGCCCCTTCCCGCTGGCCGACCACTTTAACTCGACCAATCACGCCGTGGTGCAGACCCTGGTCAACAATATGAATCCCGGCAAGGTGCCGAAGGCGTGCTGCGTGCCCACGCAACTGGACAGCGTGGCCATGCTCTATCTCAACGACCAAAGTACGGTGGTGCTGAAGAACTACCAGGAGATGACCGTGGTGGGCTGTGGCTGTCGATAGATTCGCACCACCATCGCACTAGTGACCATACCACGCCATCCACTCAACCGAGTGAATGCGATGGGAAATCGCGAGCGAGAGAGCATCAAATGCTGTTTGTTCCAAGCCGTCAATGCTTTAAACACAACGCAAACAAAATGGACTGAATATTTGAATTTTAAGTGTAAATCGTTAGACTTTAGCCGTATCGAGTAACGAGCAAACAGGCGGCAGCCACGCCCACATCCACGTCCCCACCAAAACCGCCCGCCTTG

DNA sequence re-inserted in the attp site of dpp KO mutants to generate dpp^{FRT-REP}
DNA sequence re-inserted in the attP site of brk KO mutants to generate brk^{FRT} –
Appendix

This page contains a sequence of nucleotide bases, which is typical in a genetic or molecular biology context. The text is a string of nucleotides, which are the building blocks of DNA and RNA. Each nucleotide is represented by a combination of four letters: A, T, G, and C. These letters correspond to the bases Adenine, Thymine, Guanine, and Cytosine, respectively. The sequence is likely part of a larger piece of DNA or RNA, possibly involved in genetic information coding or research.