Control of growth and pattering: a novel rol of JAK/STAT in regulating morphogen production and signaling

Control del crecimiento y patrón: un nuevo rol de JAK/STAT regulando la producción y señalización de morfógenos

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Control of growth and patterning: a novel role for JAK/STAT in regulating morphogen production and signalling

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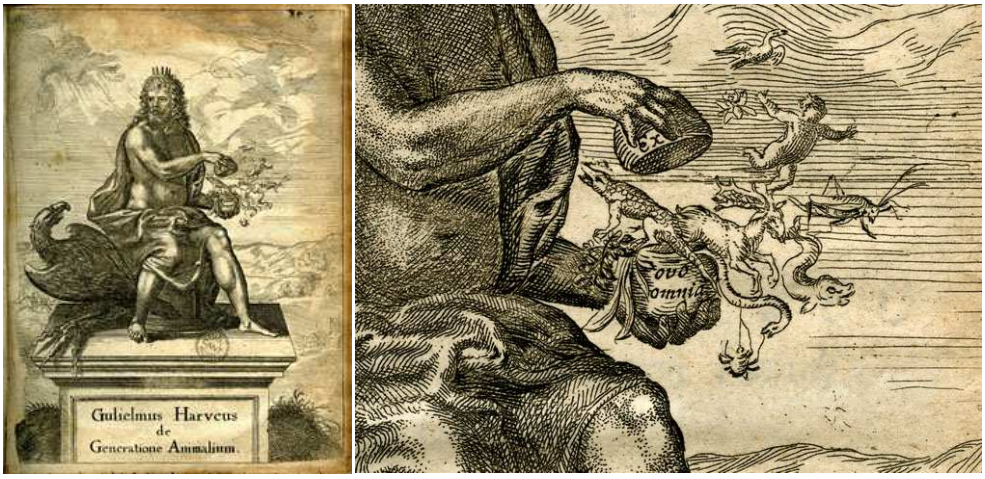
Introduction

Developmental Biology: a merge of traditions



***Ex ovo omnia* ("All from the egg").** Frontispiece of William Harvey's book *On the Generation of Animals* (1651) depicts Zeus liberating living creatures from an egg. It symbolizes the unity of the principles of development across species.

Every scientific discipline is defined by the questions it asks and by the problems it tries to solve. Developmental biology studies how an organism builds itself from a single cell zygote, how order, complexity and form emerges from initially equivalent cells, or in another way, how a relatively homogeneous material – the embryo – generates and generated the diversity of life forms. Traditionally, developmental studies focused on embryogenesis, the phase of an organism between fertilization and birth, but development does not stop at birth. Many organisms display determinate or indeterminate growth, while others experience successive developmental transitions or undergo metamorphosis after the larval stage. In general, organisms never stop developing, either by renewing old or defective cells or by regenerating lost body parts. In addition, and to some extent, disease can be viewed as a dysfunction of developmental processes or a failure to sustain tissue

homeostasis and repair, which can contribute to cancer, aging and degeneration.

Many questions that developmental biology seek to answer were formulated hundreds of years ago by philosophers and scientists, long before it defined itself as a discipline of biology. Its roots are the developmental anatomy and experimental embryology traditions that eventually combined together with other disciplines such as genetics, physiology, and cell, molecular and evolutionary biology. The integration with other areas brought new ideas and inspired concrete thinking about the cellular and molecular nature of developmental processes. In parallel, advances and applications from physics and chemistry led to the incorporation of new technologies and reagents that greatly expanded the repertory of tools available to study tissues, cells and molecules. This multidisciplinary approach revealed that despite the great diversity of sizes and shapes, the body plan of most organisms is built up by a limited and conserved set of developmental toolkit genes recurrently used for different purposes during development (Newman, 2006; Newman and Bhat, 2009; Rokas, 2008a, 2008b). The majority of toolkit genes are components of signalling pathways, encode for the production of signalling molecules and secreted morphogens, cell adhesion proteins, receptor ligands, intracellular signalling proteins and transcription factors. Nowadays developmental biology tries to describe the processes and mechanics of life at any level of detail, and occupies a central positon in biology both in basic and biomedical research.

The questions: a conceptual toolkit

Development accomplishes two major goals. First, to generate an organism from an embryo and second, to ensure the continuity of life by reproduction. Two big questions that developmental biologists conveniently divided into smaller, yet complex problems and concepts that outline some of the principles of development. In general, the formation of a complex multicellular organism encompasses three minimal requisites: an increase in size, some degree of cellular specialization and sub-functionalization as well as the spatial ordering of cells in a coherent unit according to the morphology of its species. Nonetheless, developmental processes do not function as independent entities, but they influence considerably each other.

Growth and size determination

Control of organ size is a fundamental aspect of biology and is critical for organism fitness. Developing organisms must grow up until its species-specific size whereas many organs also display homeostatic size-control mechanisms to (i) ensure that the final size is attained and (ii) to maintain-the overall body and organ size during the rest of life. In general, the number and size of the cells it contains determine the final size of an animal. The balance between cell proliferation and cell death fixes cell number, while cell size depends on cell growth. Decades of research raised and answered questions regarding the connections between growth – increase in cell mass – and cell division (Neufeld et al., 1998; Su and O'Farrell, 1998; Weigmann et al., 1997). How is cell proliferation coupled with cell growth in continuously dividing tissues and uncoupled in other cell types? How growth is coordinated within and between organs to generate well-proportioned individuals? Can growth

be pushed by driving cell proliferation or cells must attain a minimal size to progress in the cell cycle? Perhaps even more intriguing than knowing how an organ grows is to understand how its size is determined. How an organ stops growing? Does a tissue count cell numbers or cell divisions? Which are the size-sensing mechanisms that measure global dimensions and arrest growth at the appropriate size? Answers to these and other questions related to growth control during development are certainly helping to address other growth-dependent processes, such as cancer and regeneration.

Morphogenesis, on form and function

The size and shape of organs, limbs and other body parts define the organism's morphology. Form and function are tightly linked; the shape of a structure in an organism is critical to its function, even at the individual cellular level, for example the branching of neurons and elongated skeletal muscle fibres. Cell communication, adhesion and polarity are essential for morphogenesis since they explain how cells move, migrate, rearrange and change their morphology during development in a coordinated manner in order to build complex multicellular organisms (Lecuit and Le Goff, 2007).

Pattern formation, from cell fate to differentiation

The building of an organism is a process of refinement. Initially involves laying down the overall body plan defining the main axes of the animal – the anterior and posterior ends and the dorsal and ventral sides. General features of a body plan appear first and, as development proceeds, the more particular and specialized structures are progressively elaborated. This regional specification is the result of a developmental genetic program that precisely controls the spatial and temporal pattern of gene

activity to instruct cell identity and fate. During development, patterns of gene expression emerge to subdivide adjacent cell populations into distinct genetic domains and delineate specific territories in the developing tissues that will ultimately define each portion within an organ or anatomical unit (Figure 1).

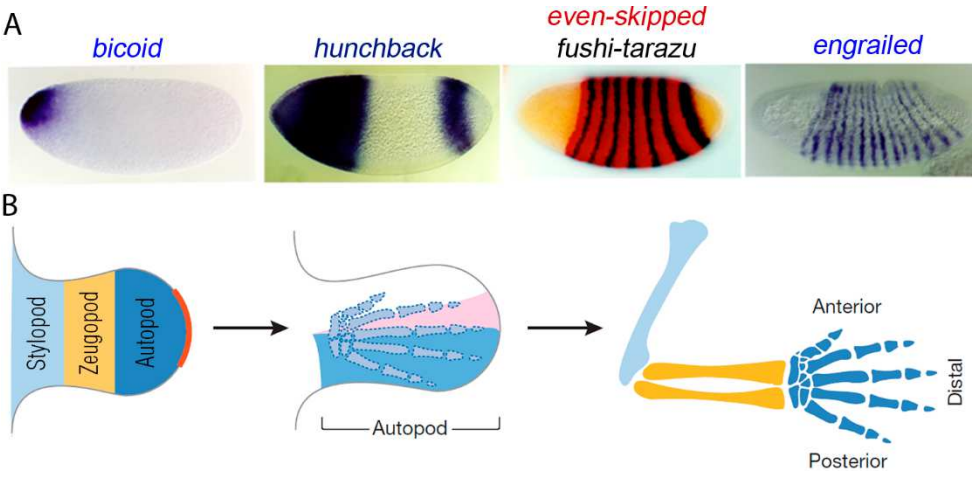


Figure 1. Patterning subdivides tissues into territories (**A**) Illustrative image of the spatially restricted expression of some genes involved in the patterning of the *Drosophila* embryo along the AP axis. The combined action of these and other genes establish the antero-posterior polarity, divide the embryo into regions and set the boundaries of segments. (**B**) In the vertebrate limb, a patterning system subdivides the growing limb along the proximo-distal axis in three main regions and along the antero-posterior axis to stablish the position of digits. Adapted from (Dekanty and Milán, 2011).

The generation of patterns from a uniform field of cells often involves the activity of **signalling centres**, which behave as **organizers** by the secretion of long- and short-range ligands as well as **morphogens**. The idea that organizers are group of cells that release **inductive signals** to regulate morphogenesis was demonstrated by Hans Spemann and his assistant Hilde Mangold (Spemann and Mangold, 1924). In a famous

transplant experiment in amphibian embryos, they showed that a secondary embryo could be formed by grafting one small region onto a new site on another embryo. This region was called the Spemann organizer, since it seemed to be sufficient to cause a global reorganization of the spatial pattern and to direct the development of a new body axis. Since then, several organizers have been described as a recurrent strategy to control pattern and morphogenesis during development (Anderson and Stern, 2016). The view that organisms are patterned by gradients of "formative substances" was anticipated by Morgan and Boveri (Boveri, 1901; Morgan, 1901) studying regeneration in worms and hydroids. Morgan observed a gradient of regenerative capacity along the antero-posterior axis in worms and conceived the existence of graded substance differences along the body axis. The word morphogen was later coined by Alan Turing (Turing, 1952) without pretending to have a very exact meaning, but simply the substance envisioned to convey the idea of a diffusible form producer. Lewis Wolpert refined later the concept as a synthesis of several ideas – organizers, induction, gradients, thresholds and diffusion – from previous embryological and theoretical studies and proposed his "French flag model" as a mechanism for morphogen gradients in pattern formation (Figure 2). He also introduced a new cellular parameter, the concept of positional information (Wolpert, 1969, 1989, 2011, 2016). Cells acquire information about their position relative to their neighbours and to an internal coordinate system, and they interpret these positional values according to their genetic constitution and developmental history to differentiate at specific positions. The current view of a morphogen retains Wolpert's core notion and is defined as a molecule that spreads out from a localized source to form a concentration gradient that provides positional information and determines the responses of all cells in the field. This

gradient creates a series of concentration thresholds along the tissue that set the transcriptional state of downstream target genes in discrete domains of expression (Figure 2). These genetic subdivisions are ultimately used to define cell identity and the pattern of differentiation. Even though at earlier stages of pattern formation cells might not look differentiated, variations between cells exist in terms of their commitment to particular fates and their developmental potential becomes restricted. Committed cells will eventually start to display marked changes in their morphology, biochemistry and function as they differentiate according to their position within an organ to generate the diversity of cell types.

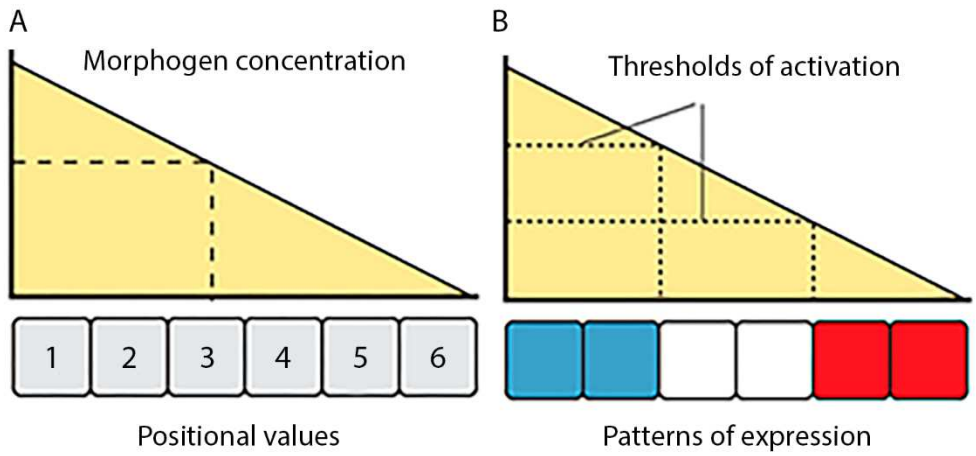


Figure 2. Morphogen gradients in pattern formation. (**A**) A gradient of morphogen concentration determines, point by point, the positional values of all cells in the field. (**B**) The morphogen gradient activates target genes in a concentration-dependent manner, thus positional values are interpreted by the cells to form a defined pattern (colours). Adapted from (Wolpert, L. Principles of Development).

As noted above, there is an interplay between developmental processes, they influence and sometimes overlap each other since they occur simultaneously and coordinately. For instance the connection

between growth and morphology. It is known that morphological changes can be driven by **local differences in growth rates,** by adjusting the **duration of the growth period** or by modulating the **direction of growth**. Randomly oriented cell proliferation result in isodiametric growth, whereas oriented cell divisions result in shape-elongated tissue growth (Figure 3A) (Baena-López et al., 2005; Desplan and Lecuit, 2003; Lecuit and Le Goff, 2007; Rolland-Lagan et al., 2003). Similarly, local variations in the growth rates within and between organs can also affect shape and proportions. The proximo-distal outgrowth of vertebrate limbs was initially suggested to rely on a gradient of proliferation rates that elongates the limb primordia from the distal tip (Fernandez-Teran et al., 2006; Niswander et al., 1993), and recently it has been proposed that oriented cell divisions are critical to promote elongation in the mice limb bud (Boehm et al., 2010). Similarly, in both the petal lobe of *Antirrhinum* and the *Drosophila* wing, the final shape is achieved by orienting cell divisions along the proximo-distal axis (Baena-López et al., 2005; Mao et al., 2011; Rolland-Lagan et al., 2003). Whereas organ-intrinsic variations in local growth may alter the shape of an individual organ, differential growth rates and timings between organs change the allometric relationship between distinct body parts. A classic example are D' Arcy Thompson's illustrations in his book *On growth and form,* 1917, which show how the transformation of one form to another of a related species can be represented by geometric distortion of a Cartesian grid (Figure 3B). In cellular terms, transformations are probably caused by changes in the rates, durations and directions of growth.

Growth and patterning are also connected. Tissue growth can influence how patterning signals exert their function and modulates the way receiving cells respond to developmental cues, by adjusting the time,

the intensity and the amount of cells exposed to patterning signals. Blocking tissue growth may cause the failure to specify cell fates and define territories, resulting in terminal phenotypes reminiscent of patterning defects rather than a mere undergrowth (Kenyon et al., 2003; Rafel and Milán, 2008; Towers et al., 2008). Conversely, patterning events generate new domains within a tissue that often behave as organizers, essential to propel further growth of the tissue (Figure 3C, D). Thus, growth can be "upstream" of pattern formation and vice versa. The interplay between morphogens, growth and patterning will be discussed further in the next sections of this thesis in the context of *Drosophila* development.

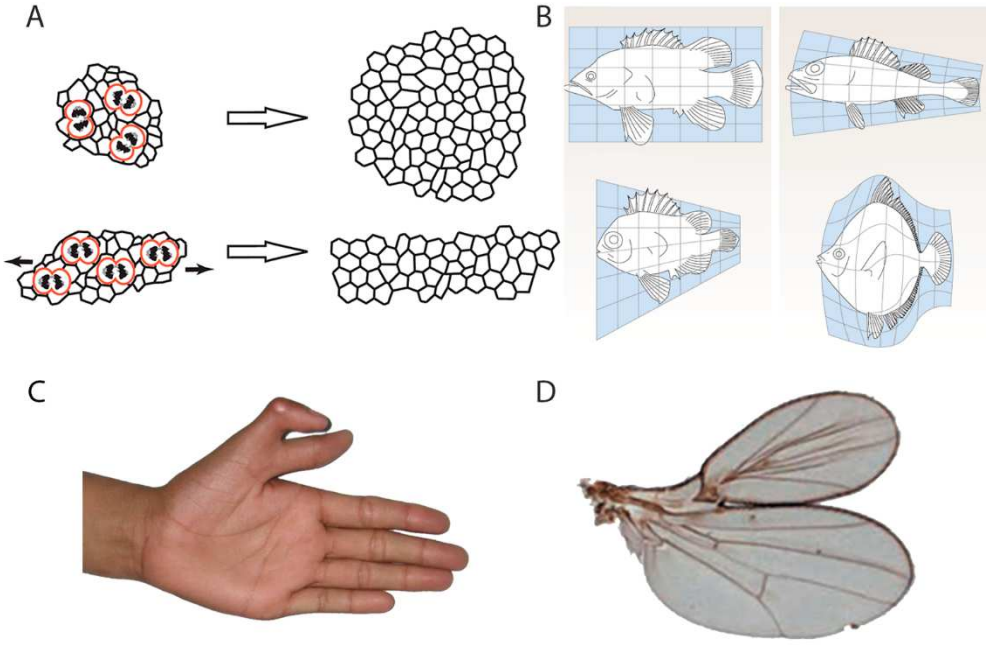


Figure 3. Interplay between growth, patterning and morphology. (**A, B**) Growth influences morphology. (**A**) The orientation of cell divisions results in shape differences in a growing tissue. (**B**) Adaptation of D'Arcy Thompson's illustrations showing morphological transformations viewed as changes in the relative growth between body regions. (**C, D**) Patterning directs growth. A patterning event such as the generation of a new organizer in developing human or *Drosophila* limb results in additional growth and duplication of structures. Adapted from (Arthur, 2006; Lecuit and Le Goff, 2007; Tabata, 2004).

Drosophila as a model organism

The universality of the genetic language and the conservation of the core structural and functional elements that govern essential biological functions makes the use of model organisms extremely useful and necessary to address a variety of biological questions, many of them relevant for human physiology. The discoveries made in one organism are expected to provide insight into the workings of other organisms but, since life is too diverse to find all the answers in a single organism, biologist adopted several model systems, each representative of particular taxa.

In this work, we used the fruit fly *Drosophila Melanogaster* as a model system. The biology of *Drosophila* is one of the best known among all animal models that, by historical and practical reasons, has been used to address a variety of biological questions for more than one hundred years. Its suitability for genetic manipulations and short generation times and its well-described developmental biology makes the fruit fly an ideal model system for research in several areas of biology. The vast collection of mutant strains previous to the genomic era, the ample repertory of tools to alter gene expression in specific tissues as well as the use of RNAi-mediated gene silencing (Dietzl et al., 2007; Ni et al., 2011) or targeted mutations (Bellen et al., 2004, 2011; Xu et al., 2015) to interrogate gene function, have characterized many of the genes involved in signalling pathways. The use of genetic mosaic techniques also allows the study of lethal recessive mutations in defined cell populations or to test the “cell-autonomy” of a mutant phenotype (Blair, 2003). The ease of genetic studies is because *Drosophila* is a less complex animal compared to vertebrates, with low genetic redundancy and streamlined versions of the major signalling pathways, making the identification of core functional elements

and epistatic analysis more straightforward. In addition, after the sequentiation of the *Drosophila* genome it was estimated that 75% of human disease-related genes have an identifiable orthologue in *Drosophila* (Adams, 2000; Reiter et al., 2001). This allows to recapitulate some of the cellular and molecular features of human physiology, model disorders and drug testing in a simplified system (Padmanabha and Baker, 2014; Singh and Irvine, 2012; Wangler et al., 2015; Yamamoto et al., 2014; Zirin and Perrimon, 2010). *Drosophila* is a holometabolous insect constructed by a linear series of repeating body segments or metameres that fuse or articulate to form the functional morphological units or tagmas, such as the head, thorax and abdomen. Its life cycle comprises four different main forms, the embryo, larva, pupa and the adult (Figure 4).

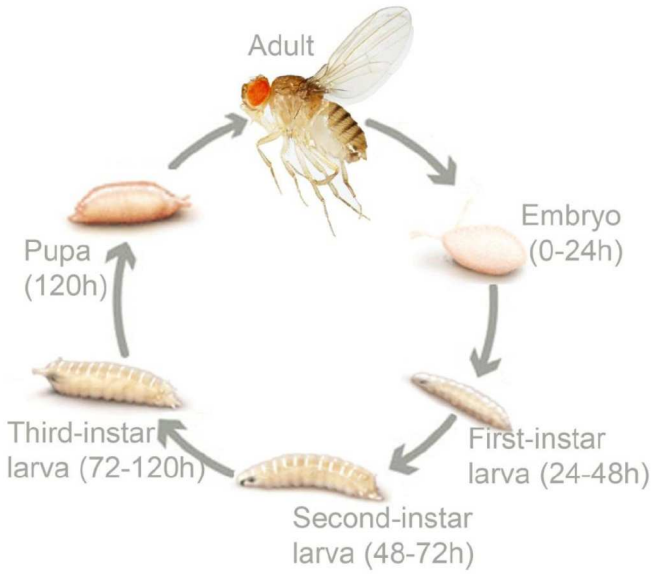


Figure 4. The life cycle of *Drosophila Melanogaster.* The duration from the embryo to the adult stage extends about ten days at 25ºC. Its life cycle starts with a 24-hour embryogenesis, followed by four days of larval development and five days of pupal stage where metamorphosis occurs and most of the larval tissues are histolyzed and replaced by adult tissues. The larval stage is divided in three instars (L1, L2 and L3) spaced out by ecdysis, or cuticle molts.

The larvae are composed of two types of tissues: the polyploid endoreplicative and the diploid proliferative imaginal tissues. The first go through successive rounds of genome duplication without cell division (G-S cycles) leading to an increase in cell size but not cell number. This is the case of the gut, salivary glands, trachea, malpighian tubes, fat body and larvae integument. By the end of embryogenesis, specific groups of cells invaginate and are set aside from the embryonic ectoderm in fixed positions that will give rise to the imaginal tissues, the primordia of the adult cuticle (Bate and Arias, 1991; Cohen, 1990; Cohen et al., 1993; Mandaravally Madhavan and Schneiderman, 1977). While the epidermis of the adult abdomen originates from histoblast nests, the rest of the external adult structures derive from imaginal discs (Figure 5). There are nineteen imaginal discs, nine pairs precursors of the head, thorax and appendages, and a single genital disc (Aldaz et al., 2010). Imaginal discs grow and proliferate throughout larval stages and they differentiate and fuse during metamorphosis to form adult structures. Given that the adult cuticle does not grow neither molts, any future increase in body size is constrained by the rigid exoskeleton. Consequently, the rate and the duration of growth during the feeding period restricts body growth, and the transition from larva to pupa fixes the future final adult size. In this context of extensive growth, an autonomous and genetically determined series of signalling cascades pattern imaginal discs along the main axes to assign cell identities and positions. Indeed, allograft transplantations of disc fragments into mature larvae showed that the important events of pattern formation have already occurred in late imaginal discs and as such, the fate of each territory is strictly determined to give rise to the different parts of the adult cuticle and its corresponding histotypes (Bryant, 1971, 1975).

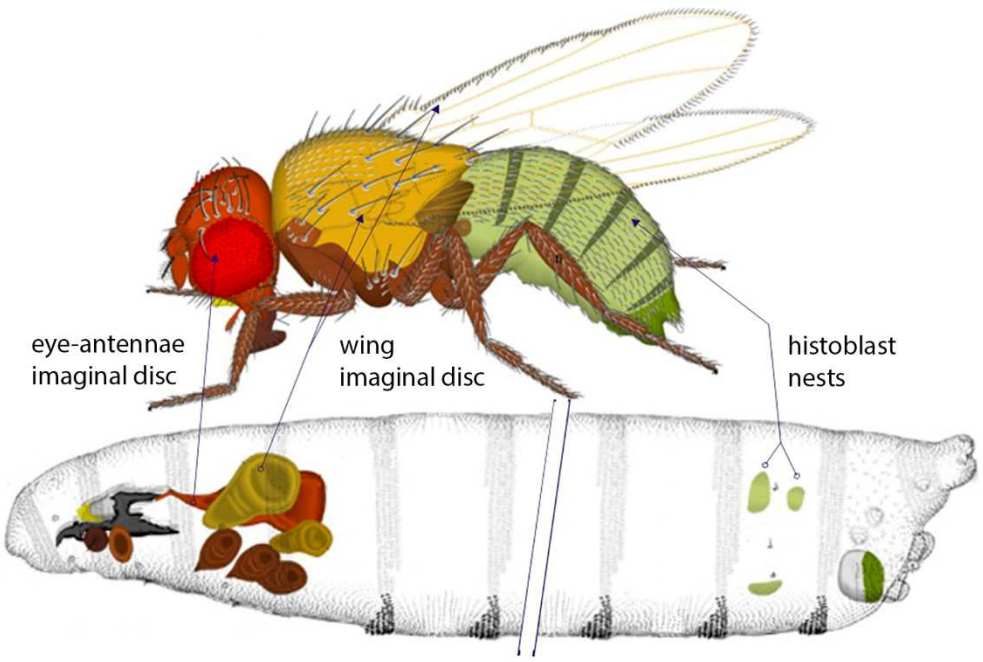


Figure 5. Imaginal discs allocation in the larva and adult derivatives. Schematic representation of a *Drosophila* adult (top) and mature larva (bottom). The head capsule, eyes and antennae derive from the eye-antennae imaginal discs (red) while the thorax and wings originate from the wing imaginal discs (yellow). The epidermis of the adult abdomen derives from imaginal tissues called histoblast nests that proliferate during metamorphosis (green).

In this thesis, we focused on the wing imaginal disc as a model system to analyse the developmental roles the JAK/STAT signalling pathway in limb development. The *Drosophila* wing imaginal disc has substantially contributed to the genetic dissection of the developmental pathways controlling growth, patterning and programmed cell death (Andersen et al., 2013; Beira and Paro, 2016; Blair, 1995; Hariharan, 2015; Neto-Silva et al., 2009), and has proven to be a fruitful model for investigations in limb development (Morata, 2001; Serrano and O'Farrell, 1997; Shubin et al., 1997). The developing limbs of *Drosophila* have been extensively used to study morphogens and members of their corresponding signalling pathways, and have been influential in our current understanding of the

interplay between morphogen function, growth and patterning (Affolter and Basler, 2007; Arias, 2003; Baena-Lopez et al., 2012; Dekanty and Milán, 2011; Lawrence, 2001; Restrepo et al., 2014). Work in this model system has also contributed to the discovery of the developmental role of apoptosis in correcting developmental defects, sculpting differentiated limbs as well as the impact of tissue growth in cell fate specification (Adachi-Yamada et al., 1999; Dekanty and Milán, 2011; Kenyon et al., 2003; Manjón et al., 2007; Milán, 2002; Rafel and Milán, 2008)

Drosophila limb development: origin and fate map

Embryonic origin of the limb primordia

The second thoracic segment of the adult fly contains a pair of wings and legs, each located dorsally and ventrally, respectively. Precursors of these appendages, the wing and the leg imaginal discs, originate in a lateral position of the embryo from a common embryonic limb primordia (Cohen et al., 1993; Wieschaus and Gehring, 1976). This cluster of founder cells of polyclonal origin segregates together by invagination from the parasegments 4 and 5 in the embryonic ectoderm, which correspond to the second thoracic segment (T2) (Crick and Lawrence, 1975; Kornberg, 1981; Martinez-Arias, 1986). In the embryo, a Dpp stripe runs laterally along the anterio-posterior axis and bisects the embryonic ectoderm into dorsal and ventral sides, whereas Wingless is expressed in the ventral-anterior compartment of the second thoracic segment (Cohen et al., 1993). The intersection between these two stripes is critical for the activation of *Distalless* (*Dll*), the developmental and molecular marker of the embryonic limb primordia that functions as a developmental switch to promote the development of limb structures (Cohen et al., 1993, 1989). In principle, all the segments have the potential to develop appendages, but genes of the Bithorax Complex (BX-C) repress *Dll* transcription in abdominal segments of insects. In the absence of BX-C function, *Dll* is activated in an equivalent position in all abdominal segments to form limb primordia (Gebelein et al., 2002; Vachon et al., 1992). This fits well with the idea that insects derive from multi-legged ancestors that subsequently lost legs in their abdominal segments (Galant and Carroll, 2002; Levine, 2002; Lewis et al., 2000; Ronshaugen et al., 2002). Interestingly, *Dll* is expressed in the primordia

and later in the distal regions of the developing appendages of all arthropods (Cohen, 1990; Panganiban et al., 1994, 1995; Popadic et al., 1996). More important, its ortholog *Dlx* is expressed in fish fins and tetrapod limb buds, as well as along the proximo-distal axis in the developing appendages of different taxa such as polychaete, onychophoran, ascidian and even the echinoderm tube feet (Panganiban et al., 1997). The structural and functional conservation of the *Dll* gene in such diverse appendages could be convergent, but this would have required the independent co-option of *Dl/Dlx* several times in evolution. Alternatively, *Dll/Dlx* might have originated once in a common ancestor and might represent a member of an ancestral proximo-distal patterning system recurrently used to generate body wall outgrowths (Morata, 2001; Pueyo and Couso, 2005).

The nascent wing and haltere imaginal discs originate from a subset of *Dll*-expressing cells that migrate dorsally from the embryonic limb primordium at the time they start to ubiquitously express *vestigial* (*vg*), which becomes the developmental marker of wing and haltere discs (Cohen et al., 1993; Goto and Hayashi, 1997). The wing and haltere are homologous dorsal appendages of the second (T2) and third (T3) thoracic segments, respectively, and their differences are the direct consequence of the position where they originate in the embryo (Figure 6). The halteres are small modified hindwings, a derived state of the posterior wings present in the four-winged ancestor of Diptera (Carroll et al., 1995; Wootton and Kukalová-Peck, 2000). In *Drosophila*, the Hox gene *Ultrabithorax* (*Ubx*) suppresses wing disc development in the dorsal T3 imaginal disc and confers haltere identity to this primordium, whereas the absence of Ubx in the dorsal T2 imaginal disc allows the development of the wing primordium. Total loss of *Ubx*

function in the developing halteres results in the complete transformation of halteres to wings, giving rise to a fully-formed four-winged adult fly (Carroll et al., 1995; Lewis, 1978), suggesting that this structure (the wings) represent the ground plan for the dorsal appendage (Mann and Carroll, 2002). Thus, the *Ubx* gene functions as a selector gene, a developmental switch on top of the genetic pathway that selects between two alterative developmental fates. The Ubx-dependent patterning event that selects between wing or haltere fate is one example in which patterning acts upstream of growth, since it defines two distinct developmental units that intrinsically carry information about their final size.

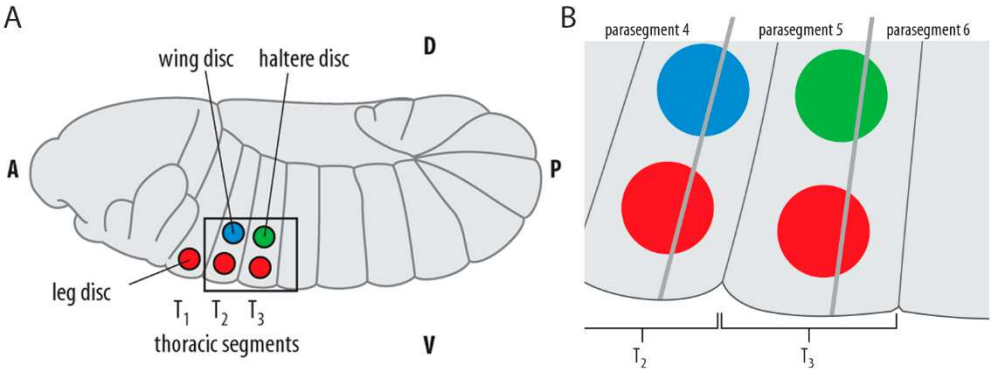


Figure 6. Embryonic origin of imaginal discs. (**A**) Cartoon depicting the embryonic origin of the wing (blue) and haltere (green) imaginal discs from the second and third thoracic segment (T2 and T3, respectively), located dorsal to the three ventral leg imaginal discs (red). (**B**) Both the wing (blue) and second leg (red) imaginal discs in T2 arise in parasegments 4 and 5, whereas the haltere and third leg disc develop from the parasegments 5 and 6.

Fate map of the wing imaginal disc

The wing primordium consists of a continuous epithelial monolayer that forms a two-sided epithelial sac, which surrounds the disc lumen. One side of the disc is a columnar pseudostratified epithelium, namely the disc proper (DP), and the other side is the peripodial membrane (PM), a squamous epithelium of wide and flat cells. The PM does not contribute to the formation of any adult structure but facilitates the process of wing disc eversion and thorax fusion during metamorphosis (Pastor-Pareja et al., 2004; Tripura et al., 2011). The DP will give rise after metamorphosis to the adult wing blade, the mesothoracic body wall or notum, the ventral meso- and pteropleura and the wing hinge, a structure that joints and articulates the wing blade to the body wall (Figure 7).

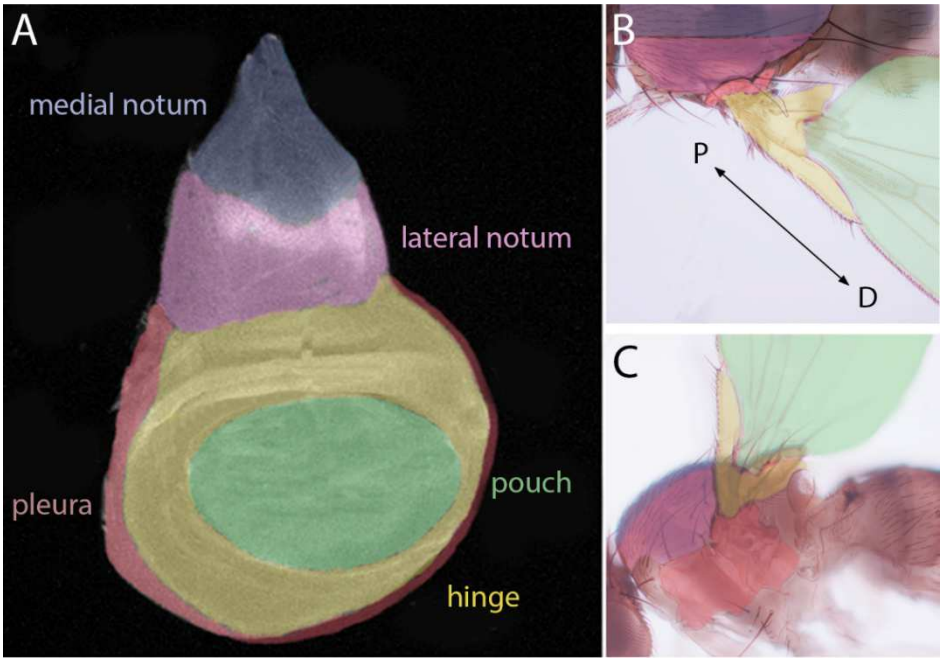


Figure 7. The wing imaginal disc territories and adult derivatives. (**A**) Mature third instar wing primordia with the major territories colour-coded. Dorsal (**B**) and lateral (**C**) views of a *Drosophila* adult with the same colour-code indicating the cuticle derivatives. P, proximal; D, distal. Adapted from (Hatini et al., 2013).

As an epithelial monolayer, the wing disc is considered a two-dimensional structure, but it gives rise to an adult appendage patterned along three axes; anterio-posterior (AP), dorso-ventral (DV) and proximo-distal (PD). The PD axis superimposes to the AP and DV axes during larval stages and consists of three main territories, from proximal to distal: notum, hinge, and the wing pouch – the precursor of the adult wing blade. The most central regions give rise to the distal structures and the surrounding the proximal ones (Figure 8).

During wing disc eversion in metamorphosis the dorsal (D) and ventral (V) surfaces fold and become apposed, revealing the third axis (Figure 9). Importantly, all the wing disc derivatives will appear as genetic domains that prefigure adult organization.

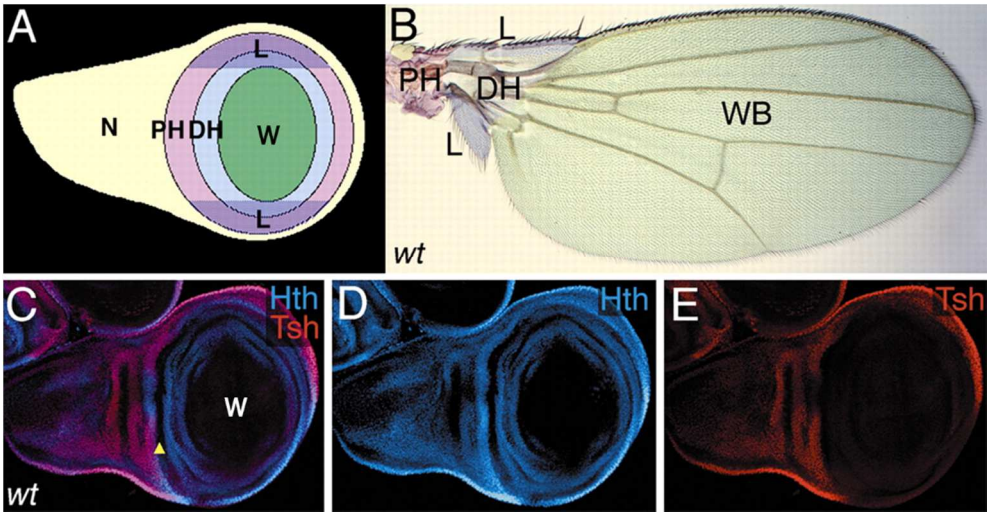


Figure 8. Subdivision of the wing disc along the proximo-distal (PD) axis. (**A**) Cartoon of a third instar wing disc showing the territories that define the PD axis; W, wing pouch; DH, distal hinge; PH, proximal hinge; L, lateral hinge; N, notum. (**B**) Wild type adult wing showing PD subdivisions. Labels are the same as in A except for the wing blade (WB). (**C-E**) Late third instar wing disc stained for Tsh (red) and Hth (blue). Hth is expressed throughout the hinge and the notum, while Tsh is restricted to the PH and the notum. Neither are expressed in the wing pouch (W) and are considered hinge and body wall markers. Yellow arrowhead marks a deep fold between the DH and PH. Adapted from (Zirin and Mann, 2004).

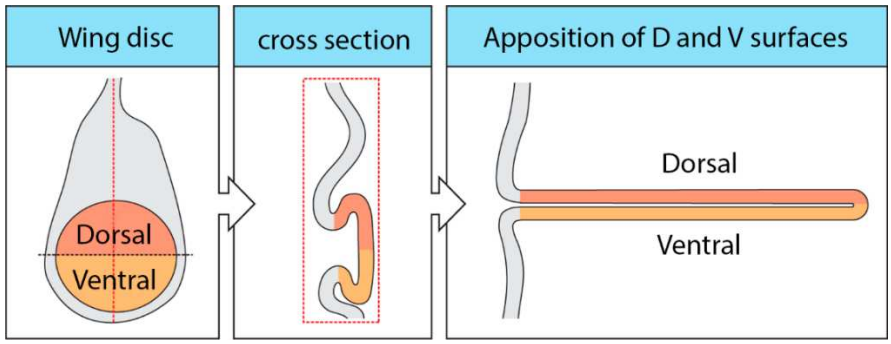


Figure 9. Wing imaginal disc eversion. In metamorphosis, the dorsal (D) and ventral (V) surfaces of the primordia fold and become apposed to give rise to the adult wing. Adapted from (Wolpert, L. Principles of Development).

Growth, cell proliferation and cell death

Mechanisms of growth control

The final size of the wing primordia depends on the growth rate – the net increase in tissue size over time – and the duration of growth during the feeding period. The net value of both parameters is computed from genetic and environmental components, such as nutrition, temperature, crowding, tissue damage, infections etc. Multiple growth regulators have been identified and can be broadly grouped in two categories: organ-intrinsic and organ-extrinsic regulators of size. Organ-extrinsic regulators act in a humoral fashion to scale the size of multiple organs within an organism. They provide systemic information about the organismal status, such as nutrition and developmental stage. They include the nutrient-sensing pathways such as the TOR and Insulin signalling. Organ-intrinsic mechanisms rely on genetically determined patterning cues that control growth in a local fashion and provide information about the local cellular environment, such as the position of cells within the field as well as cell-to-cell contacts/interactions. Their expression and activity follow a disc-intrinsic genetic program. This program continues to function even when immature wing imaginal discs are transplanted into growth-permissive hosts – such as adult female abdomens – where pattern and growth continues autonomously until they reach a determined species-specific size. Examples of this are disc-secreted factors and patterning genes such as Dpp, Wg, Hh, Upd, EGF as well as the Hippo pathway and Myc.

Cell proliferation and cell death

In *Drosophila*, different regulators drive cell cycle progression. One of the most important groups of cell cycle regulators is the CDK family of serine-threonine protein kinases, that are activated sequentially. Their activity is controlled by reversible post-translational modifications and by the association with proteins called Cyclins (Cyc), which act as the regulatory subunits of the kinase complex. The Cyclins family is divided into two main classes: the 'G1 cyclins' which comprise Cyclin D and Cyclin E, and their accumulation is rate-limiting for progression from the G1 to S phase; and the 'mitotic or G2 cyclins' which include Cyclin A and Cyclin B and are involved in the control of G2 to M transition and mitosis. Cyclins bind to and activate the CDKs, which lead to phosphorylation and inhibition of different cellular substrates.

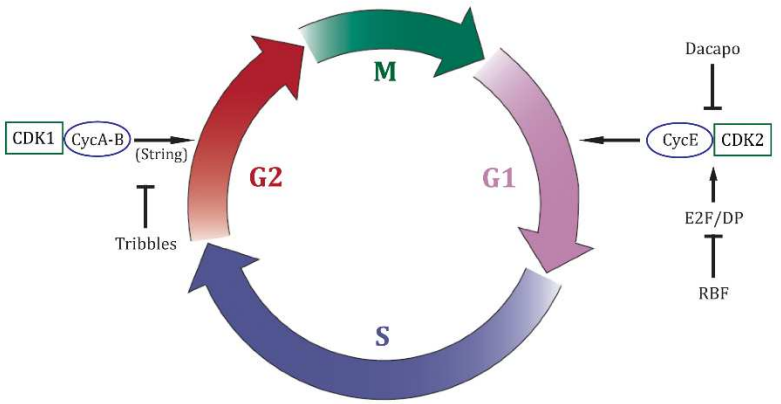


Figure 10. Cell cycle of *Drosophila melanogaster*. The cell cycle is subdivided into four phases: G1, S, G2 and M phases. Two key regulatory checkpoints are represented. In G1, Cyclin E limits S phase initiation by binding to CDK2. CycE is regulated by Dacapo/p21 and by E2F which is repressed by the retinoblastoma-like protein RBF. In G2, Cyclin A-B bind and activate CDK1 which induce String/Cdc25 thus allowing mitosis initiation. Tribbles functions as a negative regulator

Several transcription factors have a role on cell cycle regulation. The E2F transcription factor and its co-factor DP form a protein complex that induces Cyclin E (CycE) expression and thus transition from G1 to S phase [(Dynlacht et al., 1994), see Figure 10]. The protein phosphatase *String/Cdc25* is critical for the activity of the CycA-B/CDK1 complex, is rate limiting for entry into mitosis (O'Farrell et al., 1989), see Figure 10]. Different checkpoint mechanisms ensure that cells do not progress through the cell cycle when defects occur. The *Drosophila* gene *dacapo* (*dap*), which is a member of the p21/p27 family of CDK inhibitors, inhibits CycE/CDK2 activity therefore blocking G1 to S transition (Lane et al., 1996; De Nooij et al., 1996). The *Retinoblastoma-family protein* (RBF) is a family of tumor suppressor proteins known to bind to E2F, thus preventing CycE activation and blocking G1/S transition (Du et al., 1996). *tribbles* represses the cell cycle by inducing the degradation of String protein and consequently inhibits G2/M transition (Mata et al., 2000). Despite cell cycle manipulations, the resulting wings often attain the normal size. It seems that the wing discs adjusts cell numbers and cell divisions to achieve a final target size. In fact, the final adult wing can be composed either by fewer but larger cells (if cell cycle is blocked), or more but smaller cells (if cell cycle is induced) (Neufeld et al., 1998; Weigmann et al., 1997). Therefore, despite the close relationship between cell proliferation and cell growth, they are independently controlled.

The wing primordia are set aside during embryonic development as small clusters of cells that invaginate from the embryonic ectoderm and proliferate exponentially up until the end of larval stages. The wing disc starts to proliferate by the end of the first instar, about 40 hours after egg laying (AEL) (Mandaravally Madhavan and Schneiderman, 1977). The number of founder cells in the wing primordia of a newly hatched first instar larva was initially estimated as 11-38 cells (Bryant, 1970; Garcia-

Bellido and Merriam, 1971; Lawrence and Morata, 1977; Mandaravally Madhavan and Schneiderman, 1977) and the final cell number at pupariation as 50000 (Garcia-Bellido and Merriam, 1971). A more recent study reported 34 and 30000 cells for the initial and final cell numbers respectively, encompassing around 9-10 cell divisions during the larval stages (Martín et al., 2009). Moreover, during pupal stages cell proliferation resumes for two additional cell divisions (Milan et al., 1996). Cell proliferation during larval stages is uniform, as analysis of cell cycle patterns showed that cell divisions are randomly distributed throughout development, with small clusters of synchronously dividing cells (Milán et al., 1996).

During development, cells sense different extracellular signals that not only drive cell growth and cell division but also promote cell survival or cell death. Most animal cells have the ability to self-destruct by undergoing apoptosis, a form of programmed cell death. The proper regulation of apoptosis is critical for both development and tissue homeostasis, and inhibition of apoptosis contributes to the development and progression of cancer (Fernald and Kurokawa, 2013). In flies, in response to apoptotic stimuli, the pro-apoptotic genes *reaper* (*rpr*), *head involution defective* (*hid*), and *grim* are necessary and sufficient to induce apoptosis through inhibition of the caspase inhibitor *Drosophila* inhibitor of apoptosis protein 1 (dIAP1) (Goyal et al., 2000; Ryoo et al., 2002) . Subsequently, the initiator caspase Drosophila Nedd2-like caspase (Dronc, Caspase-9-like) is activated which in turn activates by proteolysis the two major effector caspases, *Drosophila* interleukin-1 converting enzyme (DrICE, Caspase-3-like) and death caspase-1 (Dcp-1, Caspase-7-like) (Figure 11) (Mills et al., 2005). Caspases are a highly specialized class of cell-death proteases. After effector caspases

activation, these caspases will cleave different cellular substrates that will promote cell death (Xu et al., 2009). The average amount of cell death during larval stages is very low without any noticeable pattern, except at the L2-L3 larval molt where there is a moderate increase in dying cells and at the hinge-notum border during late third instar (Milán et al., 1997)

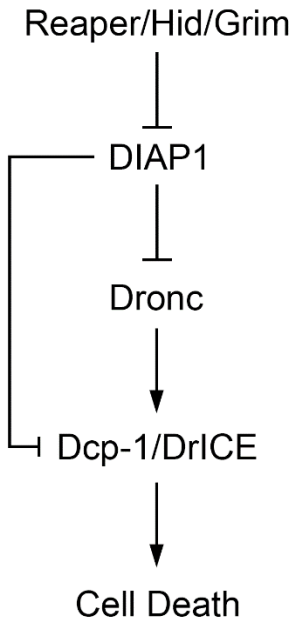


Figure 11. *Drosophila* apoptotic pathway. Initiator caspase Dronc and effector caspases DrICE and Dcp-1 are negatively regulated by DIAP1, which in turn is inhibited by the activity of the pro-apoptotic genes *hid*, *reaper* and *grim*.

Even though caspase activity and function is mainly linked with the maintenance and regulation of the apoptotic pathway, there are several evidences for non-apoptotic functions of the activated caspases (Miura, 2012). Not only in *Drosophila* but also in vertebrates, caspases have been reported to regulate cell proliferation in different ways and contexts . In the *Drosophila* wing imaginal disc, irradiation-induced cell death

triggers cell proliferation to compensate for the loss of cells and the adult wing ends up with its normal size and shape (Haynie and Bryant, 1977). In fact, caspases activate a compensatory proliferation program upon induction of cell death. In particular, Dronc, drICE and DCP-1 induce the production of morphogens in dying cells, which stimulate proliferation of adjacent cells (Fan and Bergmann, 2008). Moreover, feedback loops involving the caspase Dronc and the pro-apoptotic genes *hid* and *reaper* amplify and maintain a mechanism of compensatory proliferation (Shlevkov and Morata, 2012; Wells et al., 2006). Therefore, both cell death and cell proliferation are critical processes for the maintenance of tissue homeostasis and they are strictly linked.

Developmental subdivision of the wing: overview

Compartments and tissue territories

The wing primordium is subdivided into distinct functional units along the three major axes, AP, DV and PD. Short- and long-range patterning cues generate the three axes and provide cells within the epithelium with positional information and identity, with concomitant effects on growth, survival, adhesion and differentiation. The first subdivision occurs during segmentation in embryogenesis and is therefore inherited in the nascent wing primordia. Subsequent subdivisions of the wing disc arise *de novo* during larval stages. There are two mechanistically distinct modes of regional specification: developmental compartments and non-compartment based territories.

Developmental compartments are cell populations that do not mix during development and function as lineage restriction barriers (Garcia-Bellido et al., 1973). These subdivisions are based on a heritable pattern of selector genes, transcription factors that confer a specific identity and affinity properties on each compartment (Crickmore and Mann, 2008). The wing disc is subdivided along the AP axis into the anterior (A) and posterior (P) compartments, and along the DV axis into the (D) and ventral (V) compartments. The AP compartment subdivision is inherited from the embryonic ectoderm by the restricted expression and activity of the selector gene *engrailed* (*en*) in P cells. The DV compartment subdivision occurs later in development, during second instar, by the restricted expression and activity of the selector gene *apterous* (*ap*) in D cells. Moreover, short-range interactions between cells in adjacent compartments creates signalling centres at the interface, the AP and DV boundaries. These signalling centres behave as an internal coordinate

system that provides positional information to the field of cells at both sides of the boundary. Moreover, these boundary cells act as developmental organizers by the secretion of the long-range morphogens Decapentaplegic (Dpp) and Wingless (Wg) that control growth and pattering along the different axes of growth (Lawrence, 2001). Engrailed through Hedgehog (Hh) induces the expression of Decapentaplegic (Dpp) at the AP boundary and Apterous, through Notch (N), induces the expression of Wg at the DV boundary (Lawrence and Struhl, 1996). The intersection of the AP and DV represent the most distal point of the wing pouch and correspond to the distal tip of the adult wing blade. These two ligands are essential for proper wing development since the loss of any of them has dramatic effects in the final adult wing in terms of size and shape.

Non-compartment tissue territories are more common in development and defined by the restricted expression and activity of a gene or different combinations of gene products. These genetic domains appear as development proceeds and do not function as cell-lineage restriction boundaries. Examples of these are the wing pouch, the hinge and the notum. Even these three major territories are further subdivided into smaller genetic domains, each controlling the development of a part of the future adult structure. Thus, the wing pouch will eventually be subdivided into vein and intervein territories along the AP axis, and along the PD axis into a series of ring-like nested subdomains centred in the distal-most region of the wing pouch. The hinge also contains distal, proximal and lateral regions, each defined by a combination of gene activities. The notum is initially a single genetic unit but later becomes subdivided into medial and lateral domains as well as different portions therein (Mann and Morata, 2000)

In general, segregation of cells into distinct territories (compartments or not) confers particular properties to the cells belonging to each domain. Tissue territories have a defined identity and behave as coherent units within the tissue. In cellular terms, this is translated in affinity and adhesion properties that allow cells to recognize each other, keeping them together and avoid or limit intermixing with other cell populations. Cells between compartments cannot mix and a straight and sharp border maintains these populations apart (Dahmann et al., 2011). However, intermixing between non-compartment territories is allowed and cells can freely move from one domain to another by just switching their identity according to their new position. Even in these permissive cases, cells tend to avoid crossing between adjacent territories, as in the wing-hinge or the hinge-notum borders (Villa-Cuesta et al., 2007; Zirin and Mann, 2007).

Another important feature of developmental fields is that they selectively and differentially respond to patterning cues depending on the domain they belong. For instance, Dpp is expressed along the AP compartment boundary in a stripe that straddles the notum, the hinge and the wing pouch, however Dpp is only able to promote tissue growth in the pouch and lateral hinge cells. Similarly, Wingless overexpression has a potent mitogenic effect in the proximal wing and the hinge region (Giraldez and Cohen, 2003; Neumann and Cohen, 1996), but only low or moderate levels of Wg cause proliferative growth in the pouch (Baena-Lopez et al., 2009). An excess of Wg in the wing pouch elicits the opposite response, it triggers premature cell cycle arrest and differentiation to wing margin fate (Herranz et al., 2008; Johnston and Edgar, 1998; Johnston and Sanders, 2003). Thus, cells in the wing disc exhibit position-dependent responsiveness to patterning cues.

Compartments and tissue domains also behave as a coherent units of growth. In the case of compartments two properties have been observed (Martín and Morata, 2006; Mesquita et al., 2010; Morata and Herrera, 2010). In one view, compartments are considered autonomous units of growth control in which the growth of one compartment is independent to the adjacent one. Thus, each compartment would behave as a size-sensing unit. Experiments that generate wing discs in which compartments growth at different rates without affecting final compartment nor overall wing disc size exemplify this property. In the fast-growing compartment, the progression in the expression of key patterning genes usually shows a more advanced state compared to the slow-growing compartment, reflecting that growth and patterning of each territory is autonomous according to its intrinsic developmental program. Interestingly, once the fast-growing compartment reaches its final wild type size, growth is arrested and it gives enough time to the slow-growing compartment to attain its normal size and pattern. The final wing disc and adult wing are completely normal in terms of size and patterning elements. This observations led to the proposal that wing discs possess autonomous mechanisms to arrest growth in anterior and posterior compartments when they reach a predetermined size and therefore behave as independent developmental units (Martín and Morata, 2006; Morata and Herrera, 2010).

Other observations suggest that compartments communicate their developmental status to the neighbouring territory and the tissue responds as a whole to give rise a fully-functional adult structure. Depletion of the Insulin pathway or the protein biosynthetic machinery in one compartment autonomously reduces the growth rates and the final size of the targeted territory. Interestingly, this local perturbation is

accompanied by a non-autonomous response of the adjacent compartment, which also reduces its growth and proliferation rates in a coordinated manner. The net result of this buffering mechanism is an overall reduction in tissue size, but maintaining the relative proportions and the shape of the wing (Mesquita et al., 2010).

Developmental organizers: morphogens

The AP organizer: Hh and Dpp

During embryogenesis, the wing primordium is first subdivided into anterior and posterior compartments by the activity of the homeodomain transcription factors Engrailed/Invected in posterior cells (Kornberg et al., 1985). Engrailed generates an asymmetry by repressing the expression of the transcription factor Cubitus interruptus (Ci) in posterior cells, thus, only anterior (A) cells can respond to Hedgehog (Hh) by stabilizing Ci (Méthot and Basler, 1999). Hh coming from the posterior cells signals to cells in the anterior compartment to induce several target genes in a concentration-dependent manner (Vervoort, 2000) (Figure 12). Decapentaplegic (Dpp), the fly homologue of vertebrate bone morphogenetic proteins BMP2 and BMP4, belongs to the transforming growth factor-β (TGF-β) family and is induced by Hh in a stripe of anterior cells adjacent to the AP boundary of the wing disc (Zecca et al., 1995). Dpp spreads towards anterior and posterior cells to form a concentration gradient along the AP axis of the wing primordium with highest levels at the centre of the wing along the AP compartment boundary which decline as the distance from the source increases (Lecuit et al., 1996; Nellen et al., 1996a). Dpp acts as a long-range morphogen organizing pattern and growth symmetrically in both compartments (Figure 12).

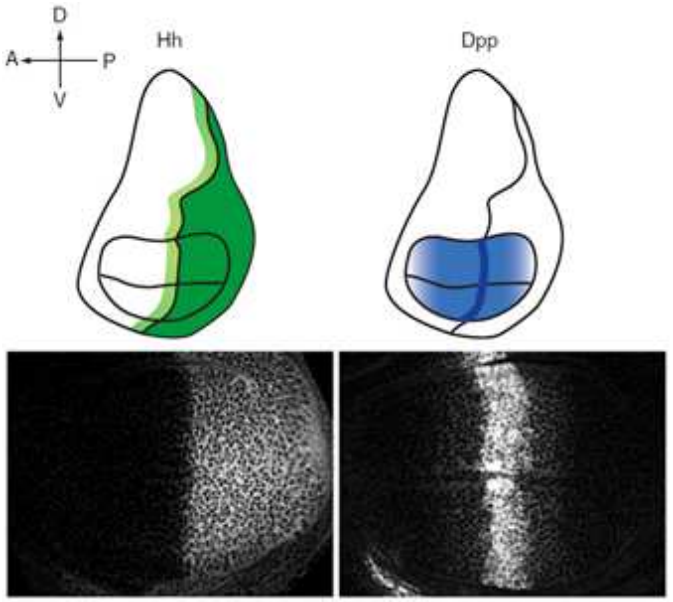


Figure 12. Distribution Hh and Dpp morphogens in the *Drosophila* wing imaginal disc. In third instar larvae wing discs, Hh is expressed in the P compartment (green) and moves into the A compartment to activate gene expression in a stripe of cells adjacent to the AP boundary (light green). Dpp is produced at the AP boundary (blue) and acts as a long-range morphogen that controls growth and patterning of wing cells along the AP axis (light blue). Adapted from (Yan and Lin, 2009).

The binding of the Dpp ligand to the type I-type II/Thick veins (Tkv)-Punt receptor complex phosphorylates and activates the intracellular signal transducer and transcription factor Mothers against Dpp (Mad) (Kim et al., 1997; Ruberte et al., 1995). Activated Mad forms a complex with Medea and enters the nucleus to inhibit the expression of the transcriptional repressor Brinker (Brk) (Figure 13). These events convert the Dpp morphogen gradient into an inverse gradient of Brk repressor activity that mediates many of the patterning and growth functions of Dpp

(Martín et al., 2004; Müller et al., 2003; Pyrowolakis et al., 2004; Schwank et al., 2008). When *brk* is ectopically expressed, cells that normally respond to Dpp become refractory to it. Therefore, in order to activate target genes, the Dpp signaling pathway must remove Brk. This downregulation of *brk* occurs at the transcriptional level. Both Brk and P-Mad regulate Dpp target genes, such as *daughters against dpp* (*dad*), *spalt* (*sal*) and *optomotor-blind* (*omb*) (Figure 13). Dad is an inhibitory SMAD that downregulates Dpp signalling in the wing primordium (Tsuneizumi et al., 1997). As it is induced by Dpp, its inhibitory function is highest in regions of high Dpp activity. This negative feedback loop might modulate the duration and intensity of the signal. The Spalt and Omb target genes are activated by P-Mad in a concentration-dependent manner, and the inverse gradient of Brk repressor is fundamental to control the expression state of these Dpp-induced genes along the AP axis (Moser and Campbell, 2005; Müller et al., 2003; Winter and Campbell, 2004). Expression boundaries of both *sal* and *omb* are set by Brk (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999); *omb* is less sensitive to Brk than *sal* and as such its domain of expression is broader. Although it is still unclear how *sal* and *omb* are repressed at different concentrations of Brk, one study suggests that different repression domains of Brk are sufficient to repress *omb* but not *sal* (Kirkpatrick et al., 2001).

Ectopic expression of Dpp in clones of cells both in the anterior or posterior compartments caused reorganizations of the wing pattern suggestive of a long-range activity of this morphogen (Capdevila and Guerrero, 1994; Zecca et al., 1995). In addition, these clones induce over-proliferation of surrounding cells and sometimes lead to organ duplications (Zecca et al., 1995). Hence, ectopic Dpp can induce

proliferation of an extra tissue and, at the same time, patterns it in the same way as the wild type tissue. How the growth and patterning of developing tissues are controlled and coordinated has been a long-standing question in developmental biology. The Dpp morphogen, that plays a role in both of these processes by providing positional information to the cells in the tissue and by acting as a trigger for tissue growth, is of crucial importance in the coordination of growth and patterning of the wing imaginal disc.

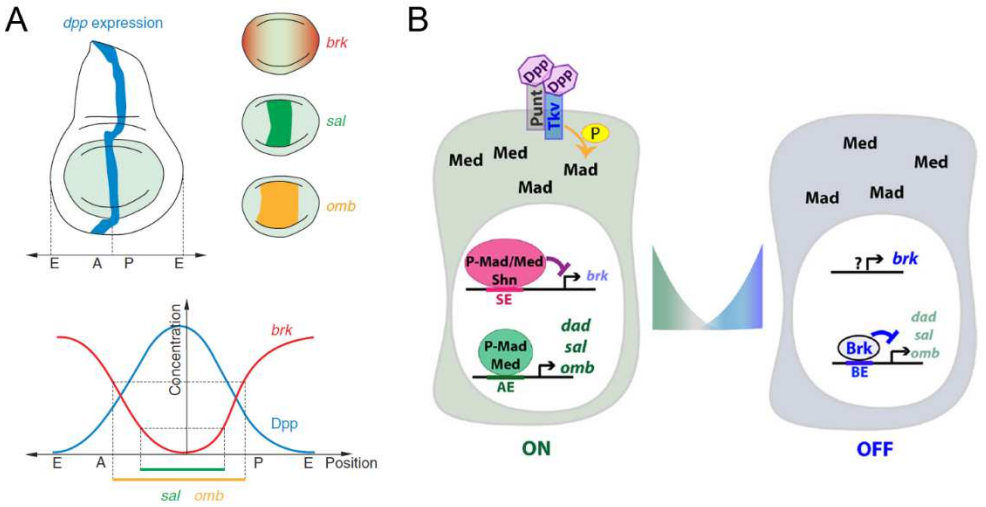


Figure 13. Dpp morphogen signalling cascade in the *Drosophila* wing imaginal disc. (**A**) In the upper panel representation of the expression patterns of *dpp* (in blue), *brk* (in red), *sal* (in green) and *omb* (in yellow). Dpp is secreted from its site of production at the center of the disc and spreads into the A and P compartments, establishing a gradient with highest levels in the center and lowest in the lateral regions. Brk forms an inverse gradient to the Dpp gradient. Brk levels are important, together with Dpp activity levels, to set the expression boundaries of sal and omb. As omb is repressed by high levels of Brk and sal by lower levels, omb is expressed in a broader domain than sal (lower panel). (**B**) Representation of the transcriptional activity of the Dpp signalling cascade in cells with two extreme levels of Dpp: a medial cell with high Dpp activity levels in green and a lateral cell with low Dpp activity levels in blue. The question mark highlights the unknown transcriptional activator of *brk* and pent. SE: Silencer Element, AE: Activating Element, BE: Brinker Element. Details are described in the main text. Adapted from (Hamaratoglu et al., 2014; Schwank and Basler, 2010).

In the mature wing of the fly, the positioning of the veins along the anteroposterior axis are manifestations of different Dpp patterning outputs. Together with Brk, Sal and Omb, encoding transcriptional regulators, Dpp is involved in the vein positioning of the adult wing. Sal and Brk are required for the specification of the longitudinal vein 2 in the anterior compartment, while Omb and Brk are important in the establishment of the vein 5 in the posterior compartment (De Celis, 2003)

The conclusion that Dpp is critical for growth of the wing imaginal disc comes from the observation that mutant flies lacking Dpp expression in the wing imaginal disc fail to form wings (Zecca et al., 1995) and clones of cells unable to transduce Dpp are eliminated from the wing blade (Burke and Basler, 1996). In an opposite way, overexpression of Dpp in its own domain causes overgrown wing imaginal discs (Burke and Basler, 1996). However, how the Dpp gradient drives uniform growth is still a matter of intense debate.

The DV organizer: Wg

Later in development, in second instar, EGFR signalling triggers the establishment of a dorsal-ventral axis by inducing the expression of the transcription factor and selector gene Apterous in dorsal cells (Wang et al., 2000; Zecca and Struhl, 2002a, 2002b). Apterous activates the expression of a Notch ligand, Serrate, in dorsal cells and restricts the expression of another ligand, Delta, in ventral cells (Diaz-Benjumea and Cohen, 1993; Doherty et al., 1996; Irvine and Vogt, 1997). This leads to the symmetric activation of Notch on both sides of the DV boundary. Notch activation leads to expression of Wg at the DV boundary that plays a fundamental role in patterning and growth along de DV axis (Diaz-Benjumea and Cohen, 1993, 1995; Zecca et al., 1996). Wg secreted from

the DV border of the wing disc acts as a long-range morphogen (Figure 14) by inducing the expression of its target genes, including *senseless* (*sens*), *distalless* (*dll*), and *vestigial* (*vg*) (Neumann and Cohen, 1997; Zecca et al., 1996). Clones of cells ectopically expressing Wg are capable or organizing growth and patterning of the surrounding tissue generating supernumerary limbs (Ng et al., 1996; Struhl and Basler, 1993; Zecca et al., 1995). Conversely, loss of Wg activity in the developing wing leads to growth defects and loss of wing tissue (Zecca et al., 1996). However, the role of Wg as a genuine morphogen it is matter of debate, as recent experimental evidence indicates that Wg secretion is dispensable for growth and patterning (Alexandre et al., 2014; Morata and Struhl, 2013). Moreover, graded distribution of Wg is not required to stimulate cell proliferation at least during the later stages of wing development (Baena-Lopez et al., 2009).

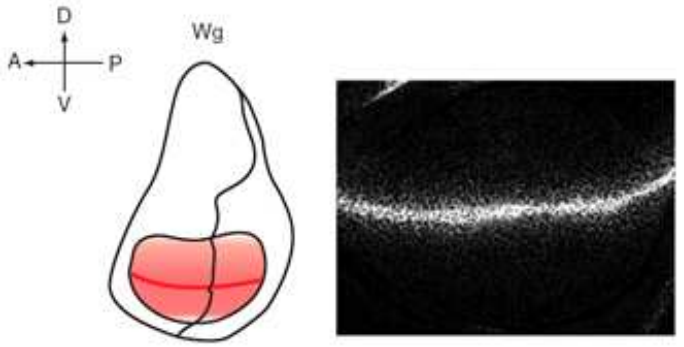


Figure 14. Distribution of the Wg morphogen in the *Drosophila* wing imaginal disc. In third instar larvae wing discs, Wg is expressed at the DV boundary and (red) acts as a long-range morphogen to organize patterning along the DV axis (light red). Adapted from (Yan and Lin, 2009).

Proximo-distal patterning of the wing disc

Notum and wing fate determinants are expressed in opposing domains

The nascent wing primordium comprises the progenitors of the adult body wall (notum) and the wing, and the segregation between these territories is the first event in the proximo-distal patterning of the wing disc. During second instar, the localized expression of Vein (Vn) and Wingless (Wg) signalling molecules in opposing domains subdivides the primordium into the presumptive body wall and the wing field, respectively (Figure 15A). These two ligands antagonize each other, Wg induces wing fate and prevents *vn* to be expressed in the distal domain, while Vn, an EGFR ligand, induces notum fate and suppresses wing development by blocking the responsiveness of cells to Wg (Figure 15B) (Wang et al., 2000).

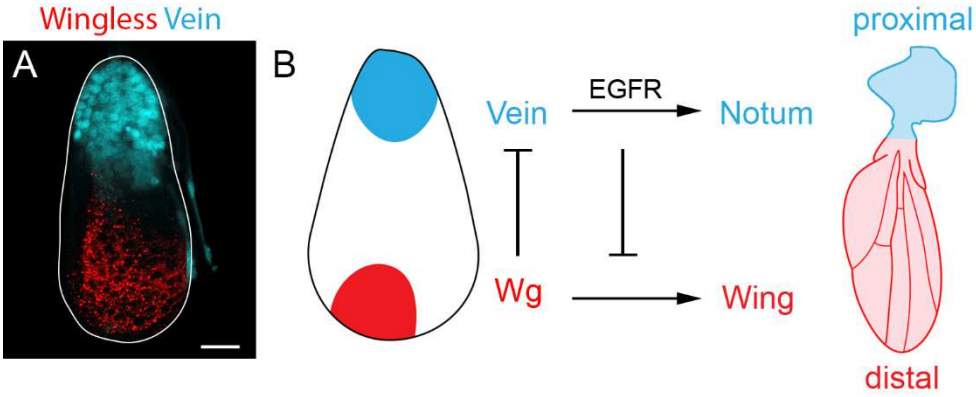


Figure 15. Segregation of wing and notum developmental fields. (**A**) Expression of *vn* transcript (cyan) and Wg protein (red) in mid-second instar wing primordia. (**B**) Simplified cartoon depicting the expression and the antagonistic functions between Vn/EGFR and Wg in second instar and the corresponding adult structures they induce. Scale bar, 20µm.

Wing fate specification by localized expression of Wingless protein

wingless, the founder member of the Wnt family of glycoproteins, is expressed at the opposite edge of *vn* in the early wing primordia, in distal cells, and is essential for wing formation (Figure 16) (Couso et al., 1993; Morata and Lawrence, 1977; Sharma and Chopra, 1976; Williams et al., 1993). *wingless* is initially and transiently induced by paracrine Hh signalling at the ventral-anterior edge of the wing primordium during early second instar and its expression is lost in *hh* mutants (Ng et al., 1996). Once the wing is specified, the *wg* locus becomes refractory to Hh signalling and symmetric Notch signalling between dorsal (D) and ventral (V) cells further restricts *wg* expression in two-three rows of cells along the dorso-ventral compartment boundary (DV), where it exerts its organizing activity during the third instar larval stage (Diaz-Benjumea and Cohen, 1993, 1995). Thus, the specification of the wing depends on the AP patterning system, but not the DV system. However, the specification of the wing pouch is required to define a field of cells in which the DV patterning system functions to control growth through the activation of Vg (Kim et al., 1996).

Some *wg* mutant alleles do not respond to Hh and behave as null or strong hypomorphic alleles only in second instar, the developmental stage when the wing field is specified by Wg protein. In these mutant animals, the wings fail to specify and consequently this structure is lost in adult flies (Figure 16). Very often, an ectopic notum develops from the ventral pleura leading to a mirror-image duplication of the endogenous body wall structures (Figure 16). In general, the *wingless* phenotype is usually mentioned as a “wing to notum transformation” because an additional notum replaces the wing (Morata and Lawrence, 1977; Sharma and Chopra, 1976). Wingless has two main functions in second instar. First, its range of activity defines the presumptive

wing field territory and promotes wing fate specification by positively regulating a set of wing-determining genes (Ng et al., 1996; Wu and Cohen, 2002). Second, it antagonizes notum development by inhibiting *vn* expression in the distal territory (Wang et al., 2000). Consequently, the terminal phenotype observed when the early function of Wg is suppressed is the manifestation of two phenotypes, the failure to specify the wing and the ectopic expression of *vn* in the distal territory, which is assumed to be responsible for the notum duplication (Figure 16) (Wang et al., 2000).

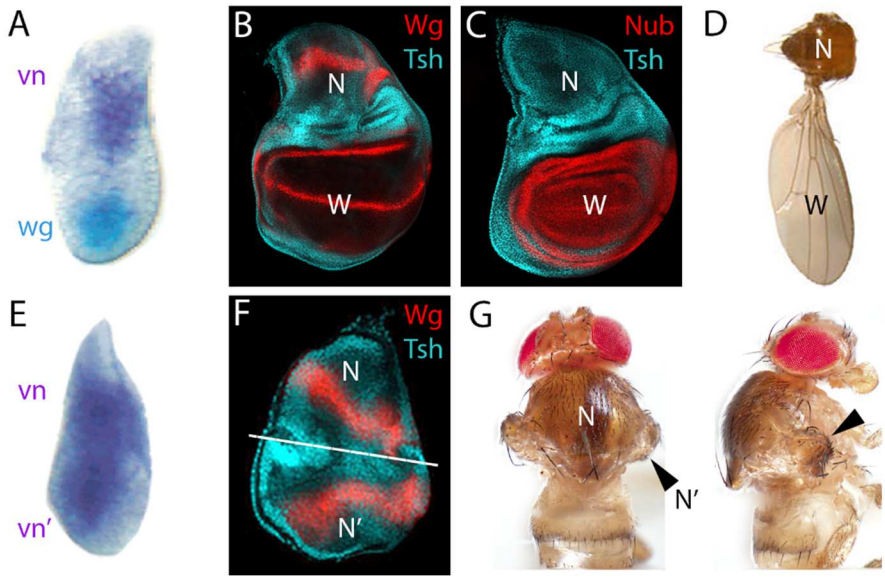


Figure 16. Wg is required for wing fate specification. Wild type (**A-D**) and *wg* LOF phenotypes (**E-G**). (**A**) Expression domains of *vn* (purple) and *wg* (blue) in second instar wing discs. (**B, C**) Late third instar wing primordia stained for Teashirt (Tsh, cyan), Wingless and Nubbin (Wg, Nub, red). Wg is expressed in a characteristic pattern in the notum (N) and the wing pouch (W) and Nubbin is expressed in the pouch. (**D**) Wild type adult wing (W) and notum (N). (**E**) In a *wg*-LOF, *vn* is ectopically expressed (vn') in the distal territory during second instar. (**F**) Consequently, in late third instar, the wing pouch is absent and the entire disc expresses Tsh with symmetric expression of the Wg notal stripe, indicating that the notum is duplicated (N and N', white dashed line marks the approximate separation between endogenous and ectopic notum). (**G**) The resulting adult flies lack the wings and show ectopic nota (N', arrowheads). Adapted from (Rafel and Milán, 2008; Wang et al., 2000).

Besides the localized expression of *wg* and *vn* fate determinants in the early wing primordia, the nascent wing imaginal discs inherit from the embryo ubiquitous expression of *teashirt* (*tsh*), *homothorax* (*hth*) and *vestigial* (*vg*) genes, and no overt territorial subdivision exists at this developmental stage along the PD axis (Wu and Cohen, 2002). Both Tsh and Hth transcription factors contribute to proximal wing disc development, notum and hinge. They are incompatible with the acquisition of distal wing positional values and must therefore be repressed in the presumptive wing field (Aldaz, 2005; Azpiazu and Morata, 2000; Casares and Mann, 2000; Zirin and Mann, 2004, 2007). Indeed, ectopic expression of *tsh* or *hth* in the developing wing pouch causes undergrowth and impairs its development either by "proximalizing" the wing or inducing ectopic hinge structures. Interestingly, *Meis1*/*2,* the vertebrate homologues of *hth*, are expressed in analogous positions of the developing vertebrate limb, from the trunk to the proximal limb bud, and implement a similar function in promoting proximal limb development (Mercader et al., 1999). This and other striking parallelisms in the molecules and mechanisms that distinguish between the trunk and appendage, support the view that both arthropod and vertebrate limbs originated from a common ancient genetic module recurrently used to form outgrowths of the body wall (Morata, 2001; Panganiban et al., 1997; Shubin et al., 1997). In contrast, *vg* is required for distal wing growth and confers the cells the identity to differentiate into the particular wing blade histotype. In *vg* mutant animals the wing blade is lost or residual (Williams et al., 1993, 1991), and *vg* mutant clones in the wing pouch have poor viability and upregulate hinge marker genes such as *hth* (Azpiazu and Morata, 2000; Kim et al., 1996; Zirin and Mann, 2004). Consequently, the retraction of *tsh* and *hth* to the hinge/notum and *vg* to the wing pouch is a requirement for the proximo-distal outgrowth of the wing.

In mid-second instar, repression of *tsh* by Wg at the time that *vg* retracts to the presumptive wing field marks the initiation of wing development, and these events are the earliest signs of wing specification (Figure 17). Dpp gain-of-function experiments led to the proposal that Dpp collaborates with Wg in repressing Tsh from the presumptive wing field in second instar (Wu and Cohen, 2002). It was suggested then, that the combined actions of Wg and Dpp to specify the wing territory would be analogous to the way they cooperate in the leg disc to establish the proximo-distal axis (Lecuit and Cohen, 1997). However several loss-of-function experiments concluded that this is not the case and only Wg mediates the repression of Tsh during second instar (Cavodeassi et al., 2002; Zirin and Mann, 2004). Soon after the repression of *tsh*, Wg induces *nubbin* (*nub*), the earliest positive marker for wing identity. Nubbin is a POU-homeodomain transcription factor required for proliferation and patterning of the wing pouch and the distal hinge, and its mutations cause reduced and shortened wings along the proximo-distal axis (Cifuentes and García-Bellido, 1997; Ng et al., 1995). Nub can be repressed by Tsh if ectopically expressed in the pouch (Wu and Cohen, 2002; Zirin and Mann, 2007). It is not clear the exact moment at which the first Nub positive cells can be detected by immunolabeling, the lack of consensus standards to stage the developing larvae at precise time points usually results in discrepancies. In general, most would agree that Nub starts to be expressed at some point from mid-second instar onwards (60 h AEL) and all its descendants comprise the wing pouch and the distal wing hinge. While the repression of *tsh* is a requirement to define the wing field and delimit the group of cells that will express *nub*, the repression of *hth* seems to occur well after *tsh* retraction (Figure 17). Since Hth and Nub proteins overlap during late second and early third instar, it is likely that Hth retraction from the wing pouch, although necessary for distal wing outgrowth, is rather a consequence of the specification of the wing than a causative event. Thus,

the specification of the wing pouch versus the surrounding body wall involves a primary event, the delimitation of the wing field by the absence of Tsh, and the induction of a positive marker for wing fate, Nubbin, which is therefore considered a secondary event. Once the wing field has been specified, *tsh* is heritable silenced from the wing pouch by Polycomb-group proteins (PcG) and no longer requires the Wingless input (Zirin and Mann, 2004). Similarly, *nub* expression becomes Wg-independent and it is proposed that epigenetic mechanisms maintain its active state through cell divisions (Johnston and Sanders, 2003; Widmann and Dahmann, 2009; Zirin and Mann, 2007). In contrast, Hth is repressed continuously and actively from the wing by a repertory of wing pouch genes such as *dpp*, *wg*, *vg* and *nub* (Azpiazu and Morata, 2000; Zirin and Mann, 2004, 2007). Whether they act in a linear cascade or in parallel has not been addressed in detail.

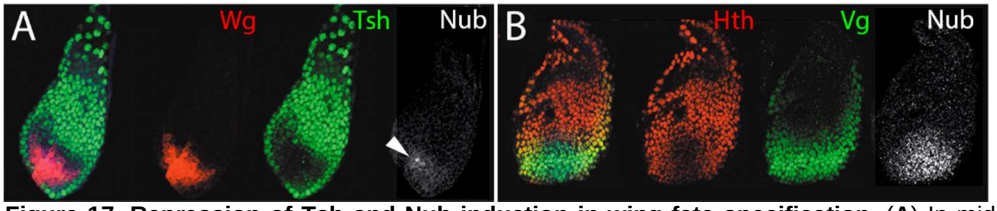


Figure 17. Repression of Tsh and Nub induction in wing fate specification. (**A**) In mid second instar (mL2) Tsh (green) is already repressed from the distal wing primordia by Wg (red) and defines the future wing pouch territory. Nub (white) is faintly expressed at this stage in few cells. (**B**) In late second instar (lL2) the Nub domain (white) fills the entire wing pouch at the time that Hth (red) starts to be repressed from this region and Vg (green) retracts into a larger area foreshadowing its expression along the DV boundary in the body wall as well as in the wing. Adapted from (Wu and Cohen, 2002).

Wingless is not only required for wing fate specification, but also capable to trigger wing development “de novo” in other locations of the wing disc (Figure 18). However, not all the regions and moments are equally suitable or competent to develop a wing when an ectopic source of Wingless is

provided, indicating that the wing-inducer function of Wg is conditioned by spatial and temporal constraints. It is widely accepted that the developmental stage at which Wg can induce wing fate is restricted to second instar, the developmental stage when the endogenous appendage is specified (Couso et al., 1993; Ng et al., 1996; Williams et al., 1993). In general, activation of the Wg signalling pathway by ectopic expression of Wg or other *Drosophila* Wnt ligands can bypass EGFR repression in the notum and cause the same effect (Collins and Treisman, 2000; Gieseler et al., 2001; Klein and Arias, 1998; Ng et al., 1996; Silver et al., 2007a). In these cases, the terminal phenotype is stated as supernumerary wings, ectopic wings or commonly as a "notum to wing transformation" because the additional wing structures arise, in part, at the expense of reprogramed notum cells. The supernumerary wing phenotype seems the reverse to the "wing to notum transformation" in the *wingless* mutants, because the additional wing structures replace normal notum tissue where they originate with an excess of wing tissue, indicating excess of proliferation as well as cell fate reprograming.

There is one particular "sweet spot" located at the scutellar region – the posterior edge of the notum – that by yet unknown reasons is promiscuous to be the point of origin where Wg signalling can elicit the development of ectopic wings (Figure 18D) (Klein and Arias, 1998). A further confirmation of this spatial restriction comes from the analysis of *osa* mutants. Osa is a member of the Trithorax-Group proteins (TrxG) and integral component of the Brahma complex, a chromatin-remodelling complex homologous to the SWI/SNIF in yeast (Collins et al., 1999; Papoulas et al., 1998; Treisman et al., 1997). Osa-containing Brahma complexes are implicated in both positive and negative transcriptional regulation, and in collaboration with the corepressor Groucho (Gro) repress Wg target genes in the absence of Wg

signalling. (Collins and Treisman, 2000; Treisman et al., 1997). In *osa* mutant animals, the wing imaginal discs often develop ectopic wings emerging from the notum, originating from the same spot in the scutellum that is especially sensitive to ectopic Wg (Figure 18H). Thus, despite that the entire wing primordium is mutant for *osa*, only a subset of cells seem to activate the Wg responsive genes that trigger wing development (Collins and Treisman, 2000).

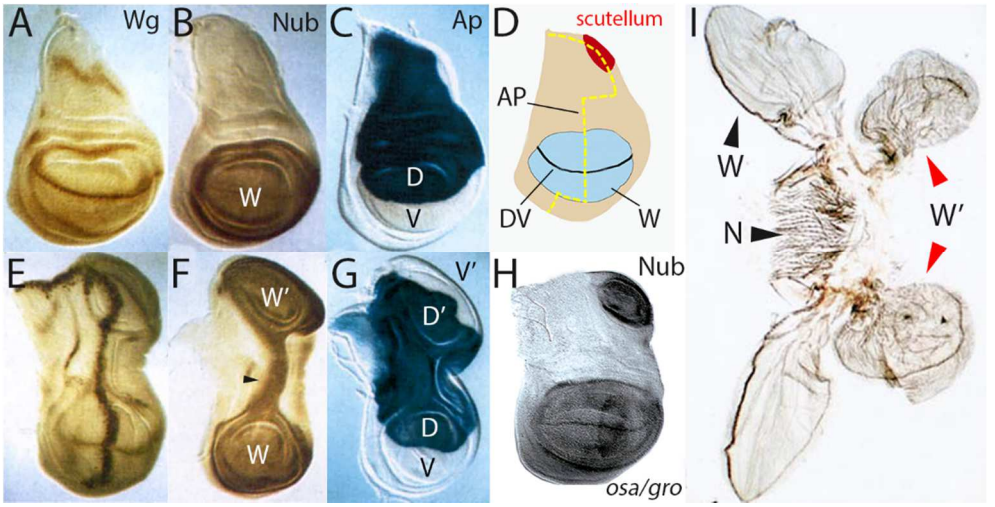


Figure 18. Ectopic activation of the Wg pathway induces supernumerary wings. Wild type (**A-C**) and experimental (**E-H**) third instar wing discs stained for Wg (brown, **A**, **E**), Nub (brown, **B, F** and black, **H**) and Apterous (blue, **C, G**). (**D**) Cartoon of a third instar wing imaginal disc. The wing pouch (W, blue), DV boundary (black line), AP boundary (yellow dashed line) and the presumptive scutellum (red) are indicated. The scutellum itself or a region close to it seems to be the point of origin of the ectopic wing. (**E-F**) Ectopic Wg expression in a stripe along the AP compartment boundary (**E**) induces a notum to wing transformation, marked by Nub expression (W, W', **F**). Nub is also induced along the AP boundary in a wider region due to Wg spreading (arrowhead, **F**). (**G**) Expression of Apterous bisects endogenous and supernumerary wing into dorsal (D, D') and ventral compartments (V, V') of inverted polarity. (**H**) In *osa/gro* transheterozigous wing primordia, the ectopic wing pouch originates in the same area as in **E-G**, presumably from the scutellum or a region close to it. (**I**) Ectopic expression of DWnt4 phenocopies the Wg gain-of-function phenotypes described in **E-H**. Endogenous wing and notum (black arrowheads, W, N, respectively) and ectopic wing blades (red arrowheads, W'). Adapted from (Collins and Treisman, 2000; Gieseler et al., 2001; Jönsson and Knust, 1996; Ng et al., 1996).

Vn/EGFR promotes notum specification and growth

Vn is a neuregulin-like ligand of the EGF receptor (EGFR) expressed in the proximal territory and is required directly for notum development and indirectly for wing outgrowth (Simcox et al., 1996; Wang et al., 2000; Zecca and Struhl, 2002a). The expression of *vn* is regulated by paracrine and autocrine mechanisms. Induction of *vn* in the first instar transiently relies on Dpp signalling coming from the peripodial membrane and transmitted across the disc lumen (Figure 19A). During second instar *vn* expression is maintained in proximal cells by autocrine Vn/EGFR signalling via the ETS transcription factor Pointed-P2 (PntP2), creating a positive feedback loop that sustains *vn* expression throughout development (Figure 19B) (Golembo et al., 1999; Paul et al., 2013; Wang et al., 2000). Mutations affecting *vn* or *egfr* lead to a "notumless" phenotype – the complement to the *wingless* phenotype – where body wall structures are either absent or reduced (Figure 19C, D). Clones of cells mutant for *egfr* or *ras* have, in general, impaired growth and viability, but are even more hardly recovered in the notum territory, where EGFR function is critical for its development (Diaz-Benjumea and Garcia-Bellido, 1990; Diaz-Benjumea and Hafen, 1994; Prober and Edgar, 2000; Zecca and Struhl, 2002b). Moreover, overexpression of Vn or Spitz (another EGFR ligand) in a *vn* mutant background can rescue the notumless phenotype, demonstrating that regardless of the nature of the ligand, a minimal EGFR signal is necessary (Austin et al., 2014; Zecca and Struhl, 2002a). Besides promoting notum development, Vn/EGFR signalling antagonizes wing development, as EGFR overexpression represses the wing specific gene *vestigial* (*vg*) and the resulting adult wings are reduced to a stump (Figure 19D). Moreover, local reductions of EGFR signalling in the presumptive body wall territory lead to the generation of ectopic wing structures emerging from the notum (Figure 19E) (Wang et al., 2000).

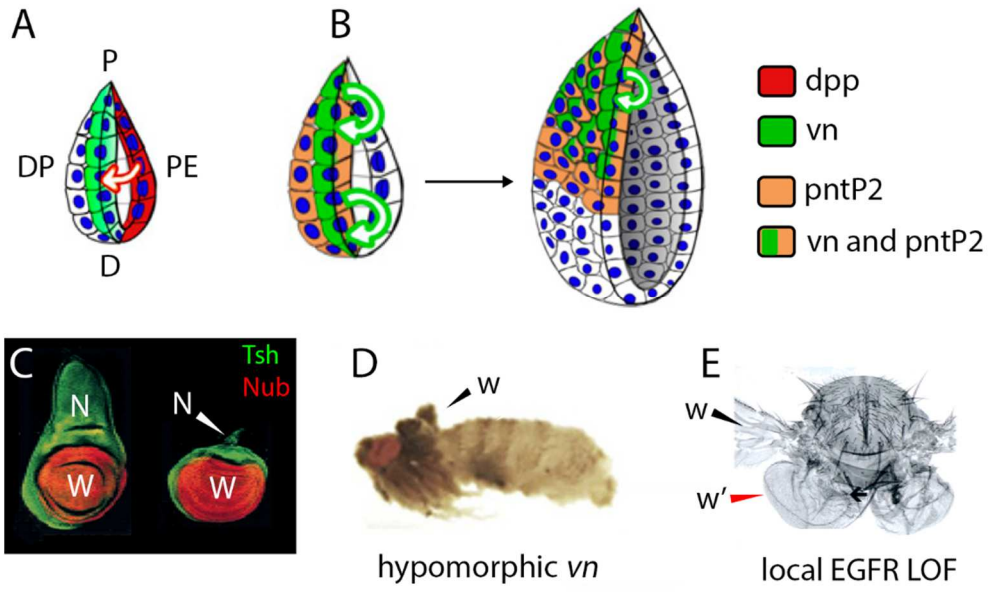


Figure 19. Regulation and function of Vn/EGFR in early wing development. (**A**) Paracrine Dpp signalling from the peripodial membrane (red) induces *vn* in the first instar (green). (**B**) After Dpp induction, *pntP2* (orange) mediates autocrine Vn/EGFR signalling to establish a positive feedback loop (green arrows). In second instar (right panel), *pntP2* retracts distally and limits productive autocrine Vn/EGFR signalling to the proximal region. (**C**) Wild type (left) and *vn* hypomorphic (right) third instar wing primordia labelled in the notum by Tsh (green, N) and in the wing pouch by Nub (red, W). The notum is almost completely lost in the mutant (arrowhead, N) and the wing pouch remains surrounded by a ring of proximal hinge tissue. (**D**) The notum structures are lost in the adult mutant and only the wings remain. (**E**) Local reduction of EGFR signalling in the notum generates ectopic wing structures emerging from the notum (red arrowhead, W'). P, proximal; D, distal; DP, disc proper; PE, peripodial epithelium. Adapted from (Austin et al., 2014; Paul et al., 2013; Wang et al., 2000).

Besides its requirement for notum growth, Vn/EGFR signalling instructs proximal cells to acquire the notum fate by inducing the expression of the three genes of the Iroquois complex (Iro-C), namely *araucan*, *caupolican* and *mirror* (*ara*, *caup* and *mirr*, respectively), three clustered homeodomain transcription factors required to specify the notum structures (del Corral et al., 1999; Wang et al., 2000). A similar genomic organization exists in vertebrates for their orthologues (*Irx1-Irx6*), grouped in two clusters of three

genes that probably originated from a duplication of an ancestral three-member cluster (Bosse et al., 2000). The *Iro-C* genes are expressed in a similar, almost overlapping pattern throughout development and confer notum identity to proximal cells that differentiate into the particular cuticle histotypes of this territory (del Corral et al., 1999). Additionally, they provide positional cues for the expression of the *achaete-scute* (*ac-sc*) proneural genes that prefigure the positions of each macrochaete, the large bristles of the thorax (Cavodeassi et al., 2001; Gómez-Skarmeta et al., 2003, 1996). There is some degree of redundancy between the *Iro-C* genes as overexpression of one single *Iro-C* gene can rescue defects in imaginal discs associated with the removal of all of them (del Corral et al., 1999). EGFR signalling is persistently required to sustain Iro-C expression*, egfr/ras* mutant clones autonomously loss Iro-C expression and ectopic EGFR/Ras is sufficient to induce these genes throughout development (Zecca and Struhl, 2002b). Patches of tissue mutant for Iro-C are associated with extensive malformations in the notum and the presence of naked or corrugated cuticle with sclerotized structures characteristic of the hinge. These transformations indicate that in the absence of Iro-C function the fate of these cells changes to wing hinge or impedes their terminal differentiation. Indeed, direct visualization of molecular markers for wing, hinge and notum identity supports this notum to hinge transformation (del Corral et al., 1999). Consistently, ectopic expression of EGFR or its downstream targets *ara* or *caup* in distal cells is sufficient to impose notum fate in this territory (Aldaz et al., 2003; Barrios et al., 2015; Wang et al., 2000). However, forced EGFR signalling only partially transforms distal tissue into notum, and the wings, albeit reduced and distorted, are still present. This suggests that Iro-C-dependent notum fate specification may rely on other additional factors that collaborate with EGFR input to sustain appropriate Iro-C levels. Alternatively, asymmetric distribution of EGFR repressors along the PD axis might dampen

the capacity of EGFR to specify notum fate in the distal territory. It is also possible that artificially provided levels of EGFR might not be strong enough to achieve the minimal Iro-C threshold that imposes notum identity. Both *egfr/ras* and *Iro-C* mutant clones are poorly recovered in the notum and this may reflect either reduced viability and/or different cell assortment that would exclude viable mutant cells from the notum. While the function of EGFR/Ras in promoting survival and growth is well described, Iro-C seems to constrain growth by negatively regulating cell cycle progression in both the eye and wing imaginal discs (Barrios et al., 2015; Bergmann et al., 1998; Kurada and White, 1998; Prober and Edgar, 2000). Both *egfr/ras* and *Iro-C* mutant clones – the latest still transduce EGFR signal – have smooth and rounded borders in the notum, suggesting that they tend to minimize contacts with the surrounding wild type cells due to a differential cell-cell affinity. In fact, randomly generated *Iro-C* mutant cells tend to fuse and join together in a single, large clone when recovered in the notum. Similarly, clones ectopically expressing *Iro-C* in the wing pouch try to contact each other and arrange in filaments separating large, roundish islands of nonexpressing cells (Villa-Cuesta et al., 2007). The fact that apposition of Iro-C expressing and non-expressing cells leads to cell sorting and fold formation demonstrates that cell affinity relies on patterning rather than direct signalling of the EGFR pathway. It is likely that growth and patterning downstream of Vn/EGFR are independent functions of the pathway and that EGFR-mediated notum growth is not a subproduct of Iro-C patterning. While EGFR signalling would directly provide proliferation and survival cues, Iro-C would confer cell identities and affinities particular of the notum.

Vn/EGFR regulates the dorsal selector gene *apterous*

The Vn/EGFR pathway has a dual role early in wing development, it promotes notum fate and suppresses wing development when ectopically expressed

in the distal portion of the wing disc but, paradoxically, also promotes wing formation. The notumless phenotype is characteristic of *vn* hypomorphs but in null *vn* alleles and some *egfr* alleles both the wing and notum fail to develop (Figure 20B) (Clifford and Schupbach, 1989; Simcox et al., 1996). Vn/EGFR is on top of the genetic pathway that subdivides the early wing primordia into the D and V compartments, and therefore establishes the Notch-Wg-Vg DV patterning system responsible for the proximo-distal outgrowth of the wing. EGFR initiates DV boundary formation by inducing the expression of the D selector *apterous* (*ap*) in second instar, simultaneously with the specification of the wing by Wg (Figure 20A) (Wang et al., 2000; Zecca and Struhl, 2002a). This positive regulation is transient and only occurs in second instar, once activated, *ap* expression is refined by an autoregulatory loop and maintained by Trithorax-Group proteins (TrxG) (Bieli et al., 2015). It is proposed that a gradient of Vn ligand generates different concentration thresholds; high levels would induce *Iro-C* and *ap*, while lower levels would only activate *ap*, thus forming two nested expression domains (Figure 20C) (Wang et al., 2000). Two observations support this proposal. First, the early Ap domain extends more distally than the Iro-C domain, cells far from the source sense lower Vn levels and activate the low-threshold target *ap* but not *Iro-C*, which would be a high-threshold target. Second, the serial phenotypes observed in *vn* or *egfr* mutants are the expected for a graded mode of action. In hypomorphic *vn* mutants, the first structure lost is the Iro-C/notum domain, which requires high levels of signalling, but the wings are present because *ap* and therefore the DV boundary are properly established. In a stronger mutant situation, both the notum and the wing are absent, the latest due to the loss of *apterous* – a putative low-threshold target – and subsequent failure to initiate DV boundary organizing activities which are responsible for distal wing outgrowth.

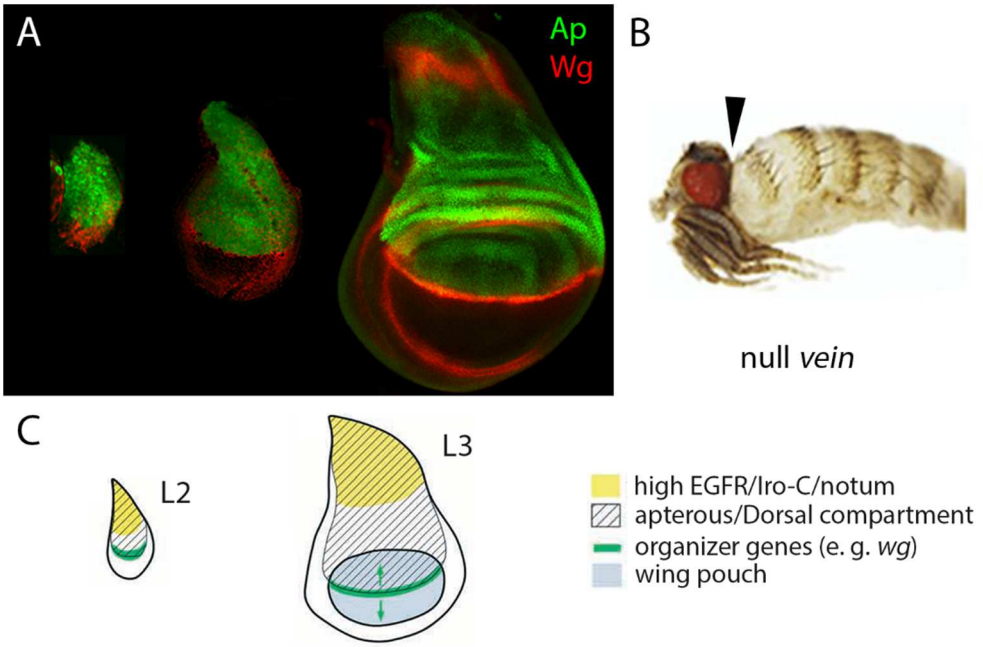


Figure 20. Early EGFR signalling regulates *apterous* expression. (**A**) Wild type third instar wing primordia from second, early third and late third instar labelled for Apterous (Ap, green) in the dorsal compartment and Wg (red). (**B**) In null *vein* mutants, the body wall and the wings are missing (arrowhead) due to the loss of *ap* and therefore the DV patterning system. (**C**) Model of ap regulation by EGFR. In second instar (left, L2), EGFR induces Iro-C and *apterous* at high and low concentration thresholds, respectively. As development proceeds, the expression of *ap* does no longer depend on EGFR input. In third instar (right, L3) the Iro-C and *ap* form two nested domains. Adapted from (Austin et al., 2014; Bieli et al., 2015; Zecca and Struhl, 2002a).

Notch signalling coordinates tissue growth and wing fate specification

Tissue growth and patterning have to be tightly coupled to generate a correctly sized and shaped structure (Dekanty and Milán, 2011; Lecuit and Le Goff, 2007). Notch activity was proposed to have a role in wing fate specification because some Notch mutant alleles result in phenotypes reminiscent of the loss of Wg signalling during second instar, the failure to induce wing fate and concomitant duplication of body wall structures (Couso

and Arias, 1994). This question was revisited later and confirmed previous observations by tissue specific targeting of Notch function during second instar (Figure 21A-D) (Rafel and Milán, 2008). Although Notch is required for proper wing fate specification, it neither does so cell autonomously nor behaves as a Wg-like molecule since Nub – a Wg target gene in second instar – is unaffected when Notch activity is blocked in a subset of wing disc cells (Figure 21E). Moreover, compromising Notch function does not influence the initial localization of Vn and Wg in opposite domains. Thus, Notch does not regulate Wg expression in the distal-anterior edge in second instar, nor collaborates with Wg in restricting Vn to the most proximal region of the primordium. However, Notch is likely genetically upstream of Wg, as increasing Wg levels rescues the notum duplications produced by loss of Notch. Consistently, Notch overexpression does not rescue the Wg mutant phenotype, indicating that Wg is a downstream component in the developmental pathway that determines the wing field. Notch is thought to promote growth in the early wing imaginal disc (de Celis and García-Bellido, 1994) and growth induced by Notch is required for specification of the eye within the *Drosophila* eye-antenna primordium (Kenyon et al., 2003). Interestingly, second instar wing discs are significantly smaller when Notch signalling is compromised. In this context, overexpression of a collection of cell cycle regulators and growth promoters rescues the undergrowth as well as Nub expression and wing fate specification (Figure 21F). Conversely, the solely overexpression of growth inhibitors such as PTEN or Hippo is sufficient to suppress wing development and generate notum duplications (Figure 21G) (Rafel and Milán, 2008). It is important to notice that the early function of Notch promoting growth and wing specification takes place long before the requirement of Notch to establish the DV organizer.

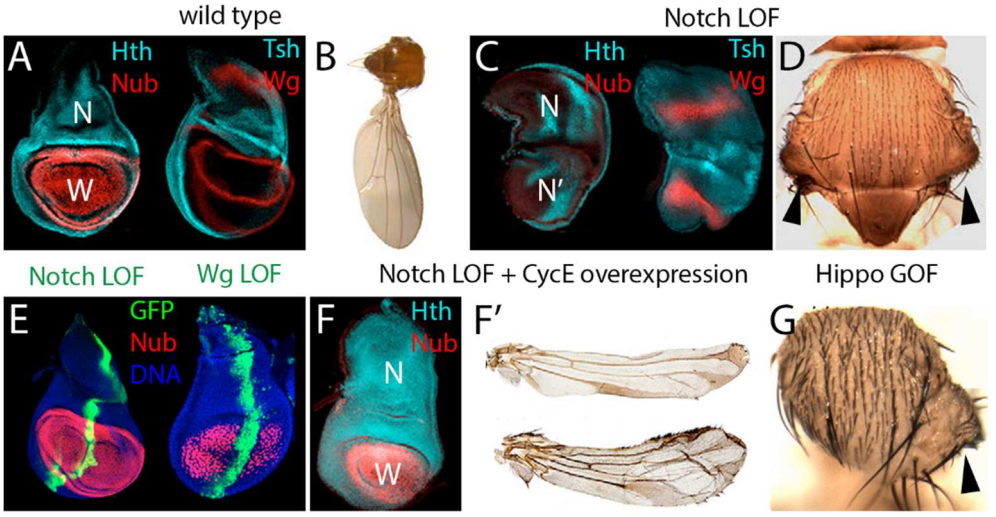


Figure 21. Notch signalling coordinates tissue growth and wing fate specification. (**A, B**) Wild type third instar wing primordia (**A**) and adult wing (**B**) labelled for Hth, Tsh (cyan) and Wg, Nub (red) that mark the body wall (N) and wing (W), respectively. (**C, D**) In a Notch loss-of-function situation (LOF) the wing is not specified and body wall structures are duplicated (N' in **C** and arrowheads in **D**). The wing marker Nub is absent (red, left panel, **C**) and Hth, Tsh and Wg form a symmetric duplication of the endogenous body wall. (**E**) Blocking Notch activity in a subset of cells (green) does not modify Nub levels (red) whereas inhibition of Wg signalling in the same domain autonomously downregulates Nub protein levels. (**F, F'**) CycE overexpression rescues wing fate specification upon Notch loss-of-function. Nub expression (red, **F**) is restored as well as adult wing formation (**F'**). (**G**) Overexpression of the growth suppressor Hippo phenocopies the notum duplications observed in Notch loss-of-function experiments. Adapted from (Rafel and Milán, 2008).

In the current model, the expression of Vn and Wg in opposite domains of the early wing disc instruct cells to acquire the proximal notum fate and distal wing fate, respectively. Wg restricts Vn to the proximal part, whereas Vn blocks the responsiveness of body wall cells to Wg (Ng et al., 1996; Wang et al., 2000). Thus, the relative concentration of Wg and Vn experienced by disc cells directs their wing versus body wall fate. Importantly, expression of these two ligands is established long before the wing field is specified in the primordium, shown by the retraction of Tsh that precedes Nub activation (Wu and Cohen, 2002). It is proposed that tissue growth acts as a "clock" and modulates the time and signalling levels that every cell is exposed to the instructive functions of Vn and Wg. In the early wing disc, Vn may reach

every cell and make them “blind” to Wg signalling, thereby blocking wing fate specification (Figure 22A). If the tissue does not grow enough during second instar, distal cells continuously transduce the Vn/EGFR pathway and wing specification is suppressed. Only when Notch-mediated growth pulls the sources of Wg and Vn apart, distal cells may not sense sufficient levels of Vn, allowing Wg to trigger wing development (Figure 22B, C) (Rafel and Milán, 2008). Thus, in highly proliferative tissues like imaginal discs, tissue growth may increase the distance from the source of morphogens and modulates the range of activity of these signalling molecules. In cellular terms, this is translated into the signal duration and intensity experienced by cells as well as to the amount of cells sensing a particular combination of patterning cues in a developmental field. This is one of the examples in which growth acts upstream of patterning. This elegant manner to couple tissue growth and patterning also operates in the eye-head imaginal primordium of *Drosophila*. In this developmental context, specification of eye and head structures also depends on the antagonistic activities of two morphogens expressed at opposite sides of the tissue. Dpp determines the eye field and Wg the head structures. It is proposed that tissue growth pulls the sources of these two morphogens apart and ensures the response of cells to the eye-inducing activity of Dpp (Kenyon et al., 2003). Insects have recurrently lost and recovered wings during the course of evolution suggesting that wing developmental pathways are conserved in wingless insects (Whiting et al., 2003). It is speculated that if the developmental potential to generate a wing is maintained, adaptive changes in animal or organ size might drive some of these reversible evolutionary transitions simply by modulating the cellular response to morphogens (Dekanty and Milán, 2011; Rafel and Milán, 2008).

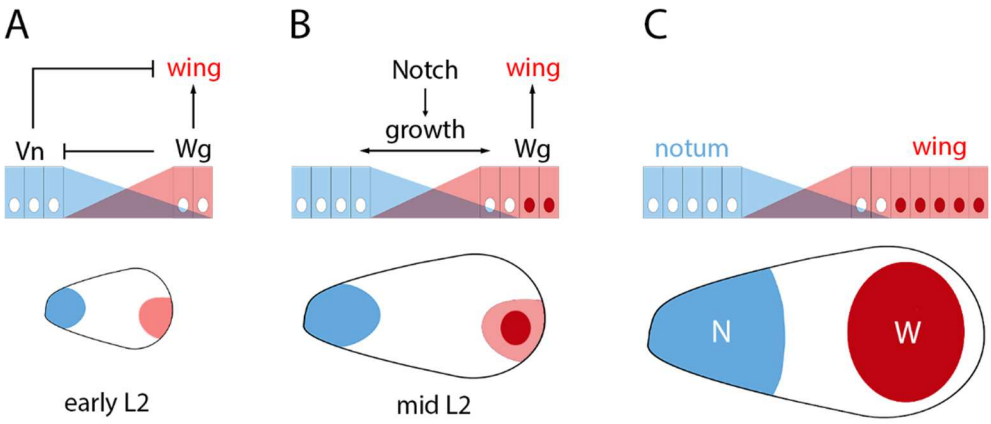


Figure 22. Coupling growth and wing fate specification. (**A-C**) Cartoon depicting the subdivision of the wing imaginal disc into wing (W, red) and body wall (N, blue) territories by Vn and Wg signalling molecules (blue and pink, respectively). Distally expressed Wg induces wing fate, whereas proximally expressed Vn blocks Wg responsiveness. Wg restricts Vn to the proximal region. (**A**) In early second instar (early L2) Vn diffuses and reaches every wing cell. Distal cells are not able to respond to Wg and do not acquire wing fate (pink cells, white nucleus). (**B**) Growth of the tissue promoted by Notch pulls the sources of Wg and Vn apart. Now, some distal cells do not sense sufficient levels of Vn and therefore Wg induces wing fate (pink cells, red nuclei) in mid second instar (mid L2). (**C**) The number of cells out of the range of Vn increases as the tissue expands and Wg induces wing fate in more cells. N, notum; W, wing. Adapted from (Rafel and Milán, 2008).

JAK/STAT signalling pathway

The JAK/STAT core signalling cascade

The JAK (Janus Kinase)/STAT (Signal Transducer and Activator of Transcription) signalling pathway is highly conserved from flies to humans and plays important and diverse roles in several biological processes relevant to development and disease. Despite the same signal transduction mechanism between vertebrates and flies, *Drosophila* has the advantage of fewer family members for each component of the pathway. The Unpaired cytokines are Interleukin-6 (IL-6)-like secreted proteins produced and released from a localized source that spread along the tissue to activate the JAK/STAT signalling cascade. The *Drosophila* genome encodes for three IL-6-like cytokines, Unpaired (Upd) – also called Outstretched (Os) – Upd2 and Upd3 (Agaisse et al., 2003; Gilbert et al., 2005; Harrison et al., 1998; Hombría et al., 2005). The binding of the extracellular ligand to the single transmembrane gp130-like receptor Domeless (Dome) (Brown et al., 2001) results in the activation of the only *Drosophila* receptor-associated JAK, Hopscotch (Hop) (Binari and Perrimon, 1994), which is most similar to the mammalian JAK2. The JAK tyrosine kinase then phosphorylates itself and the associated Dome receptor generating docking sites for the SH2 domains of the transcription factor STAT92E (Hou et al., 1996; Yan et al., 1996a), the single *Drosophila stat* gene, homologous to the mammalian STATs 3 and 5. STAT when phosphorylated forms dimers, which are stabilized by the interaction between the SH2 domain of one molecule and phospho-Tyr of the other molecule, and translocate to the nucleus to activate transcription of its target genes (Figure 23).

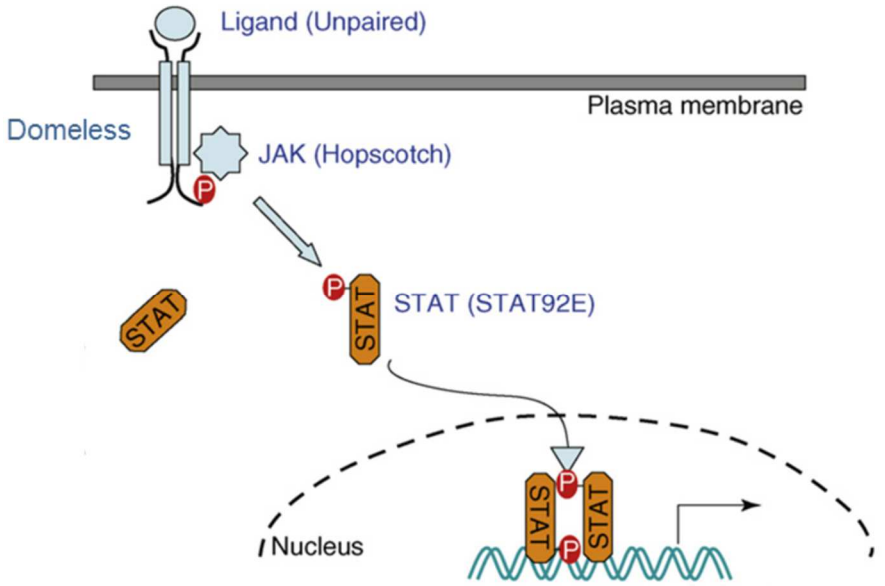


Figure 23. Canonical JAK/STAT signalling. Schematic representation of the JAK/STAT signalling pathway in *Drosophila*. Upon binding of the ligand Unpaired to the Domeless receptor, JAK phosphorylates itself and the Domeless receptor creating docking sites to STAT. The cytoplasmic unphosphorylated STAT is then phosphorylated and translocate into the nucleus where it dimerises to activate transcription of target genes. Names of the *Drosophila* homologs are in parentheses. Adapted from (Li, 2008).

Although dimerization of STATs via an N-terminal domain interaction can occur without pathway stimulation, only complexes activated by phosphorylation appear to induce target gene expression (Braunstein et al., 2003). Before the recruitment to the receptor/JAK complex, STATs are normally in the cytoplasm as inactive monomers. However, it was also shown that STATs are constitutively shuttling between the cytoplasm and nucleus before being retained in the nucleus after activation (Vinkemeier, 2004). In fact, studies in *Drosophila* have demonstrated a non-canonical mode of JAK/STAT signalling (Shi et al., 2006, 2008). In the non-canonical mode of signalling, a pool of unphosphorylated-STAT is localized in the nucleus on heterochromatin in association with HP1. This association is essential for maintaining HP1 localization and heterochromatin stability (Shi et al., 2008).

Activation of STAT by phosphorylation causes STAT dispersal from heterochromatin, which in turn leads to HP1 displacement and heterochromatin destabilization. This disruption allows derepression of genes that are not direct targets of STAT (Shi et al., 2006).

Several STAT92E target genes have been identified. Briefly, they include the Suppressor of Cytokine Signalling 36E (SOCS36E), which encodes a negative regulator of the pathway (Callus and Mathey-Prevot, 2002). *Socs36E* introns contain multiple high affinity STAT binding sites (Karsten et al., 2002). In some contexts, the receptor Dome is subject to a positive feedback since expression of *dome* is upregulated by STAT92E activation (Bach et al., 2003; Flaherty et al., 2009).

While *Drosophila* has a single *jak* and a single *stat* gene, in mammals the signalling cascade is much more complex. It comprises a wide and diverse range of extracellular ligands and receptors, four JAKs and seven STATs genes (Kisseleva et al., 2002). Therefore, the lack of redundancy in *Drosophila* makes it an excellent model for studying this signalling pathway.

JAK/STAT regulators

While the activation of JAK/STAT signalling pathway in mammals is triggered by a wide range of interleukins, interferons and growth factors, the characterisation of *upd* mutations in *Drosophila* suggest that the canonical requirements for JAK/STAT activity are likely to be mediated exclusively by the three Upd ligands (Agaisse et al., 2003; Gilbert et al., 2005; Harrison et al., 1998; Hombría et al., 2005). The *Drosophila* homologue of human BRWD3 is a large WD40- and bromo-domain-containing protein and was identified as a positive modulator of JAK/STAT signalling. It was found to strongly suppress the transcription of STAT92E-dependent reporters in cell culture (Müller et al., 2005). Moreover, mutations in BRWD3 decrease the

frequency of the melanotic tumour phenotype associated with a *hop* gain-of-function mutation (Müller et al., 2005).

Some negative regulators of the JAK/STAT pathway have been identified. The Suppressor of Cytokine Signalling (SOCS), originally identified in vertebrates, are the best known suppressors of JAK/STAT signalling. In *Drosophila*, three members of the SOCS family of proteins were shown to act as negative modulators of the JAK/STAT pathway (Stec and Zeidler, 2011). Among them, SOCS36E is part of a feedback loop since it not only acts an inhibitor of JAK/STAT signalling but it is also a target of STAT92E (Callus and Mathey-Prevot, 2002). The Protein Inhibitors of Activated STAT (PIAS) family represent another group of JAK/STAT suppressors that bind STATs and target them for degradation (Wormald and Hilton, 2004). A single *Drosophila* PIAS-like protein, ZIMP, has been shown to physically interact with STAT92E and to supress the formation of haematopoietic tumours caused by ectopic activation of the pathway (Betz et al., 2001). Phosphatase activity is likely to account for an important regulatory mechanism of JAK/STAT activity. In fact, the tyrosine phosphatase Ptp61F was identified as a suppressor of STAT92E-dependent transcription (Baeg et al., 2005; Müller et al., 2005). However, the exact mechanism by which PTP61F can modulate JAK/STAT signalling is not entirely clear. Ken & Barbie (KEN), that belongs to the family of BTB/POZ domain-containing transcriptional repressors, is a selective negative regulator of STAT92E activity. While *in vitro* experiments show that KEN recognises a DNA sequence that partially overlaps the one of STAT92E, tissue culture assays indicate that it specifically downregulates JAK/STAT activity reporters containing the consensus KEN DNA-binding site (Arbouzova and Zeidler, 2006). *In vivo*, KEN can only downregulate a subset of STAT92E target genes (Arbouzova and Zeidler, 2006). Moreover, its human homologue BCL6 has also been shown to repress STAT6-dependent

transcription in cell culture (Harris et al., 1999). The *eye transformer* (*et*) also called *latran* (*lat*) encodes a transmembrane protein found to physically interact and form heterodimers with Dome and antagonize JAK/STAT signalling probably because its short intracellular domain lacks a JAK/Hop binding motif. *In vivo* studies show that the eye overgrowth phenotype caused by excessive JAK activation was enhanced by downregulation of Et/Lat (Kallio et al., 2010) and that Et/Lat is required for JAK/STAT downregulation in hemocytes precursors (Makki et al., 2010).

Functions of JAK/STAT

Not only JAK/STAT structural components have been conserved during evolution, but there is also substantial conservation of JAK/STAT function between human and *Drosophila* systems, despite lower redundancy compared to the mammalian system. Although the JAK/STAT pathway was originally discovered as a cytokine-induced signalling cascade required for immune functions, decades of research in *Drosophila* has implicated JAK/STAT signalling in several biological processes in embryonic, larval and adult stages.

The first clues about JAK/STAT function in flies came from the identification of Hop as a maternally supplied protein required for patterning of the embryonic cuticle and proliferation of imaginal cells (Binari and Perrimon, 1994; Perrimon and Mahowald, 1986). Likewise, mutations in *upd*, *dome* and *stat92E* consistently show defects in morphogenetic segmentation of the embryo (Brown et al., 2001; Wieschaus et al., 1984; Yan et al., 1996a). Null mutations of *upd* show segmentation phenotypes less severe than those of mutants for the more downstream components of the pathway, indicating that other ligands might partly compensate for the loss of *upd* (Hombría et al., 2005). Evidence for the molecular mechanisms underlying the role of

JAK/STAT signalling in embryonic segmentation came from the identification of STAT92E binding sites present in the promoter of the pair-rule gene *evenskipped* (*eve*) (Yan et al., 1996a), showing the requirement of JAK/STAT signalling as a transcriptional regulator of patterning genes. However, the role of JAK/STAT signalling in embryonic development is extended beyond its role in segmentation. JAK/STAT was also implicated in sex determination. It reinforces the mechanism by which the information about sex chromosome content is translated into the activation of male or female genetic program, through the regulation of *Sex lethal* (Sxl) (Avila and Erickson, 2007; Sefton et al., 2000). Moreover, in late embryogenesis, JAK/STAT also plays an important role in male germ line sexual development (Wawersik et al., 2005) (Wawersik et al., 2005). Among others, JAK/STAT has also been involved in the embryonic development of the tracheal system, in the process of gut elongation as well as in the formation of the spiracles (Hombría and Sotillos, 2013).

JAK/STAT as a modulator of cell proliferation

Several evidences indicate that JAK/STAT signalling is a wide regulator of cellular proliferation, a process that is essential to many aspects of normal development and disease.

Analysis of loss-of-function mutations indicate that JAK/STAT is required for normal proliferation of imaginal cells. While loss of *hop* leads to small imaginal discs (Mukherjee et al., 2005; Perrimon and Mahowald, 1986), several hypomorphic *upd* alleles display small eye phenotypes (Bach et al., 2003; Tsai and Sun, 2004). Conversely, ectopic expression of Upd in the eye enhances proliferation resulting in enlarged and overgrown adult structures, phenotype shown to be sensitive to the dose of the downstream elements of the pathway (Bach et al., 2003; Mukherjee et al., 2005; Tsai and Sun, 2004).

Consistent with this pro-proliferative function, JAK/STAT also regulates the competitive status of proliferating cells in the eye and wing imaginal discs. Cells lacking *stat92E* are eliminated from the epithelium through cell competition (Rodrigues et al., 2012), a process by which slow-dividing cells (so called losers) are detected and removed through apoptosis by fast growing cells (so called winners) (Morata and Ripoll, 1975). Furthermore, cells with hyperactivated STAT92E become winners and manifest supercompetitor features such as their trigger the non-autonomous induction of apoptosis in the surrounding wild type cells and their subsequent elimination (Rodrigues et al., 2012). The competitive capacity of JAK/STAT is dependent on the pro-apoptotic gene *hid*, but independent of dMyc, Yorkie (Yki), Wg signalling and ribosome biogenesis (Rodrigues et al., 2012).

Insights into the role of JAK/STAT signalling in *Drosophila* blood cells development, called hemocytes, have proven to be important in the understanding of mechanisms relevant to human disease. Gain-of-function mutations of Hop result in the overproliferation of hemocytes within the developing larva and their premature differentiation causing the formation of large melanotic tumour cell masses (Hanratty and Dearolf, 1993; Harrison et al., 1995; Luo et al., 1995, 1997). In fact, these tumours have invasive potential since transplantation of the hematopoietic organ of flies carrying the gain-of-function mutation of Hop into a wild type host produces the appearance of melanotic masses in the host (Hanratty and Ryerse, 1981). Interestingly, constitutive activation of several STATs has been observed in multiple human cancers, including blood malignancies (Calò et al., 2003).

JAK/STAT signalling has also an essential role in stem cell maintenance and proliferation within the gonads of both sexes. It has been proposed that the Upd ligand, expressed by a small group of somatic cells, sustains stem cell state and/or proliferation of adjacent germline stem cells (GSCs). As these

cells divide, the more distal daughter cell is further away from the source of the ligand and it starts to differentiate since is no longer exposed to sufficiently high levels of Upd necessary for pathway stimulation. Consistently, *hop* and *stat92E* mutant testes prematurely lose their GSCs during larval development (Kiger et al., 2001; Tulina and Matunis, 2001), while ectopic expression of *upd* results in the expansion of GSCs at the expense of differentiated cells (Kiger et al., 2001; Tulina and Matunis, 2001). So, JAK/STAT appears to have a pro-proliferative function in several different contexts in normal development. Not only activation of the pathway appears to be necessary to modulate proliferation but it is also sufficient to drive proliferation in multiple tissues. However, the mechanisms by which JAK/STAT regulate cell proliferation remain poorly understood. In humans, STAT activates *cyclin D1*, which encodes a regulatory subunit of the CYCD/CDK4 complex that promotes G1/S transition, and *c-myc*, which functions as a transcriptional regulator of cell cycle progression. Moreover, activation of both genes by JAK/STAT account for the proliferative effect of the pathway (Bowman et al., 2000; Calò et al., 2003). In *Drosophila*, STAT92E has been reported to interact with CYCD/CDK4 and CYCE/CDK2 complexes (Chen et al., 2003). Moreover, activation of the JAK/STAT pathway induces upregulation of *cycD* in the eye imaginal disc (Tsai and Sun, 2004) and increases CycB levels in wing imaginal disc cells (Mukherjee et al., 2005). However, these observations do not explain the mechanism by which JAK/STAT controls proliferation. Loss of a single copy of *cycD* does not reduce the eye overgrowth phenotype caused by ectopic activation of the JAK/STAT pathway (Mukherjee et al., 2006), and whether CycB regulation by JAK/STAT contributes to its role in proliferation has not been addressed. In addition, activation of JAK/STAT in wing imaginal cells does not increase dMyc protein levels (Rodrigues et al., 2012). In this sense, although some links between JAK/STAT pathway and cell cycle have been reported, the

exact mechanisms by which JAK/STAT controls proliferation remain to be elucidated.

JAK/STAT was also demonstrated to function as a pro-survival factor in response to tissue stress (Betz et al., 2008; La Fortezza et al., 2016; Verghese and Su, 2016). High JAK/STAT activity can protect cells from radiation-induced apoptosis (Betz et al., 2008; Verghese and Su, 2016). STAT92E, when activated directly increases expression of the *Drosophila* inhibitor of apoptosis (Diap1) through binding to two STAT92E binding sites in the *diap1* promoter (Betz et al., 2008). Activation of *upd* transcription and JAK/STAT signalling upon tissue damage has been associated to compensatory proliferation and regeneration in imaginal discs and adult guts (Jiang et al., 2009; Katsuyama et al., 2015; Santabárbara-Ruiz et al., 2015).

JAK/STAT signalling in *Drosophila* appendage development

Several evidences implicate the JAK/STAT signalling pathway in the development of *Drosophila* appendages, such as the eye, leg, antenna and wing primordia. Besides the role of JAK/STAT in controlling proliferation in the eye imaginal disc it was also shown to control early eye patterning by regulating regional specification of the eye primordium. Unpaired is expressed in the ventral eye primordium at the first larval instar stage and activation of the pathway represses *wingless* expression (Ekas et al., 2006). As a result, the Iroquois-complex genes are only activated by Wg in dorsal cells. The dorsal-ventral subdivision induces the activation of a Notch organizer along the equator of the eye primordium, which is responsible for growth of the eye disc. JAK/STAT was shown to control proliferation of the eye primordium downstream of the Notch organizer. Upon ectopic expression of *upd*, the derepression of Wg creates a second organizer in the

dorsal half of the eye (Gutierrez-Aviño et al., 2009). In fact, ectopic activation of JAK-STAT is unable to restore eye size when Notch signalling is attenuated (Gutierrez-Aviño et al., 2009). Later in development, *upd* expression becomes restricted to the most posterior edge of the eye, the so called firing point as it is the point from where the eye morphogenetic furrow begins its anterior differentiation wave-like movement. The retraction of *upd* expression is mediated by Notch-induced activation of Eyegone (Eyg) (Chao et al., 2004). Several experiments indicate that Upd diffusion from the firing point is required for *wg* repression and proper progression of the morphogenetic furrow in the dorsal eye. *Stat93E* mutant clones show ectopic *wg* expression and impede the progression of the furrow, while ectopic Upd represses *wg* and induces precocious furrow initiation that is prevented by co-expressing Wg (Ekas et al., 2006; Tsai et al., 2007). Thus, the main JAK/STAT pathway function in eye development is to regulate Wg.

JAK/STAT plays a similar role in the proximo-distal patterning of both antenna and leg imaginal discs. Wg and Dpp are expressed in opposing domains of the antenna and leg discs and interactions between these two signalling pathways creates a proximo-distal (PD) axis by activation of Distalless (Dll) in the centre of the disc. Udp is expressed in a pattern complementary to that of Wg and Dpp and it restricts Wg and Dpp expression to their corresponding domains to guarantee the formation of a single PD axis. Ectopic expression of Upd results in *wg* and *dpp* repression, while *stat93E* mutant clones show ectopic expression of *wg* and induce the duplication of leg and antenna structures by creating a secondary PD axis (Ayala-Camargo et al., 2007). Besides, Wg and Dpp reciprocally restrict *upd* expression (Ayala-Camargo et al., 2007) revealing that the reciprocal

interaction between these three signalling pathways is important for patterning of the antenna and leg imaginal discs.

Evidence for a role of JAK/STAT in wing development comes from the original observation of the adult outstretched wing phenotype produced by the regulatory allele *os*^{1} (Muller, 1930) which was later attributed to the late role of JAK/STAT in hinge development (Ayala-Camargo et al., 2013; Johnstone et al., 2013). In the embryo, the wing disc primordium expresses low levels of *upd* mostly restricted to the anterior domain and little or no activation of the pathway is detected at this time (Rodrigues et al., 2012). Later in development, in second instar stage, *upd* is widely expressed throughout the wing primordium with a corresponding global activation of the pathway (Ayala-Camargo et al., 2013; Hatini et al., 2013; Rodrigues et al., 2012) where it was suggested to inhibit the induction of ectopic wing fields (Hatini et al., 2013). At the beginning of the third instar larval stage activity of JAK/STAT becomes restricted to the hinge region. The downregulation from the wing pouch and notum coincides with the activation of Nub and Eyg in these regions, respectively, suggesting a causal relationship. In fact, Nub represses JAK/STAT activity and *upd* expression, as both gain- and loss-of-function of Nub modulates expression of both *upd* and a STAT92E activity reporter (Ayala-Camargo et al., 2013). The retraction of JAK/STAT activity from the pouch and notum contribute to the proportional proximo-distal subdivision and expansion of the pouch and notum regions as well as for medial-lateral patterning of the notum (Hatini et al., 2013). This is mediated by restricting the scope of Odd-skipped (Odd) function in the notum, which is required for notum AP axis organization, and by antagonizing Dpp function in the patterning of the medial-lateral axis of the notum (Hatini et al., 2013). Moreover, the retraction of JAK/STAT activity from

the pouch and notum suggests that Upd might be deleterious to wing blade and notum development. Indeed, Upd misexpression in the entire wing primordium causes aberrant development of the wing disc (Hatini et al., 2013). In addition, ectopic expression of Upd within the presumptive wing blade causes small adult wings (Ayala-Camargo et al., 2013). Characterisation of the *outstretched* alleles of the *unpaired* locus demonstrated a role of JAK/STAT signalling in wing hinge development during late larval stages. Late expression of *upd* becomes restricted to a characteristic five-dot pattern and activity of pathway is confined to the wing hinge (Ayala-Camargo et al., 2013; Johnstone et al., 2013). The defects in wing posture of os^{1} alleles arise from the lose of Unpaired expression in the proximal hinge region which results in decreased JAK/STAT activity (Johnstone et al., 2013). In fact, reduction in JAK/STAT activity within the proximal region results in abnormal hinge folds and phenocopies the held out wing defect associated with classical *os* alleles (Johnstone et al., 2013). Hinge cells lacking *stat92E* have significantly reduced proliferation suggesting that Upd acts as a growth factor in these cells. *Stat92E* mutant clones cell autonomously downregulate hinge-specific factors, such as *dachsous* (*ds*), Muscle segment homeodomain (Msh) and Zfh2, which are consistently upregulated upon ectopic STAT92E activity (Ayala-Camargo et al., 2013). Increased STAT92E activity also cell autonomously represses the Iro-C protein Araucan (Ara) (Ayala-Camargo et al., 2013). Some insights about the upstream regulation of JAK/STAT signalling come from the evidence that *hth* mutant clones show loss of STAT92E activity and Hth-overexpressing cells can induce ectopic activity of the pathway in the pouch and notum regions (Ayala-Camargo et al., 2013) The wing marker Nub represses STAT92E activity and *upd* production (Ayala-Camargo et al., 2013) which is consistent with the observation that developmentally regulated retraction of *upd* expression coincides with the induction of Nub.

In addition, Wg was also demonstrated to be upstream of STAT92E in the hinge, since absence of Wg signalling leads to loss of JAK/STAT activity (Ayala-Camargo et al., 2013). Therefore, JAK/STAT signalling is critical for hinge fate specification and growth during wing imaginal disc development, a region that articulates the proper wing with the thorax of the adult fly.

Although the JAK/STAT pathway plays important developmental roles in patterning and growth of *Drosophila* appendages, no developmental role of the conserved JAK/STAT signalling cascade has been described so far in vertebrate limbs.

Project and objectives

A discrete number of signalling pathways and morphogens of the Wnt/Wg, Shh/Hh and BMP/Dpp families regulate tissue growth and pattern formation in vertebrate and invertebrate limbs. The general aim of this thesis was to analyse the roles of the JAK/STAT signalling pathway in the development of wing imaginal disc. The objectives of this thesis can be subdivided as follows:

- Analyse the effect of loss- and gain-of-function of JAK/STAT signalling in the wing imaginal disc during early and late developmental stages.

- Characterize the relationship of the JAK/STAT pathway with other signalling pathways involved in wing imaginal disc growth and patterning.

- Identification of the molecular mechanisms underlying the function of JAK/STAT in wing development.

Results

JAK/STAT in the proximo-distal patterning of the wing

Early expression and activity of JAK/STAT signalling

Early in development, in second instar wing discs, localized expression of Wingless (Wg) and the EGFR ligand Vein (Vn) in opposing domains subdivides the wing primordium into the presumptive wing field and body wall (or notum) regions, respectively [Figure 24, (Ng et al., 1996; Wang et al., 2000)].

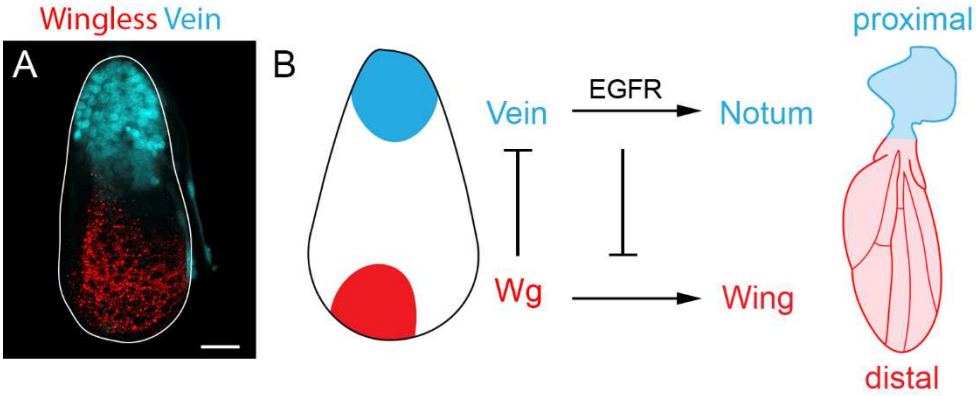


Figure 24. Segregation of wing and notum developmental fields. (**A**) Expression of *vn* transcript (cyan) and Wg protein (red) in mid-second instar wing primordia. (**B**) Simplified cartoon depicting the expression and the antagonistic functions between Vn/EGFR and Wg in second instar and the corresponding adult structures they induce. Scale bar, 20µm.

We monitored at this developmental stage the expression of the Unpaired 1 ligand (Upd) using the Gal4/UAS binary expression system (Brand and Perrimon, 1993) and by *in situ* hybridization with an antisense RNA probe complementary to the *upd* mRNA. The *upd-gal4* driver is a P-element transposon insertion in the *upd* locus that carries the Gal4 transcriptional activator and behaves as an enhancer trap, recapitulating the endogenous expression pattern of the *upd* gene (Tsai and Sun, 2004). *upd* expression can be therefore visualized when the *upd-gal4* fly strain is coupled with a *UAS-GFP* transgene, resulting in Gal4-driven GFP expression in the *upd*

expressing territories. We also monitored the activity of the pathway using the well-characterized *10xSTAT-GFP* reporter. This reporter was designed from the *Socs36E* gene, a target of JAK/STAT that functions in a negative feedback loop to attenuate signalling (Callus and Mathey-Prevot, 2002; Karsten et al., 2002). The *10xSTAT-GFP* construct contains five tandem repeats of a 441 bp fragment from the first intron of *Socs36E*, each containing at least two potential binding sites for STAT92E, that drive the expression of GFP upon pathway activation (Bach et al., 2007).

We found that the expression of *upd* was restricted to the most distal domain of the wing disc, in a broader domain than Wg (Figure 25B). We confirmed the expression of *upd* to the distal domain of second instar wing discs by *in situ* hybridization, validating the use of the *upd-gal4* driver as a faithful tool to monitor *upd* expression (Figure 25A). Expression of *upd* is dynamic and evolves during development, once the wing has been specified during second instar, *upd* retracts from the distal domain and starts to accumulate in a ring-like domain foreshadowing the presumptive wing hinge. As development proceeds during third instar, *upd* expression progressively resolves into its characteristic five-spot patern in the hinge – a region that connects the developing wing to the surrounding body wall – and the ventral pleura, which forms the lateral plate of the thorax. (Ayala-Camargo et al., 2013; Hatini et al., 2013; Mukherjee et al., 2005; Rodrigues et al., 2012). Activation of the pathway during second instar was observed throughout the wing disc, although GFP levels were clearly lower in the most proximal region of the wing primordium (Figure 25C).

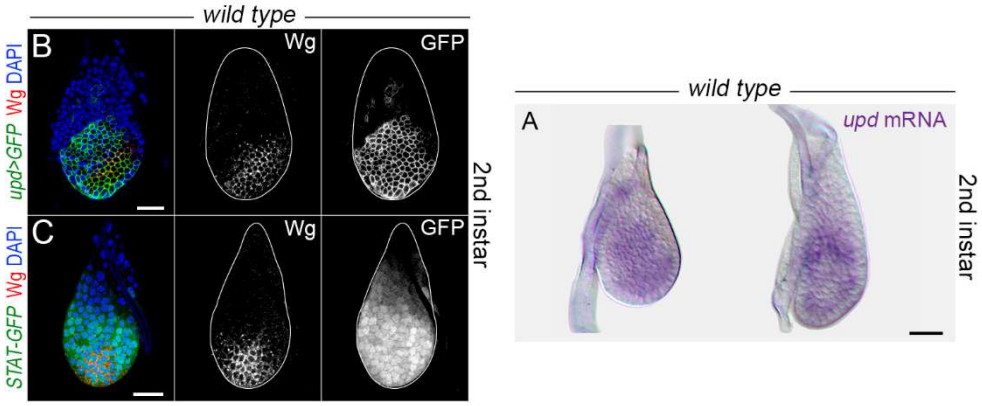


Figure 25. Expression and activity of JAK/STAT signalling components in second instar. (**A-C**) Wing primordia of second instar larvae labeled to visualize expression of *unpaired* (*upd*, purple, **A,** by ISH; green or white, **B**, in *upd-gal4, UAS-myrGFP* larvae), Wingless protein (Wg, red or white, **B, C**), the activity of the JAK/STAT pathway (green or white, **C**, in *10xSTAT-GFP* larvae), and DAPI (blue, **B, C**). A white line marks the contour of the discs. Scale bars, 20 µm.

JAK/STAT is required for the wing versus notum subdivision

We next analysed the potential developmental role of the JAK/STAT pathway at this stage of wing development. For this purpose, we used a collection of RNAi forms against the Domeless receptor, the JAK kinase Hop and the STAT92E transcription factor. We drove the expression of these RNAi transgenes with the *scalloped-gal4* (*sd-gal4*) driver, which is expressed at high levels in the whole wing primordium at this developmental stage (Rafel and Milán, 2008). Remarkably, the resulting adult wings were either vestigial or absent, and body wall structures were often duplicated (Figure 26A, B). This phenotype is reminiscent of the *wg* mutant adult phenotype (Morata and Lawrence, 1977; Sharma and Chopra, 1976). In the developing wing imaginal disc, expression of the homeodomain protein Homothorax (Hth) is restricted to the presumptive hinge and body wall (Azpiazu and Morata, 2000; Casares and Mann, 2000), while the POU homeodomain protein Nubbin (Nub) is expressed in the presumptive wing territory and the distal part of the hinge (Ng et al., 1995, 1996). Wg is expressed in the body wall, hinge and wing pouch territories of late third instar discs in a characteristic

pattern (Figure 26C). In the hinge, two rings of Wg, the inner and the outer ring (IR and OR respectively) encircle the wing pouch, which is bisected into dorsal (D) and ventral (V) compartments by a stripe of Wg expression (Neumann and Cohen, 1996, 1997). In the notum, Wg runs in a stripe from the anterior (A) to the posterior (P) notum and approximately marks the border between the lateral and medial notum territories (Calleja et al., 2000). We then analysed and compared the expression of these molecular markers in mature wing discs in which the JAK/STAT pathway had been compromised. Consistent with the adult phenotypes, Nub was absent or residual in a small group of cells, and the characteristic expression pattern of Hth and Wg in the notum showed a mirror-image duplication (Figure 26D, E). These results indicate that JAK/STAT is required for proper wing fate specification.

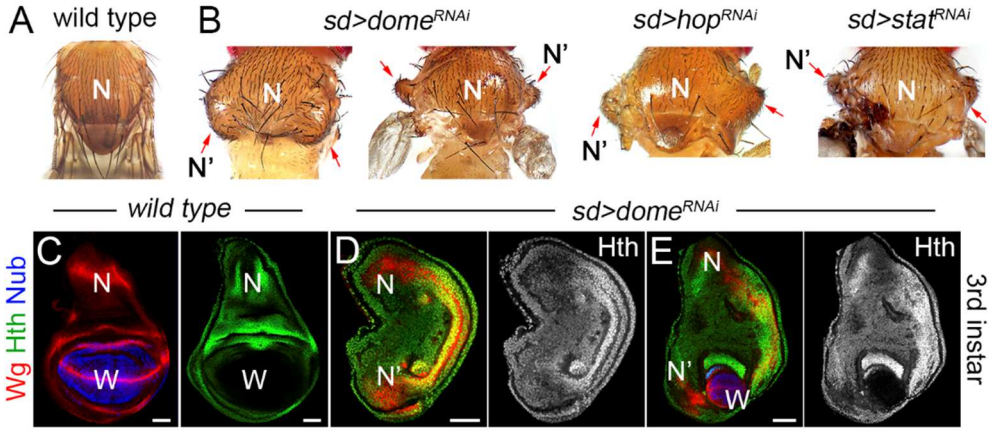


Figure 26. Failure to specify wing fate in the absence of JAK/STAT. (**A, B**) Adult thoraxes and mature wing primordia (**C-E**) of *wild type* male individuals (**A, C**) or male individuals expressing the indicated transgenes under the control of the *sd-gal4* driver (**B**, **D**, **E**). Wing primordia were stained for Wingless (Wg, red, **C**-**E**), Nubbin (Nub, blue, **C-E**) and Homothorax (Hth, green or white, **C**-**E**). Wing territory (W), endogenous nota (N) and duplicated nota territories (N') are marked. Red arrows in **B** point to the duplicated nota (N'). Adult thoraxes are illustrative examples of complete or partial duplications of the notum structures. Scale bars, 50 µm.

JAK/STAT restricts the notum fate and ensures wing fate specification

To understand the mechanism by which JAK/STAT ensures proper wing versus notum specification we hypothesised that JAK/STAT might control the expression or activity of Wg, or it might collaborate with the Wg pathway during wing fate specification. Alternatively, JAK/STAT might repress the Vn/EGFR pathway or its target genes, acting as a brake, to restrict the notum fate to the most proximal territories of the early wing primordium and thus allowing Wg-mediated appendage specification. We therefore checked the expression and activity of Wg and Vn/EGFR in JAK/STAT-depleted wing discs. As was previously shown, blocking the response to Wg by overexpression of Shaggy/GSK3 – an antagonist of the Wg pathway – in a stripe along the AP compartment boundary caused a cell-autonomous loss of Nub [Figure 27A, (Rafel and Milán, 2008)]. In contrast, blocking JAK/STAT activity in the same domain did not have this effect (Figure 23B). Moreover, the early expression of Wg was not affected upon depletion of the JAK/STAT pathway (Figure 27B). Overall, these observations indicate that this pathway does not have an active role in inducing wing fate or in regulating Wg expression and activity, and therefore JAK/STAT does not behave as a Wingless-like molecule.

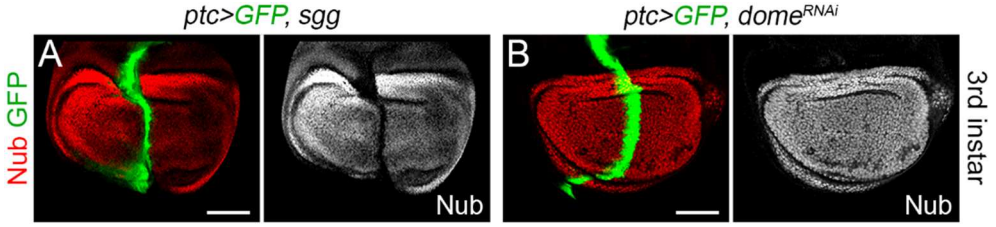


Figure 27. JAK/STAT does not regulate wing fate cell-autonomously. (**A**, **B**) Wing primordia from late third instar larvae expressing *shaggy (sgg,* **A**) or *dome*^{RNAi} (**B**) under the control of the *ptc-gal4* driver and labeled to visualized Nub protein (red or white) and GFP (green). Note that Nub is cell-autonomously downregulated in the *ptc-gal4* expressing domain (green, **A**). Scale bars, 50 µm.

The *Drosophila* Iroquois complex *(Iro-C)* consists of three genes encoding for homeobox transcription factors, namely Araucan, Caupolican and Mirror, which are expressed in the most proximal region of the wing primordium by the activity of the Vn/EGFR pathway (Figure 28A, C) and are required to specify notum structures [(del Corral et al., 1999; Simcox et al., 1996), see Introduction]. Recent experimental evidence has revealed a late role of the JAK/STAT pathway in repressing these genes in the hinge of mature wing primordia (Ayala-Camargo et al., 2013; Hatini et al., 2013). We therefore analysed whether JAK/STAT has an earlier and more extensive role in restricting the expression of Vn/EGFR targets such as the *Iro-C* genes and *apterous*, a gene encoding for a LIM-homeodomain transcription factor that specifies the dorsal (D) compartment (Diaz-Benjumea and Cohen, 1993). We monitored the expression of *mirror* using the transcriptional reporter *mirror-lacZ*, a target of EGFR in the notum throughout development that serves both as a marker for notum identity as well as a readout of EGFR signalling in the presumptive body wall. We noted that *mirror* expression was expanded distally in JAK/STAT-depleted second instar wing primordia (Figure 28A, B) as well as in the resulting mature third instar wing discs, where the expansion of mirror was even more evident (Figure 28C-E).

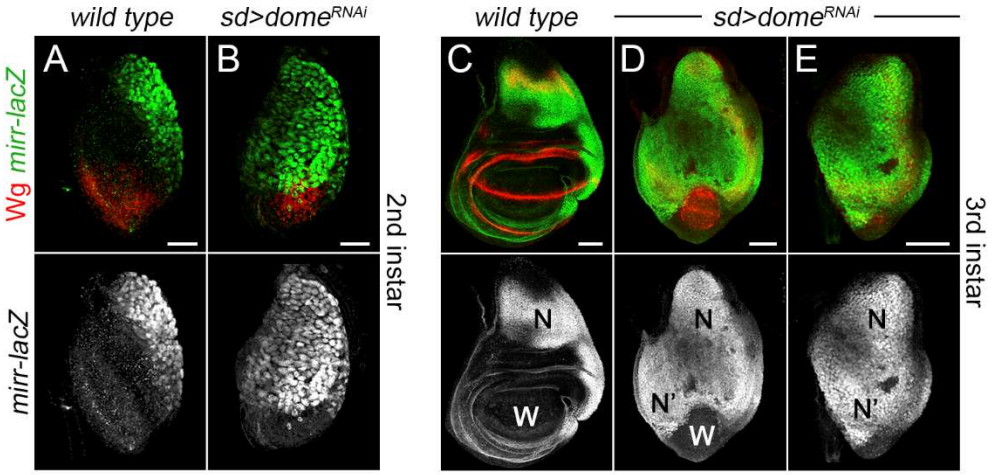


Figure 28. JAK/STAT restricts the expression of the EGFR target mirror to the body wall. (**A-E**) Wing primordia from *wild type* larvae (**A, C**) or from larvae expressing *dome*^{RNAi} under the control of the *sd-gal4* driver (**B, D, E**) labeled to visualize *mirror* (*mirr-lacZ*, antibody to β-gal, green or white) and Wg protein (red) in second (**A, B**) and late third instar stages (**C-E**). Wing territories (W), endogenous nota (N), and duplicated nota territories (N') are marked in **C-E**. Scale bars, 20 µm (**A, B**) or 50 µm (**C-E**).

We then monitored the expression of *apterous* using the transcriptional reporter *apterous-lacZ*. *apterous* expression transiently relies on EGFR signalling during second instar and later is maintained by an autoregulatory loop (Bieli et al., 2015; Wang et al., 2000; Zecca and Struhl, 2002b). In *dome*^{RNAi}-expressing third instar wing primordia, *apterous* expression was also expanded distally (Figure 29G-I), which is consistent with the duplicated nota observed in the adults, since the notum is mostly a dorsal derivative. The expansion in the expression domains of *mirror* and *apterous* was not always accompanied by the loss of the presumptive wing field (Figure 28D and 29H). In those cases where the notum duplication was partial, the remaining wing was very small and displaced ventrally, as well as apposed directly to the surrounding body wall due to the loss of the intervening hinge population (Figure 26E and28D). In the total duplications, almost the entire wing primordia expressed the notum marker *mirror* and the dorsal selector *apterous* (Figure 28E and 29I).

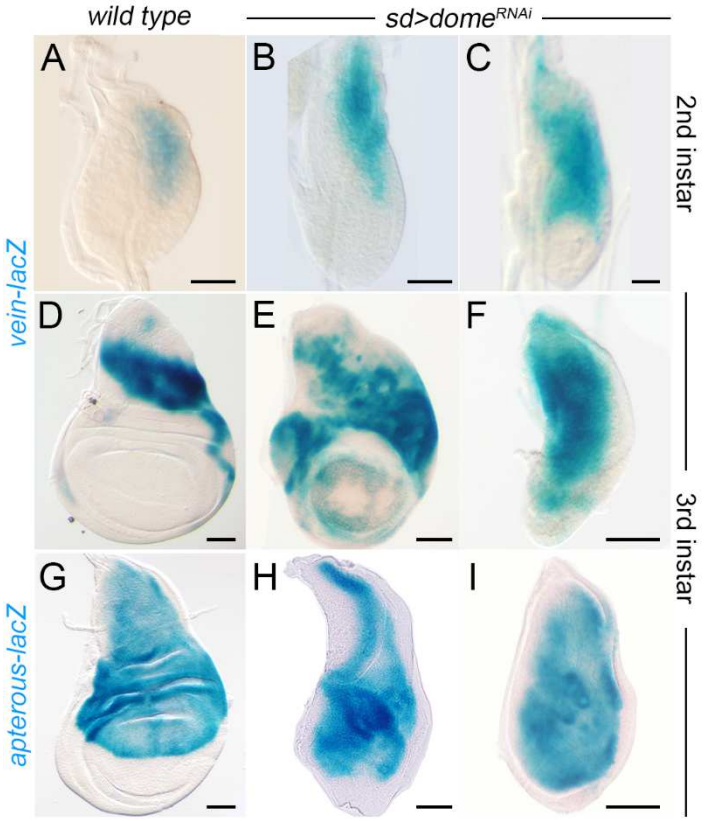


Figure 29. JAK/STAT restricts the expression of *vein* and *apterous* to the body wall. (**A-F**) Wing primordia from *wild-type* larvae (**A, D**) or from larvae expressing *dome*^{RNAi} under the control of the *sd-gal4* driver (**B, C, E, F**) labeled to visualize *vein* expression (*vein-lacZ,* X-Gal staining, blue) in second (**A-C**) and late third instar (**D-F**) stages. (**G-I**) Wing primordia from late third instar *wild type* larvae (**G**) of from larvae expressing *dome*^{RNAi} under the control of the *sd-gal4* driver (**H, I**) labeled to visualize *apterous* expression (*apterous-lacZ,* X-Gal staining, blue). Scale bars, 20 µm (**A-C**) or 50 µm (**D-I**).

In the early wing primordium, induction of Vn initially depends on Dpp signalling but slightly later is maintained by a positive feedback amplification loop through the activation of the EGFR pathway. Thus, Vn is both the ligand and a target of EGFR in the presumptive body wall [(Paul et al., 2013; Wang et al., 2000), see Introduction]. We found that the initial expression of the Vn ligand was unaffected in JAK/STAT-depleted early wing primordia (Figure 29B, compare with Figure 29A). Consistent with our observation that JAK/STAT restricts EGFR-regulated genes, we found that Vn expression was

expanded in later JAK/STAT-depleted second instar wing primordia (Figure 29C) and that this expansion remained in mature wing primordia (Figure 29E, F, compare with Figure 29D).

To further reinforce the proposal that the distal expansion of EGFR regulated genes observed upon JAK/STAT inhibition is responsible for the duplication of notum structures, we performed genetic interactions with members of the EGFR pathway and the *Iro-C* genes. We used of a null *Egfr* allele (*Egfr*^{F2}) or a deletion that covers the three *Iro-C* genes [*Iro*^{EGP7}, (Andreu et al., 2012)] in order to rescue the penetrance of the duplicated nota. Halving the doses of the *Egfr* gene or of the whole *Iro-C* reduced the frequency of duplicated nota observed in adults (Figure 30), confirming that the distal expansion of EGFR target gene expression contributes to the duplication of notum structures observed in adults. This frequency was increased in *wg* heterozygous animals (Figure 30) indicating that halving the dose of *wg* facilitates the acquisition of notum fate by ectopic *Iro-C* genes.

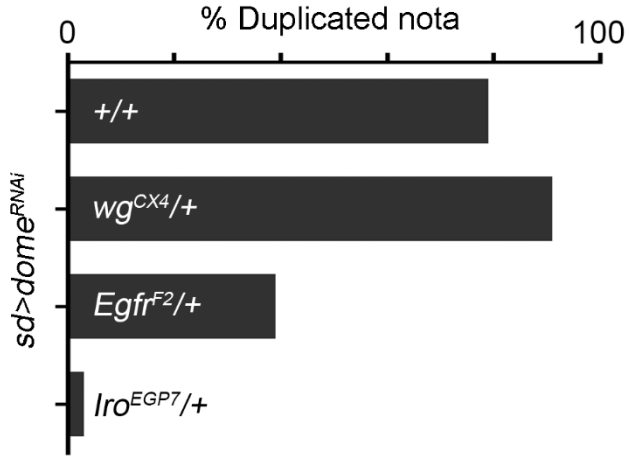


Figure 30. Reducing the *Egfr* and *Iro-C* gene doses decreases the frequency of duplicated nota. Bar graphs plotting the percentage of duplicated nota in the following genotypes: (**1**) *sd-gal4/Y; UAS-dome*^{RNAi}*/+; UAS-dcr2/+* (**2**) *sd-gal4/Y; UAS-dome*^{RNAi}*/wg*^{CX4}*; UAS-dcr2/+* (**3**) *sd-gal4/Y; UAS-dome*^{RNAi}*/EGFR*^{F2}*; UAS-dcr2/+* (**4**) *sd-gal4/Y; UAS-dome*^{RNAi}*/+; Iro*^{EGP7}*/UAS-dcr2.* Only male individuals with partial and total duplications were scored for each genotype. (n>100 heminota). See also **Table 1.**

Effects of targeted activation of JAK/STAT in the wing

We next addressed whether the ectopic activation of the JAK/STAT pathway in the presumptive notum territory might affect the expression of these genes and consequently impair notum development or cause phenotypes similar to those described for Vn/EGFR downregulation. Ectopic expression of Upd to the most proximal side of the wing primordium downregulated *mirror* expression throughout development (Figure 31A-C) and caused a reduction in the size of the notum (Figure 31E, compare with Figure 31D). These two observations resemble the behavior of *Egfr* mutant clones in the notum and the "notumless" phenotype of *vn* hypomorphs, respectively (Simcox et al., 1996; Wang et al., 2000; Zecca and Struhl, 2002b).

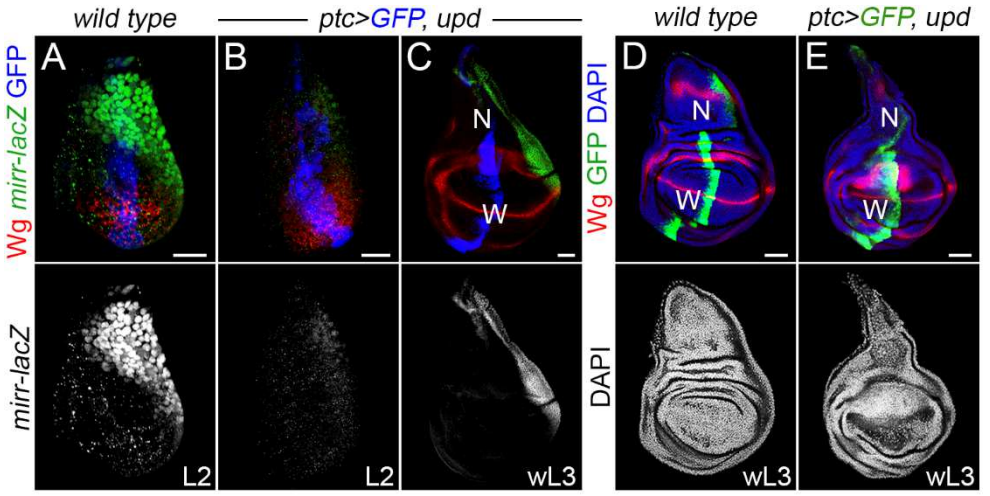


Figure 31. Ectopic expression of *upd* downregulates *mirror* throughout development and phenocopies *vn/Egfr* hypomorphs. (**A-E**) Second instar (**A, B**) and late third instar (**C-E**) wing primordia from *wild type* larvae (**A, D**) or larvae ectopically expressing the *UAS-upd* transgene under the control of the *ptc-gal4* driver (**B, C, E**). Wing discs are labeled to visualize *mirror (mirr-lacZ*, antibody to β-gal, green or white, **A-C**), Wg (red) and GFP (blue, **A-C**; green, **D, E**) to mark the domain of transgene expression. In **C-E**, wing territories (W) and endogenous nota (N) are marked. *mirror-lacZ* is downregulated in the notum from second (**A, B**) to third instar (**C**, compare with *wild type mirror* expression in **Figure 24C**) and the size of this territory is notably reduced (**E**) compared to the *wild type* (**D**). Scale bars, 20 µm (**A, B**) or 50 µm (**C-E**).

Very often, ectopic expression of Upd in the proximal territory or overexpression of the *wild type* JAK kinase (Hop) in the P compartment, led to the generation of ectopic wing structures emerging from the notum (Figure 32A, B, D, E white and red arrows). These results are reminiscent of the local reduction of Vn/EGFR activity in the notum, as expression of a chimeric protein between Vn and the secreted EGFR antagonist Argos (Vn::Aos) also induced ectopic wing structures in the same location [Figure 32C (Wang et al., 2000)]. Collectively, our results indicate that the expression of Upd and the activity of JAK/STAT during second instar correlates with a function in the process of wing versus notum subdivision. Our gain- and loss-of-function experiments show that JAK/STAT has an early role in repressing EGFR-regulated genes to restrict the notum fate to the most proximal region of the wing primordium and therefore ensuring Wg-mediated appendage specification.

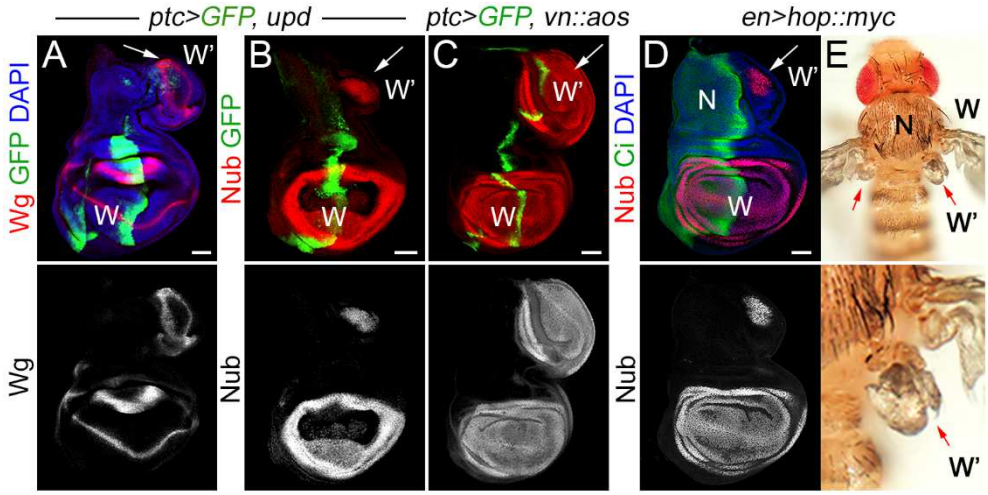


Figure 32. Targeted activation of JAK/STAT generates ectopic wings emerging from the notum. (A-E) Third instar wing primordia (**A-D**) or adult fly (**E**) from larvae expressing the indicated transgenes under the control of the *ptc-gal4* (**A-C**) or *en-gal4* drivers (**D, E**), labeled to visualize Wg protein (red or white, **A**), DAPI (blue, **A, D**), Nub (red or white, **B-D**), Ci (green, **D**), and GFP (green, **A-C**) to mark the domain of transgene expression. In **A-E**, wing territories (W), endogenous nota (N) and ectopic wing territories (W', white arrows, **A-D**; red arrows, **E**) are marked. Targeted expression of the Upd ligand (**A, B**) or the Hop kinase (**D, E**) frequently leads to ectopic wing structures emerging from the notum that phenocopy the local loss of EGFR signalling in the notum (**C,** see also Material and Methods). Scale bars, 50 µm.

Non-autonomous effects of JAK/STAT deregulation

We observed that RNAi-mediated knockdown of any element of the JAK/STAT pathway (with *dome*^{RNAi}, *hop*^{RNAi} and *stat*^{RNAi}) in the anterior (A) compartment (with either *ci-gal4* or *ptc-gal4* drivers) caused the non-autonomous induction of ectopic wing structures emerging from the adjacent posterior (P) notum (Figure 33A-C, E). The same non-autonomous effect was observed in haltere primordia (Figure 33D). These observations suggest that in this experimental condition a wing-inducer signal is produced and released from the JAK/STAT-depleted A compartment, which non-autonomously triggers wing development in the adjacent P territory of the notum.

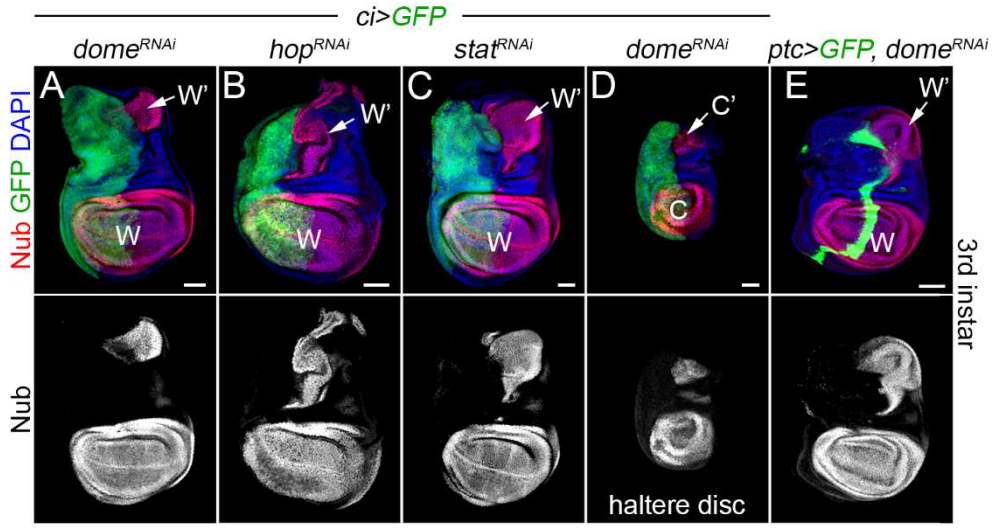


Figure 33. Depletion of JAK/STAT in the A compartment non-autonomously triggers wing development in the adjacent P compartment. (**A-E**) Late third instar wing (**A-C, E**) or haltere primordia (**D**) expressing the indicated transgenes in the anterior (A) compartment under the control of *ci-gal4* (**A-D**) or *ptc-gal4* (**E**), and labelled to visualize wing territories by Nub (red or white), DAPI (blue) and GFP (green) to visualize the domain of transgene expression. Endogenous wing (W), endogenous haltere capitellum (C), ectopic wing fields (W', white arrows, **A-C, E**) and ectopic haltere capitellum (C', white arrow, **D**) are marked. Ectopic wing fields and capitellum arise in the P compartment, adjacent to the transgene-expressing territory. Scale bars, 50 µm.

It has been shown that JAK/STAT represses Wg in the legs and antennae imaginal discs and that its downregulation in these tissues causes the ectopic expression of Wg (Ayala-Camargo et al., 2007; Ekas et al., 2006). Since Wg is absolutely required for wing fate specification and sufficient to trigger wing development *de novo* in ectopic locations (see Introduction), we wondered whether Wg was ectopically expressed upon JAK/STAT depletion in the A compartment and if so, whether it might be responsible for the non-autonomous induction of ectopic wings in the P notum. We were not able however, to detect neither an increase nor changes in the dynamic pattern of Wg protein from second to early third instar (Figure 34A-C), the developmental stage at which the endogenous wing is specified and presumably, the stage when the entire wing primordia is competent to develop ectopic wings in response to Wg (Ng et al., 1996). To ensure that our temporal analysis of the dynamic pattern of Wg was not missing a cryptic source of Wg, we performed a functional experiment to confirm that Wg was not being ectopically expressed upon JAK/STAT downregulation in the A compartment. Depletion of JAK/STAT in a subset of A cells abutting the AP compartment boundary (with the *ptc-gal4* driver) causes the non-autonomous induction of wing structures (Figure 33E and 34D). However, co-expression of a wg^{RNAi} transgene in the same domain did not rescue this non-autonomous effect (Figure 34E), indicating that the potential autonomous production of Wg upon JAK/STAT depletion is not responsible for the non-autonomous induction of supernumerary wings.

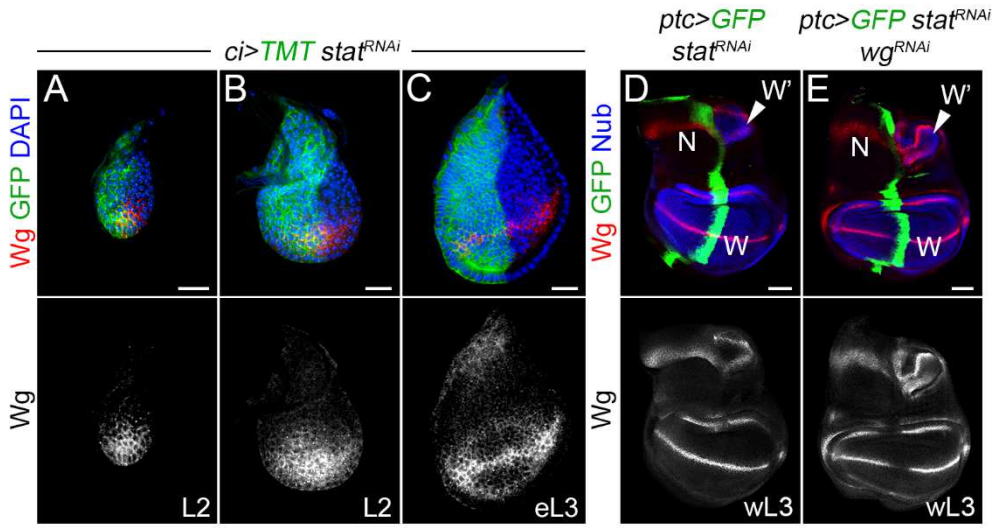


Figure 34. JAK/STAT depletion in the A compartment does not cause ectopic Wg expression. (**A-E**) Second (L2, **A, B**), early third (eL3, **C**) and late third instar (wL3, **D, E**) wing primordia from larvae expressing the indicated transgenes under the control of the *ci-gal4* (**A-C**) *or ptc-gal4* (**D, E**) drivers, labeled to visualize wing territories by Nub (blue, **D, E**), Wg protein (red or white) and myrTomato (TMT, green, **A-C**) or GFP (**D, E**) to visualize the domain of transgene expression. In **D, E** the endogenous wing (W), endogenous nota (N) and ectopic wing fields (W', white arrowheads) are marked. The second instar wing disc in **A** is slightly younger than in **B**. Notice in **D** that the ectopic wing field (W') emerges non-autonomously and adjacent to domain expressing the *stat*^{RNAi} transgene, and that coexpression of *wg*^{RNAi} in **E** downregulates Wg expression levels in the same domain but does not rescue the ectopic wings caused by *stat*^{RNAi}. Scale bars, 20 µm (**A-C**), or 50 µm (**D, E**).

Interestingly, we noticed that impairing JAK/STAT signalling in the A compartment of the wing primordium caused a non-autonomous increase in the levels of the *10xSTAT-GFP* activity reporter (Figure 35A, B, red arrow), and a non-autonomous reduction in the expression levels of *mirror* (Figure 35C, red arrow). Moreover, expression of a truncated form of the Domeless receptor (Dome^{DN}), which lacks the intracellular activator domain but is potentially able to trap the ligand Upd, did not phenocopy the non-autonomous effects of RNAi-mediated depletion of JAK/STAT (Figure 36C).

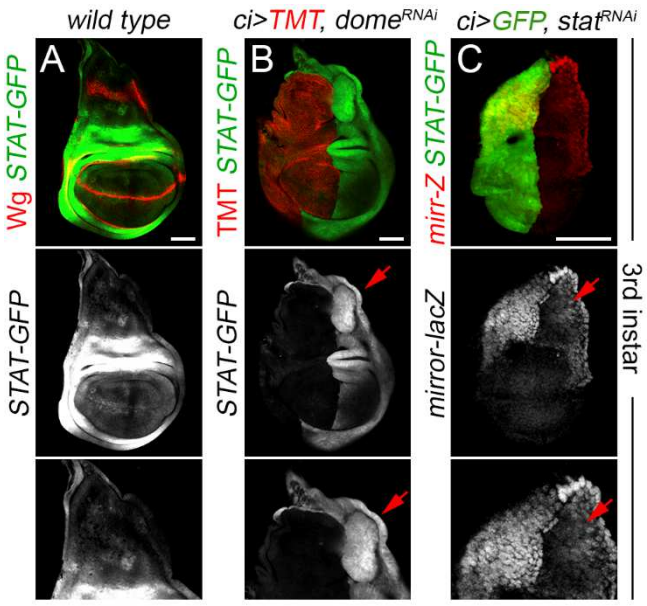


Figure 35. Autonomous JAK/STAT inhibition causes non-autonomous effects. (**A-C**) Late (**A, B**) and early third instar (**C**) wing primordia from *wild type* larvae (**A**) and from larvae expressing *dome*^{RNAi} (**B**) or *stat92E*^{RNAi} (**C**) under the control of the *ci-gal4* driver and labeled to visualize the activity of JAK/STAT pathway (*10xSTAT-GFP,* green or white, **A, B**), Wg (red, **A**), *Myristoylated-Tomato* (TMT, red, **B**), *mirror* (*mirr-lacZ*, antibody against β-gal, red or white, **C**), and GFP (green, **C**). Red arrows in **B** point to non-autonomous ectopic activation of *10xSTAT-GFP* and in **C** to non-autonomous reduction of *mirror* expression levels. Scale bars, 50 µm.

JAK/STAT restricts the expression of its own ligand Upd

Collectively, these observations together with the finding that Upd or Hop missexpression are sufficient to generate ectopic wings in the P notum, prompted us to consider whether the Upd ligand might be either ectopically expressed or its levels increased upon targeted depletion of the JAK/STAT pathway in the A compartment. To test this idea, we performed a functional experiment to test whether the ectopic wings observed upon RNAi-mediated JAK/STAT depletion were Upd-dependent. Additionally, we monitored *upd* expression in JAK/STAT loss-of-function situations. Remarkably, expression of Dome^{DN} was able to fully rescue the non-autonomous induction of wing structures caused by targeted expression of *dome*^{RNAi} in the A compartment

(Figure 36A, C). Note that *dome*^{RNAi} is not expected to affect the expression levels of the *dome*^{DN} transgene as this RNAi targets the mRNA region encoding for the C-terminal intracellular domain (see Material and Methods). This result reinforces our proposal that the ectopic wings depend on Upd since this phenotype is suppressed in the presence of Dome^{DN}, likely by its ability to trap the ligand and limit its spreading towards the adjacent P compartment.

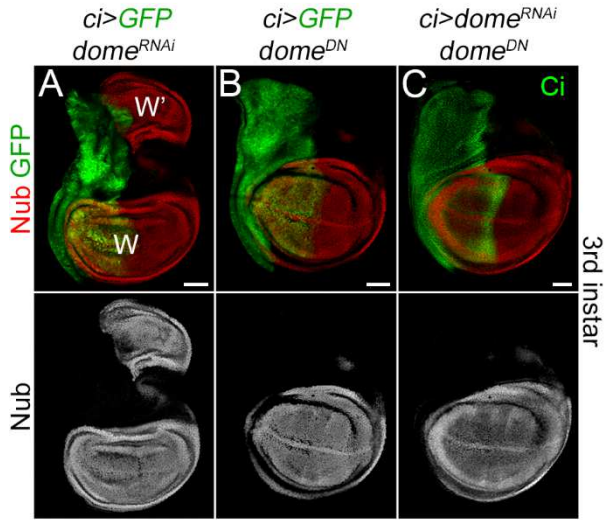


Figure 36. Non-autonomous induction of ectopic wings upon JAK/STAT inhibition is Upd-dependent. (**A**-**C**) Late third instar wing primordia from larvae expressing the indicated transgenes under the control of the *ci-gal4* driver, labeled to visualize wing territories by Nub (red or white, **A-C**) Ci (green, **C**), and GFP (green, **A, B**) or to mark the domain of transgene expression. In **A**, the endogenous (W) and ectopic wing fields (W') are marked. The ectopic wing field (W') emerges non-autonomously and adjacent to domain expressing GFP and *dome*^{RNAi} transgenes, and that Dome^{DN} in **B** does not induce ectopic wings by itself but is able to fully rescue the ectopic wings caused by *dome*^{RNAi} (compare **A** and **C**). Scale bars, 50 µm.

We therefore analysed throughout development the expression of *upd* by *in situ* hybridization in *wild type* and in wing discs with compromised JAK/STAT signalling in the A compartment. *upd* is expressed in a distal domain of the second instar wing primordium (Figure 25A, B), and once the wing has been specified, it is repressed from the nascent wing pouch by Nub (Ayala-Camargo et al., 2013). During third instar, *upd* expression evolves into its

characteristic five-spot pattern located in the hinge and the ventral pleura (Figure 37, top). We found that depletion of JAK/STAT in the A compartment clearly impaired the restriction of *upd* to these five dots and *upd* expression levels were visibly increased (arrows in Figure 37, bottom). Consistent with this observation, clones of cells homozygous mutant for a null *stat92E* allele expressed *upd* in the wing pouch of early third instar wing discs (Figure 38). Whether this *upd* results from ectopic and the novo transcription of the *upd* locus or a failure to repress the distal expression of *upd* during second instar remains unknown. We also observed that JAK/STAT depletion did not cause the ectopic expression of *upd* in the body wall of early instar (Figure 38). Taken together, these observations support the notion that the negative feedback loop between JAK/STAT signalling and its own ligand contributes to restrict the expression levels and pattern of Upd to the maturing wing hinge and that a failure to do so interferes with the wing vs body wall subdivision.

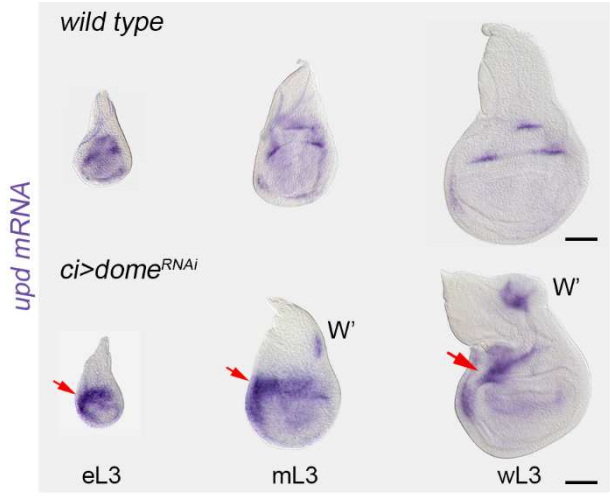


Figure 37. JAK/STAT restricts the expression of its own ligand Upd. Wing primordia from early (eL3), mid (mL3) and late wandering (wL3) third instar stages of the indicated genotypes and labeled to visualize *upd* mRNA by *in situ* hybridization (purple). Note that *upd* expression pattern fails to resolve into its characteristic five-spot pattern and accumulates at higher levels in the anterior hinge (red arrows) upon JAK/STAT depletion. Endogenous wing (W) and ectopic wing territories (W') are marked. Scale bars, 50 µm.

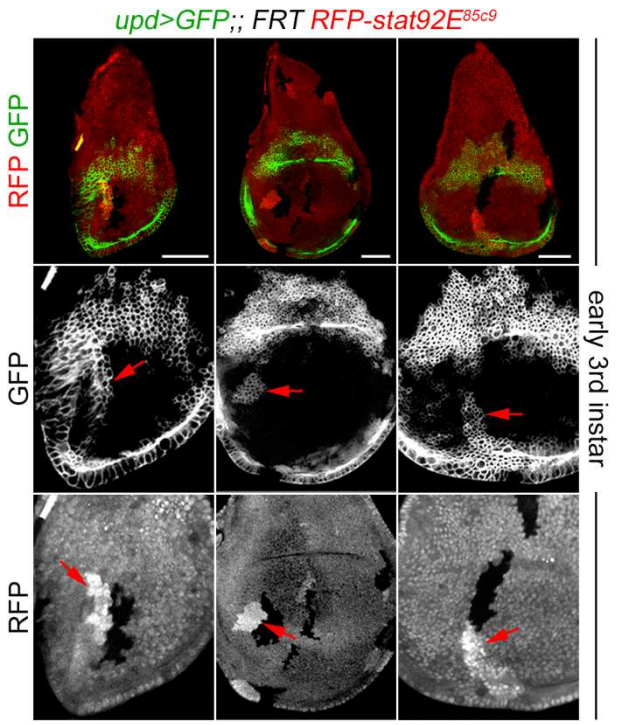


Figure 38. *stat92E* mutant cells fail to repress *upd*. Three distinct examples of early third instar wing primordia with clones of cells lacking *stat92E* activity generated at the first instar and marked by the presence of two copies of RFP. Note that clones express *upd* (monitored by *upd-gal4, UAS-myrGFP*, green or white, red arrows). Scale bars, 50 µm.

Upstream regulators of *upd* and JAK/STAT activity

During second instar, *upd* expression is restricted to the distal part of the wing primordium, in a broader domain that Wg and opposite to the proximal Vn/EGFR territory (Figure 25B and 39A). This observation prompted us to analyse whether the early expression pattern of *upd* or the activity of the pathway relies on either positive or negative inputs from Wg or Vn/EGFR, respectively. We therefore modulated the Wg and EGFR pathways and monitored the early expression of *upd* and the *10xSTAT-GFP* activity reporter. However, the distal localization of *upd* and the activity of JAK/STAT during second instar were both largely unaffected in a *wg* transheterozygous mutant background that abolishes *wg* expression specifically during second instar (wg^{CX3}/wg^{CX4}, Figure 39B, D, compare with 39A, C). Similarly,

overexpression of a constitutively active EGF receptor (*EGFR*^{CA}) or its target gene *araucan* in the whole wing primordium did not modified the activity of the JAK/STAT pathway during second instar (Figure 39E, F, compare with 39C). Collectively, these results indicate that neither Wg nor Vn/EGFR are the upstream regulators of *upd* and JAK/STAT activity at this stage. Thus, it remains to be elucidated the mechanism by which the early expression of *upd* is restricted to the distal wing primordium.

During the third instar stage, JAK/STAT is downregulated from the developing wing pouch as the ligand Upd retracts to five dots in the hinge and ventral pleura (Figure 37). These events have been correlated with the expansion of the presumptive wing pouch (Hatini et al., 2013) in which Nubbin represses distal *upd* transcription and attenuates JAK/STAT signalling in the pouch (Ayala-Camargo et al., 2013). We see a decrease in the levels of the *10xSTAT-GFP* reporter in the presumptive body wall during second instar, and we show that this is necessary to ensure the correct development of this territory since maintaining high levels of STAT92E activity interferes with notum development. However, it is unclear whether the graded activity of the pathway is exclusively the result of the decay in the spreading of Upd from the distal domain or whether a pro-notum factor also collaborates to downregulate JAK/STAT activity from the body wall to allow its expansion (Hatini et al., 2013).

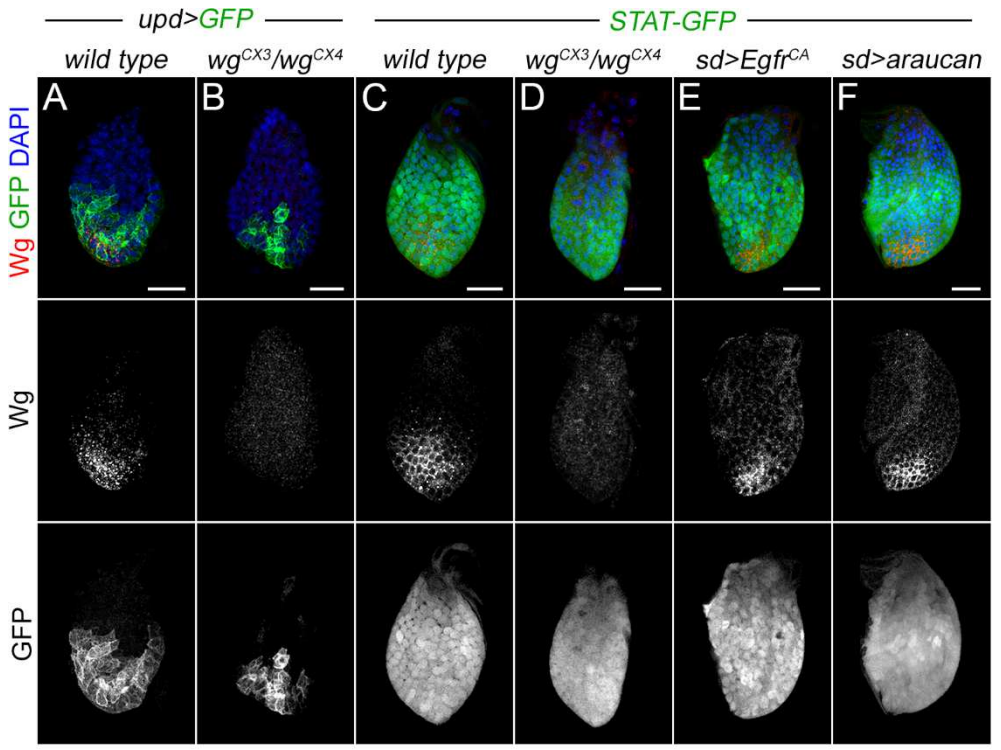


Figure 39. The early expression and activity of JAK/STAT signalling components does not depend on Wg or EGFR. Second instar wing primordia from wild type (**A, C**), *wg* trans-heterozygous mutants (**B, D**) or from larvae expressing a constitutively active EGF receptor ($Egfr^{CA}$, **E**) or its target gene *araucan* (**F**) under the control of the *sd-gal4* driver (**E, F**), labelled to visualize *upd* expression (in *upd-gal4, UAS-myrGFP* larvae, green or white, **A, B**), *STAT-GFP* activity reporter (green or white, **C-F**), Wg protein (red or white) and DAPI (blue). Scale bars, 20 µm.

Following this idea, we compromised Wg signalling (with a *UAS-notum* transgene) or overexpressed the pro-notum gene *araucan* in the whole wing primordium from very early stages and monitored JAK/STAT activity in the resulting mature third instar wing discs. In both cases, the loss of the wing was accompanied by a symmetric duplication of body wall structures as previously published [Figure 40A, C, D, see Introduction (Barrios et al., 2015; Morata and Lawrence, 1977; Sharma and Chopra, 1976; Wang et al., 2000)]. In these mature primordia, we observed a clear downregulation of the *10xSTAT-GFP* reporter from the developing ectopic nota (N', Figure 40A, C, D). We observed variable levels of JAK/STAT activity in these discs but always restricted to a central region, in between the endogenous and the

duplicated nota. This might be either a remnant activity from a residual source of *upd*, the activity of the pathway in the pleura or even vestiges of the hinge tegula [Figure 40A, C, D, see (Bryant, 1975; Morata and Lawrence, 1977) for fate maps and detailed anatomical characterization of the notum duplications]. In contrast, ectopic expression of Wg was not able to increase the levels of JAK/STAT activity (Figure 40B).These observations reinforce the idea that the expansion of the notum field is largely incompatible with high levels of JAK/STAT in this territory. Therefore, in our experimentally induced ectopic nota, the loss of JAK/STAT activity in maturing third instar discs is probably a consequence of the misspecification of the wing and concomitant duplication of body wall structures.

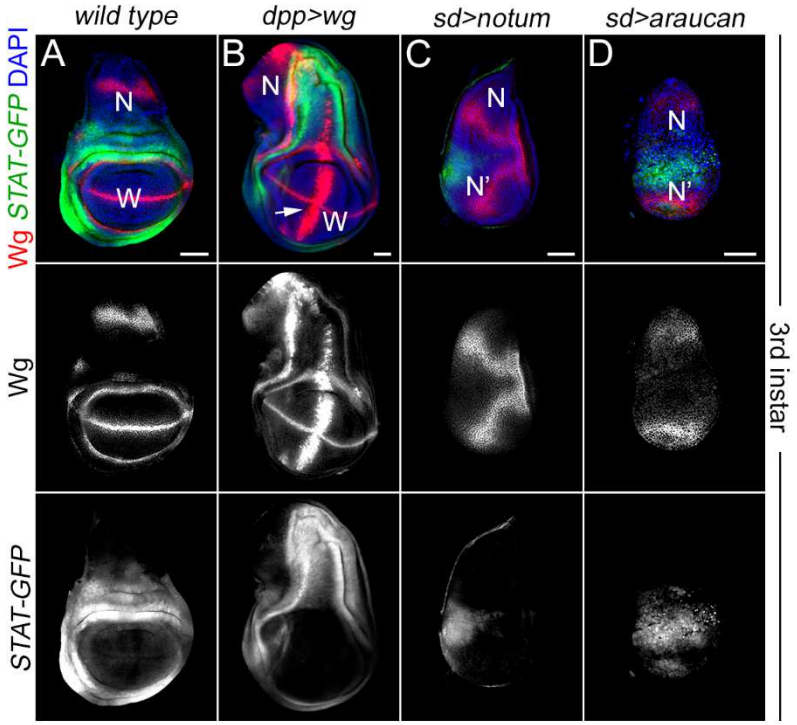


Figure 40. Late effects of Wg and EGFR deregulation on JAK/STAT activity. (**A-D**) Third instar wing primordia from *wild type* (**A**) or from larvae expressing the indicated transgenes in a stripe along the AP compartment boundary (*dpp-gal4*, **B**) or in the whole wing primordium (*sd-gal4,* **C, D**) to modulate the Wg signalling pathway (**B, C**) or the EGFR target *araucan* (**D**), and labelled to visualize *STAT-GFP* activity reporter (green or white), Wg protein (red or white) and DAPI (blue). In **B**, the ectopic Wg protein is expressed at very high levels in a stripe (white arrow) perpendicular to the endogenous Wg. Endogenous wing (W), endogenous nota (N) and ectopic nota (N') are indicated. Scale bars, 50 µm.

JAK/STAT and the control of overall organ size

JAK/STAT activity in the wing territory throughout development

Expression of Upd evolves as wing development proceeds as the ligand becomes restricted to the presumptive wing hinge and pleura, and consequently the *10xSTAT-GFP* reporter is robustly activated in these territories [Figure 35A and 40A, (Ayala-Camargo et al., 2013; Hatini et al., 2013; Johnstone et al., 2013; Rodrigues et al., 2012)]. Interestingly, we observed that mild activation of the *10xSTAT-GFP* reporter also occurs in the whole wing field at later stages and that this expression depends on the activity of the JAK/STAT pathway, as depletion of the Upd receptor Domeless induced a clear cell-autonomous downregulation of the reporter (Figure 41A, B). These observations prompted us to analyse whether the JAK/STAT pathway might have a broader developmental role during limb development, besides its reported activity in defining and promoting wing hinge growth (Ayala-Camargo et al., 2013; Johnstone et al., 2013). For this purpose, and in order to bypass the earlier requirement of JAK/STAT signalling in wing fate specification, we used genetic tools with a milder effect on the pathway.

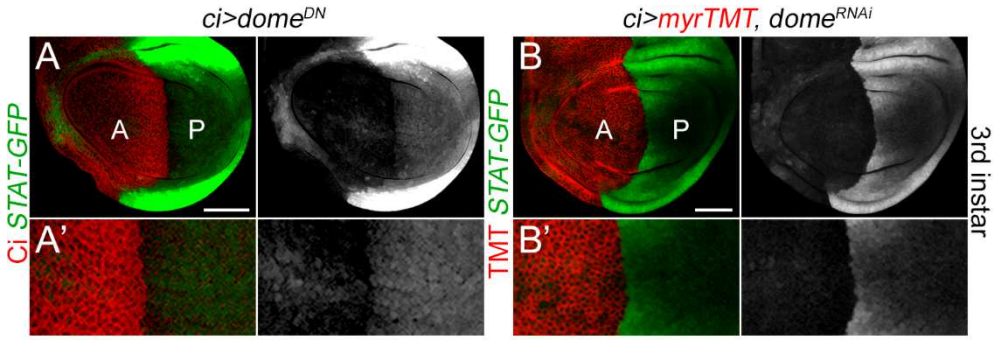


Figure 41. JAK/STAT is active in the wing pouch at later developmental stages. (**A, B**) Third instar wing primordia expressing *dome*^{DN} (**A**) or *dome*^{RNAi} (**B**) transgenes in the anterior compartment under the control of the *ci-gal4* driver, labeled with Ci (red, **A, A'**) or myrTomato (TMT, red, **B, B'**) to visualize the domain of transgene expression, and STAT-GFP reporter to monitor JAK/STAT activity (red or white, **A, A', B, B'**). STAT-GFP is autonomously downregulated in the transgene-expressing anterior compartment. The anterior (A) and posterior (P) compartments are indicated in the channel overlay (**A, B**). Scale bars, 50 µm.

JAK/STAT maintains the size of the P compartment

Expression of Dome^{DN} in the *sd-gal4* domain gave rise to a clear growth defect (Figure 42B, compare with 42A) and, most interesting, the size of the P compartment was clearly reduced (Figure 42B, white arrow). In some cases, the P compartment was virtually lost, giving rise to a stronger decrease in the size of the wing pouch, now composed entirely by A cells (Figure 42C, white arrow). The small-disc phenotype is characteristic of *hop* mutants but the anterior versus posterior compartment size has not been addressed in this case (Mukherjee et al., 2005; Perrimon and Mahowald, 1986). We therefore analysed whether the reduction in P compartment size observed in Dome^{DN}-expressing primordia was reproducible in null but otherwise larval-viable JAK mutants. The *hop^{27}* mutation is a chemically-induced amorphic allele on the X chromosome in which embryonic and larval lethality is rescued by maternal contribution until the pupal stages. In this genetic background, the wing disc was smaller and most important, the P compartment was strongly reduced in size (Figure 42E, white arrow).

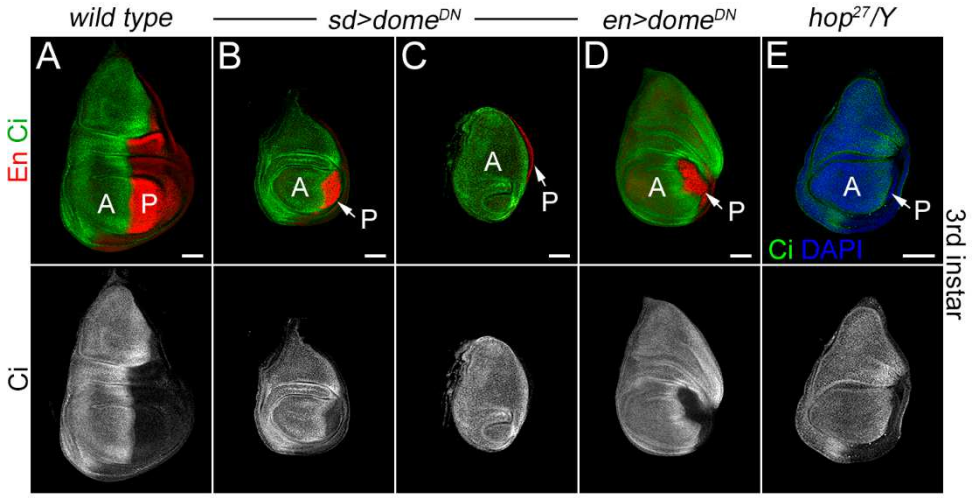


Figure 42. JAK/STAT is required to maintain the size of the posterior compartment. (**A-E**) Third instar *wild type* (**A**), *hop^{27}* hemizygous mutant (**E**) or larvae expressing the Dome^{DN} receptor (**B-D**) in the whole wing primordium under the control of the *sd-gal4* (**B, C**) or in the P compartment under the control of *en-gal4 driver* (**D**), labelled to visualize En protein (red, **A-D**), DAPI (blue, **E**) or Ci (green or white). Scale bars, 50 µm.

While depletion of Dome, Hop or Stat92E in A cells (with the *ci-gal4* or *ptc-gal4* drivers) did not have any noticeable impact on the size of this territory (Figs. 33A-C, 35B, 36 and 41), expression of Dome^{DN} as well as RNAi forms against *dome*, *hop* and *stat92E* in P cells (with the *en-gal4* driver) caused a strong reduction on the size of the P compartment (white arrow in Figure 42D and 43A-D). A similar phenotype was observed in wing discs expressing *dome^{RNAi}* in the whole wing disc using the *sd-gal4* driver (Figure 43E).

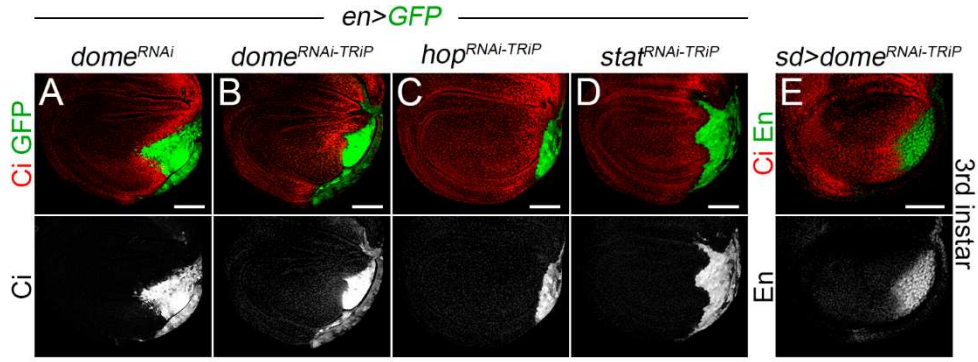


Figure 43. Canonical JAK/STAT is required to maintain the size of the posterior compartment. (**A-E**) Third instar wing primordia from larvae expressing the indicated transgenes under the control of the *en-gal4* (**A-D**) or *sd-gal4* (**E**) drivers, labelled to visualize the A compartment with Ci protein (red), En protein (green or white, **E**) and GFP (green or white, **A-D**). Scale bars, 50 µm.

To address whether JAK/STAT is also broadly required to maintain the size of the P compartment in other *Drosophila* appendages, we inhibited JAK/STAT activity in the A or P territories (with *ci-gal4* and *hh-gal4* drivers, respectively), and analysed the resulting mature leg and haltere discs. Expression of an RNAi transgene against *hop* in P cells caused strong undergrowth of the P compartment in the leg and haltere primordia (Figure 44A, B, D, E), while the A compartment was unaffected when the same RNAi was expressed in A cells of these tissues (Figure 44A, C, D, F).

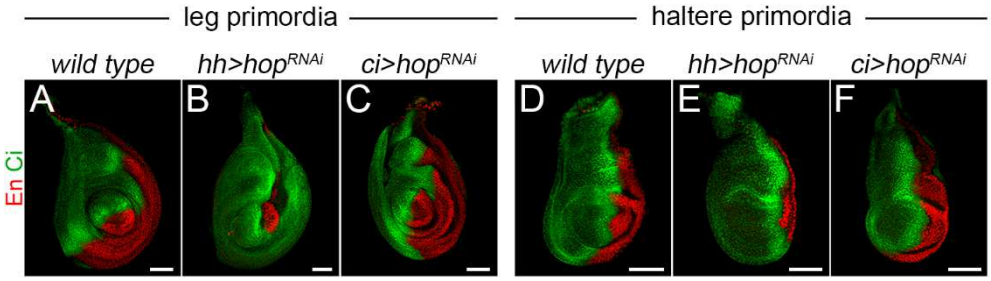


Figure 44. A general requirement of JAK/STAT in maintaining the P compartment of *Drosophila* appendages. (**A-F**) Mature larval leg (**A-C**) and haltere (**D-F**) discs from *wild type* larvae (**A, D**) or from larvae expressing hop^{RNAi} under the control of *hh-gal4* (**B**, **E**) or *ci-gal4* (**C**, **F**) labeled to visualize expression of Ci (green) and En (red) to mark the A and P compartments, respectively. Scale bars, 50 µm.

Interestingly, we observed that early L3 wing primordia, already shown a reduced P compartment (Figure 45A, C, quantified in 45F). To analyse whether JAK/STAT signalling is required to maintain the size of the P compartment throughout development, we used the thermosensitive version of the Gal4 repressor, $Gal80^{ts}$, to temporally control JAK/STAT depletion in P cells. At the permissive temperature (18 °C), the Gal4 transcriptional activator is inactive due to the $Gal80^{ts}$ repressor. Once the temperature is raised to 29 °C, the $Gal80^{ts}$ protein becomes inactive and thus allowing Gal4-driven transgene expression. We noticed that the reduction in the size of the P compartment observed in early third wing primordia (72 h after egg laying, AEL) grown at the restrictive temperature (29 °C) was restored when larvae were shifted to the permissive temperature (18 °C) from early third instar until the end of larval development (Figure 45B, D, E, quantified in 45F). These results indicate that JAK/STAT is required to maintain the size of the P compartment throughout development.

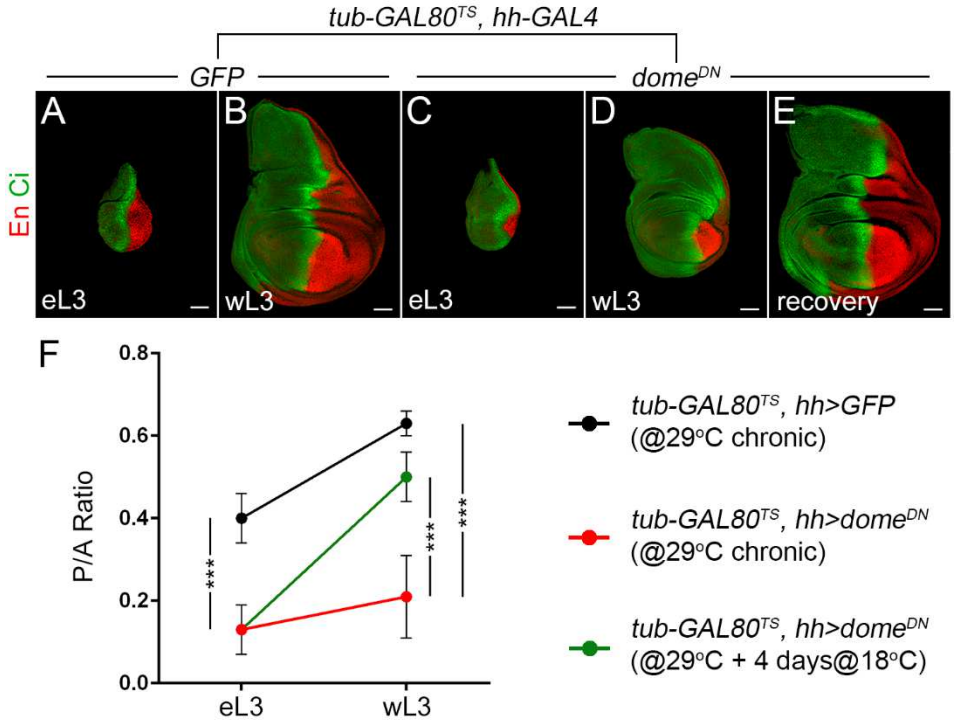


Figure 45. JAK/STAT is required throughout development to maintain the P compartment size. (**A-E**) Early (**A, C**) and late (**B, D, E**) third instar larval wing primordia expressing *GFP* (**A, B**) or *dome^{DN}* (**C-E**) transgenes under the control of the *hh-gal4* driver and labeled to visualize expression of Ci (green) and En (red). Larvae were raised at 29 ºC until dissection in early L3 (eL3, **A, C**) or late third instar stages (wL3, **B, D**). To address the capacity of the wing disc to recover, larvae were raised at 29 ºC until early third instar, transferred to 18 ºC at this stage and dissected 4 days later in late third instar (recovery, **E,** see also Material and Methods). Scale bars, 50 µm. (**F**) Graphic representation of the average size of the P versus de A compartment (P/A ratio) of the wing primordia represented in **A-E**. Error bars represent standard deviation. ***p<0,001. Number of wing discs (n>17). See also **Table 2.**

To further characterize the requirement of JAK/STAT in the maintenance of P compartment size, we analysed the size and distribution of clones of cells mutant for a *stat92E* null allele (*stat92E^{85c9}*) using the classic FLP/FRT-mediated mitotic recombination technique. With this method, single cells homozygous mutant for the gene of interest are randomly generated all over the tissue, which then grow and proliferate to form groups of cells related by lineage called clones. Since cells mutant for JAK/STAT are eliminated through cell competition [(Rodrigues et al., 2012), a process by which slow-growing cells are detected and removed through apoptosis by

fast-growing cells (Levayer and Moreno, 2013)], we gave the *stat92E*^{85c9} mutant cells a relative growth advantage using the FLP/FRT *Minute* technique to impair growth of the surrounding non-mutant cells. In a *Minute/+* heterozygous background, *wild type (+/+)* clones were similarly recovered in both the A and P compartment (Figure 46A), and the average percentage of each compartment covered by these clones was largely similar (Figure 46C). In contrast, a low number of *stat92E*^{85c9} mutant cells were recovered in the P compartment (Figure 46B). The average percentage of each compartment covered by *stat92E*^{85c9} mutant clones was smaller when compared to *wild type* clones, and this difference was highest in the case of the P compartment (Figure 46C).

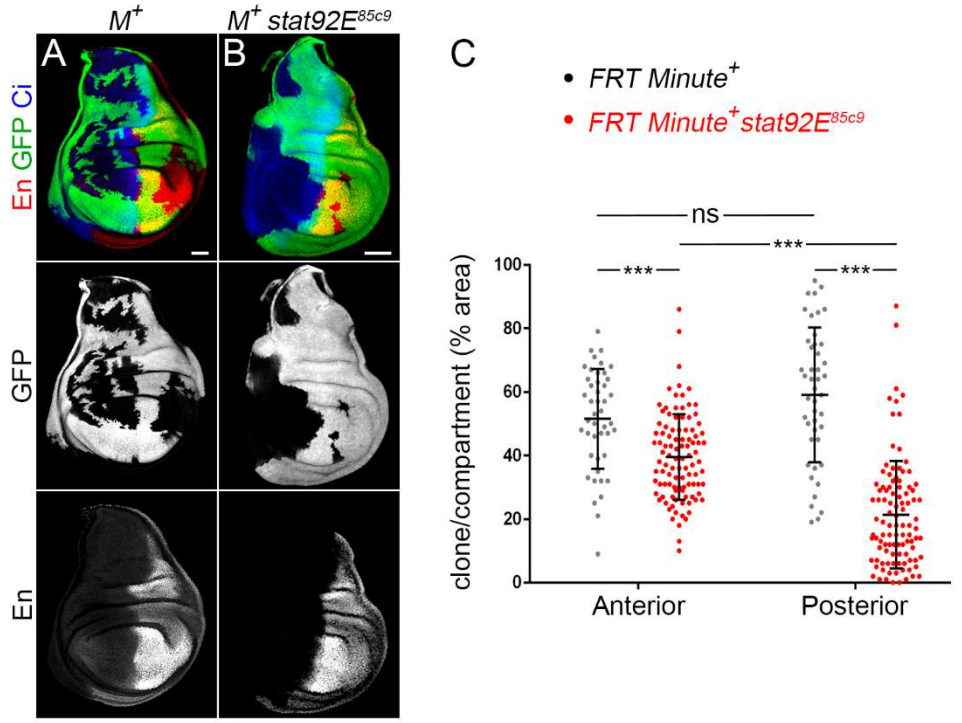


Figure 46. *stat92E* mutant clones are poorly recovered in the P compartment. (**A, B**) Examples of mature wing primordia with *wild type* clones (**A**) or clones mutant for *stat92E* (**B**), labeled by the absence of GFP (green or white), Ci (blue or white), En (red or white), and induced at first instar (see Material and Methods). Scale bars, 50 µm. (**C**) Scatter plot showing the percentage of the A or P compartments covered by *stat92E* mutant (red) or *wild type* (grey) clones. Error bars represent standard deviation. ns = not significant; ***p<0,001. Each dot represents a wing disc. Number of wing discs (n>45). See also **Table 3.**

Analysis of cell identity in JAK/STAT loss-of-function

The asymmetric recovery of *stat92E*^{85c9} mutant cells was not caused by changes in identity between A and P cells, as P mutant cells continued to express the P-selector gene Engrailed (Figure 46B and 47A). Neither was it a consequence of cells crossing from the P to the A compartment, since clones mutant for *stat92E*^{85c9} born in the P territory respected the AP compartment boundary (Figure 47A). In addition, we carried out a lineage tracing experiment to irreversibly label all cells born in the P compartment and expressing Dome^{DN} using the G-TRACE technique (Evans et al., 2009). Although a small number of cells with a P compartment origin crossed to the A compartment under these circumstances (Figure 47B), this violation does not appear to explain the observed reduction in the size of the P compartment. In this context, it is interesting to note that the cellular behaviour observed is reminiscent of the boundary transgressions that take place in regenerating wing discs upon transient induction of pro-apoptotic genes (Herrera and Morata, 2014), thereby suggesting that reduced survival cues might explain the size reduction of the P compartment (see below).

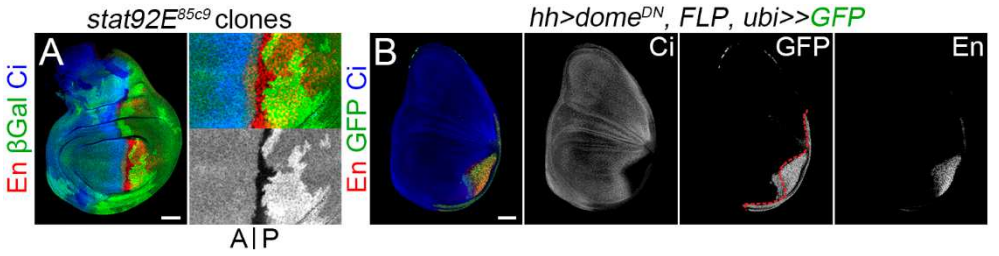


Figure 47. *stat92E* mutant clones respect the AP compartment boundary and do not switch from P to A identity. (**A**) Representative examples of mature larval wing primordia with clones of cells homozygous mutant for *stat92E*, labeled by the absence of *lacZ* expression (antibody against β-gal, green or white). Wing disc was stained for En (red) and Ci (blue). Clones were induced at first instar (see Material and Methods). Mutant clones and their corresponding twins (labeled with two copies of *lacZ*) can be monitored. (**B**) G-TRACE mediated cell lineage analysis to irreversibly label all cells born in the P compartment upon JAK/STAT depletion in P cells. Wing disc was stained for Engrailed (En, red or white), Ci (blue and white) and GFP (green or white). Dashed red line marks the AP boundary. Note that some cells of P origin (GFP) have lost their P identity (Engrailed) and belong now to the A compartment (Ci). Scale bars, 50 µm.

One important difference between the body wall and wing territories is the marked asymmetry in their respective P and A compartment sizes. In the notum, the P territory is very small relative to the A compartment, while in the wing pouch both the A and P compartments are of comparable sizes. Since we showed that JAK/STAT is involved in the wing versus notum subdivision during second instar, we wondered whether the specific reduction in the P compartment of the wing was caused by transformation of the P wing to the P notum, thus acquiring the intrinsic growth program of the P notum and decreasing its size. However, this reduction was not due to the transformation of the posterior wing to the posterior notum, as the characteristic expression pattern of wing and body wall markers (Nub and Tsh, respectively) was unaffected in *hop*^{27} hemizygous mutant animals (Figure 48A) and in Dome^{DN}-expressing wing discs (Figure 48B).

The wing and haltere primordia originate from dorsally-located imaginal discs in the embryo and are considered homologous organs, being the haltere a derived state from ancestral four-winged insects. Their differences rely on the specific expression and activity of the homeobox selector gene *Ubx* in haltere cells, which acts as a master switch that triggers haltere developmental program. If by mutation or experiment, *Ubx* function is removed from the haltere discs, this tissue fully transforms into a wing (Lewis, 1978). Conversely, ectopic expression of the Ubx transcription factor in the wing disc causes a wing-to-haltere transdetermination (Cabrera et al., 1985; White and Akam, 1985). The Ubx transcription factor is involved in several aspects of haltere development, among them, is the reduced size of the P compartment relative to the A due to low Dpp signalling in P haltere cells. Therefore, if Ubx was being ectopically expressed in the P compartment of our JAK/STAT depleted wing discs, this territory would acquire haltere-like characteristics with the subsequent decrease of the P compartment size. However, we did not detect ectopic Ubx protein neither in the A nor the P

compartments of Dome^{DN}-expressing discs besides its normal expression in the cuboidal marginal cells present at the disc posterior edge that joints the disc proper (DP) to the Ubx-expressing peripodial epithelium (Figure 48C). All together, these results reveal a cell-autonomous and compartment-specific requirement of JAK/STAT signalling in promoting the growth, proliferation, and/or cell survival of P cells during development.

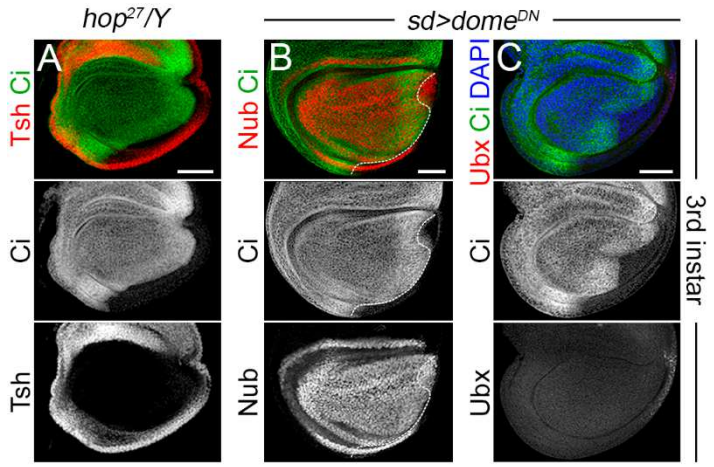


Figure 48. JAK/STAT depleted wing cells neither acquire notum identity nor transdetermine to haltere fate. (**A-C**) Third instar wing primordia from *hop^{27}* mutants (**A**) or from larvae expressing *Dome^{DN}* under the control of *sd-gal4* (**B, C**), labelled to visualize the body wall marker Tsh (red or white, **A**), the wing marker Nub (red or white, **B**), the haltere identity marker Ubx (red or white, **C**), DAPI (blue, **B**) and Ci to mark the A compartment (green or white). White dashed line in **B** marks the AP compartment boundary. Scale bars, 50 µm.

JAK/STAT promotes the cycling and survival of P cells

Based on the cell autonomous requirement of JAK/STAT signalling in P cells, we monitored the activity of the major growth promoting pathways, the expression of a collection of cell cycle markers and the activity of the apoptotic pathway in P wing cells upon depletion of the JAK/STAT pathway. Growth of the developing wing relies, among others, on the activity of the Dpp morphogen expressed along the AP compartment boundary, on the

activity of the Hippo/Yorkie signalling pathway, and on the expression levels of the proto-oncogene *dMyc* (Hariharan, 2015; Neto-Silva et al., 2009). The activity levels of the Dpp and Hippo/Yorkie signalling pathways were unaffected in P cells expressing Dome^{DN}, as monitored by the expression of Spalt [a target of Dpp (de Celis et al., 1996)] and the Hippo readout *expanded* [a target of Yorkie (Yu et al., 2010)], respectively (Figure 49A, B). The levels of dMyc protein were also unaffected in Dome^{DN}-expressing cells (Figure 49C).

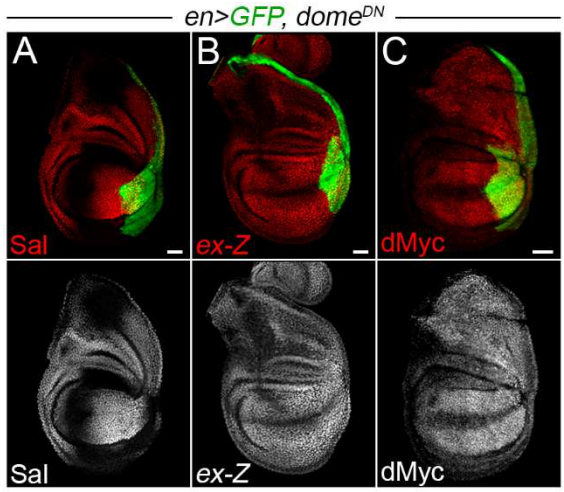


Figure 49. The reduction of P compartment size is independent of Dpp, Hippo and dMyc growth regulators. (**A-C**) Third instar wing primordia expressing the *dome^{DN}* transgene in the P compartment under the control of the *en-gal4* driver and labelled to visualize Dpp signalling with an antibody against Spalt (Sal, red or white, **A**), the Hippo readout *expanded-lacZ* (*ex-Z*, red or white, **B**), dMyc protein levels (red or white, **C**) and GFP (green) to mark the domain of transgene expression. Expression of these markers is unaffected in the P territory. Scale bars 50 µm.

These results are consistent with the fact that cells mutant for JAK/STAT are eliminated through cell competition in a Yorkie- and dMyc-independent manner (Rodrigues et al., 2012) and that *stat92E^{85c9}* mutant clones were hardly recovered in the P compartment in spite of being conferred a growth advantage with the *Minute* technique (Figure 46).

The amount of cell death during larval development is minor, except at the L2-L3 transition where there is a modest increase in dying cells and at the hinge-notum interface during late third instar (Milán et al., 1997). We thus monitored the activity of the apoptotic pathway. A TUNEL assay to label DNA strand breaks induced by apoptotic cell death revealed an increase in the number of apoptotic cells in the P compartment of *hop*^{27} mutant wing discs during development when compared to *wild type* controls (Figure 50A-D). The *Drosophila* inhibitor of apoptosis dIAP1 protects cells from apoptosis by inhibiting active caspases (Ryoo et al., 2002; Wilson et al., 2002), and STAT92E, when activated, has been previously shown to directly regulate dIAP1 expression in imaginal discs (Betz et al., 2008). Consistent with this report, overexpression of the JAK kinase Hop led to a cell-autonomous increase in the expression levels of a *diap1* enhancer-trap (Figure 50E, F). However, the role of JAK/STAT signalling in maintaining the physiological levels of dIAP1 expression appeared to be specific to the P compartment, as depletion of JAK/STAT signalling gave rise to a clear reduction in *diap1-lacZ* and dIAP1 protein levels in P cells but not in A cells (Figure 50G, I, J compare with 50E, H).

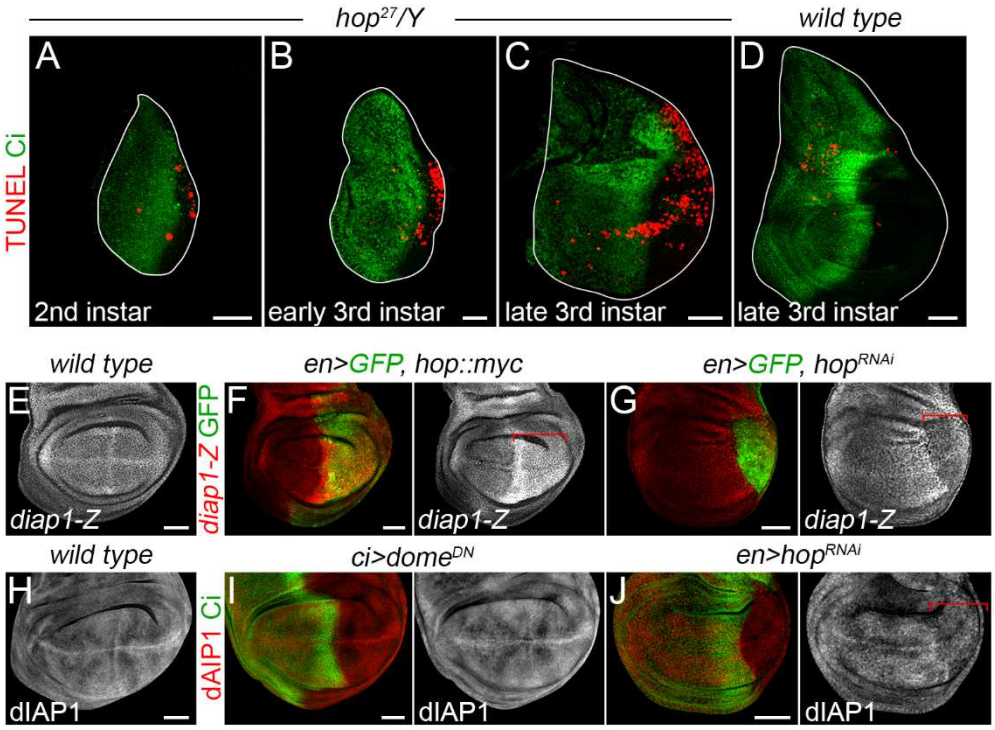


Figure 50. JAK/STAT regulates diap1 and protects P cells from apoptosis. (**A-D**) Wing primordia from *hop*^{27} mutant (**A-C**) or from *wild type* (**D**) larvae dissected at second (**A**), early third (**B**), or late third (**C, D**) instars, and labeled to visualize apoptotic cells by TUNEL staining (red) and Ci (green) to label the A compartment. A white line marks the contour of the wing discs. (**E-J**) Late third instar wing primordia from larvae of the indicated genotypes labeled to visualize expression of the *diap1-lacZ* enhancer trap (*diap1-Z*, red or white, **E-G**) and dIAP1 protein levels (red or white, **H-J**). Wing discs were labeled with either Ci (green, **I, J**) or GFP (green, **F, G**), to mark the A and P compartments, respectively. Red brackets mark the domain of transgene expression. Scale bars, 20 µm (**A, B**) or 50 µm (**C-J**).

We then inhibited cell death by expressing of a collection of *UAS-transgenes* that target different elements of the apoptotic machinery, thus impairing the initiation or execution of the apoptotic pathway. Interestingly, blocking apoptosis by overexpression of dIAP1 or an RNAi form against the initiator caspase Dronc, rescued the reduction in the size of the P compartment caused by Dome^{DN} expression (Figure 51A-C, quantified in 51E, F) as well as the amount of cell death observed (Figure 52A-C). A similar rescue of the size of the P compartment was observed upon expression of the baculovirus

protein P35, which blocks the activity of the effector caspases Dcp1 and drICE (Figure 51A, D, quantified in 51G).

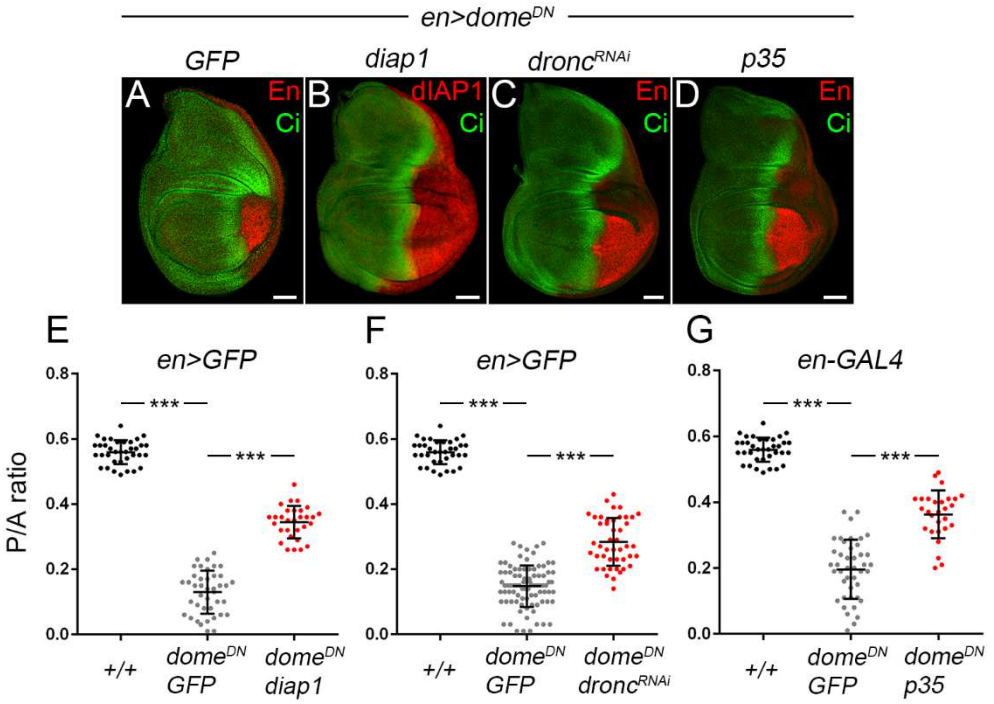


Figure 51. Blocking apoptosis rescues the size of the P compartment. (**A-D**) Late third instar wing primordia from larvae expressing the indicated transgenes in the P compartment under the control of the *en-gal4* driver, and labelled to visualize dIAP1 protein (red, **B**), En (red, **A, C, D**) and Ci (green). Scale bars, 50 µm. (**E-G**) Scatter plots showing the size of the P versus the A compartment (P/A ratio) of wing primordia expressing the indicated transgenes under the control of the *en-gal4* driver. Error bars represent standard deviation. ***p<0.001. Number of wing discs in **E** (n>30), **F** (n>36), **G** (n>28). See also **Tables 4-6.**

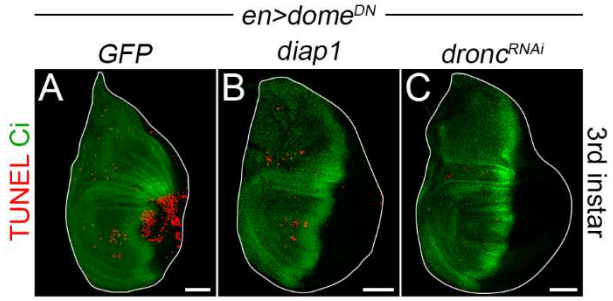


Figure 52. Expression of *diap1* or *dronc*^{RNAi} rescues the apoptosis of the P compartment. (**A-C**) Late third instar wing primordia expressing the indicated transgenes in the P compartment under the control of the *en-gal4* driver, and labelled to visualize apoptotic cell death by TUNEL (red) and Ci protein (green) to mark the A compartment. Scale bars 50 µm.

Previous studies have found that clonal overexpression of the *wild type* kinase Hop in the wing imaginal disc upregulates CycB protein levels (Mukherjee et al., 2005). We then analysed the expression levels of the G1/S rate-limiting Cyclin E (CycE) and the G2/M rate-limiting Cyclins A and B (CycA and CycB) in P cells expressing $Dome^{DN}$. The expression levels of CycE were not affected (Figure 53E). In contrast, CycA and B levels were visibly reduced in P cells expressing $Dome^{DN}$ (Figure 53A, B, F). Expression of $Dome^{DN}$ in A cells did not cause any overt reduction in the levels of these two G2 cyclins (Figure 53C, G). Interestingly, overexpression of CycA was able to largely rescue the reduction in the size of the P compartment caused by $Dome^{DN}$ expression (Figure 53D). This observation therefore indicates that the downregulation of this G2 cyclin is partially responsible for the reduction in the size of the P compartment caused by loss of JAK/STAT signalling. Surprisingly, CycB overexpression did not rescue the size reduction of this compartment (Figure 53H). All together, these results indicate that JAK/STAT signalling maintains the size of the P compartment by regulating the cycling and survival of these cells. This pathway does so by regulating the levels of the G2/M rate-limiting CycA and the inhibitor of apoptosis dIAP1.

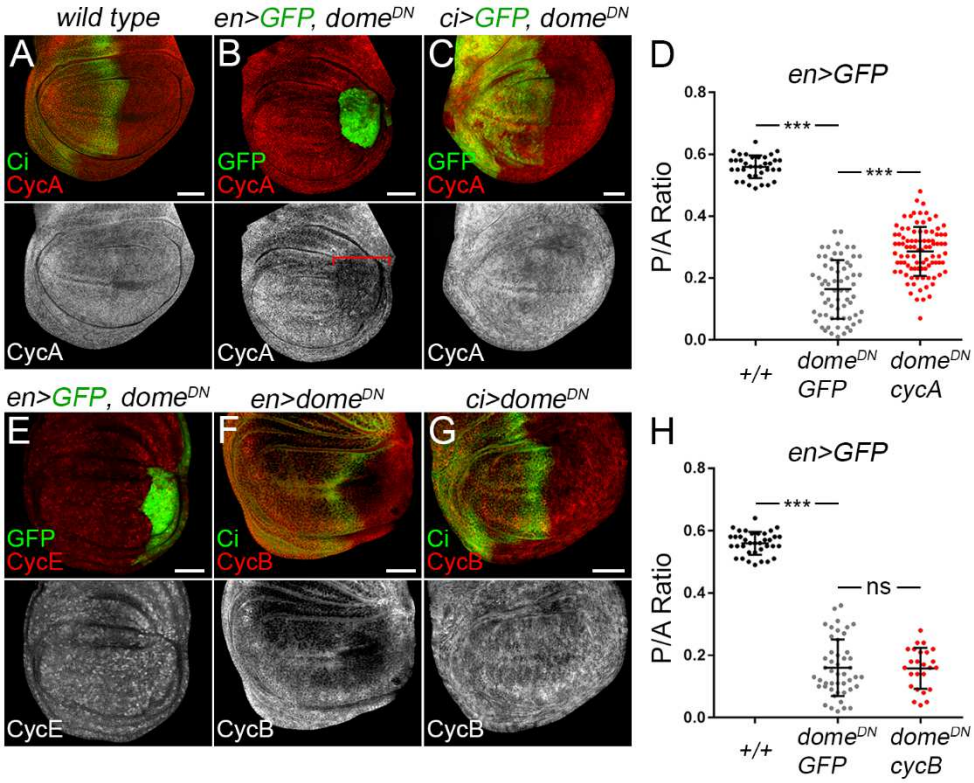


Figure 53. Impaired cell cycling of P cells contributes to the P compartment reduction. (**A-C**) Late third instar wing primordia from *wild type* (**A**) or from larvae expressing Dome^{DN} (**B, C**) under the control of *en-gal4* (**B**) or *ci-gal4* (**C**) drivers, labelled to visualize Ci protein (green, **A**), GFP (green, **B, C**) and CycA protein levels (red or white). Red bracket in **B** marks the domain of transgene expression and CycA downregulation. (**E-G**) Late third instar wing primordia from larvae expressing the Dome^{DN} under the control of *en-gal4* (**E, F**) or *ci-gal4* (**G**) drivers, labelled to visualize CycE (red or white, **E**), GFP (green, **E**), CycB (red or white, **F, G**) and Ci (green**, F, G**). Scale bars, 50 µm. (**D, H**) Scatter plots showing the P/A ratio of wing primordia expressing the indicated transgenes under the control of the *en-gal4* driver. Error bars represent standard deviation. ***p<0,001. Number of wing discs in **D** (n>66), **H** (n>25). See also **Tables 7, 8.**

JAK/STAT counteracts the negative effects of Engrailed on cell cycling and survival

Stable subdivision of the wing primordium into A and P compartments is a consequence of asymmetric signalling by Hedgehog (Hh) from P to A cells (Lawrence, 1997). The activity of the homeodomain protein En in P cells helps to generate this asymmetry by inducing expression of Hh in the P compartment and at the same time repressing the essential downstream component of the Hh pathway Cubitus interruptus [Ci, (Dominguez et al., 1996)]. Thus, only A cells that receive the Hh signal across the compartment boundary will respond by stabilizing Ci. We thus analysed whether the specific requirement of the P compartment for JAK/STAT to drive cell cycling and survival might be due to the (1) absence of Hh signalling or (2) the presence of En in these cells. In the first case, the combined activities of two signalling molecules (Upd cytokines and the Hh morphogen) might provide survival and proliferation cues to A cells, whereas in the second case, the sole action of Upd through the JAK/STAT signalling pathway might exert a similar action in P cells. The first hypothesis would explain why blocking JAK/STAT in the A compartment does not have any noticeable impact on the size of this territory, as the Hh pathway would compensate for the lack of STAT92E activity. However, depletion of Hh signalling together with JAK/STAT in A cells did not cause any obvious phenotype in terms of compartment size (Figure 54A). These observations indicate that the sensitivity of P cells to a decrease in JAK/STAT activity is not due to the lack of Hh signalling in these cells. We next addressed the alternative hypothesis and analysed whether JAK/STAT signalling counteracts the potential negative effects of the En transcriptional repressor in cell cycling and survival. In order to test this hypothesis we use different genetic tools to downregulate En protein levels in P cells simultaneously expressing $Dome^{DN}$.

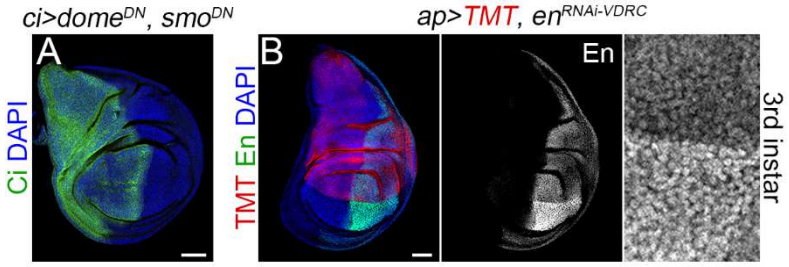


Figure 54. Simultaneous inhibition of JAK/STAT and Hh signalling does not cause undergrowth of the A compartment. (**A**) Coexpression of Dome^{DN} and Smo^{DN} in the A compartment under the control of *ci-gal4* driver and labelled for Ci (green) and DAPI (blue). (**B**) Expression of an *en^{RNAi}* transgene in the dorsal compartment (red) under the control of *apterous-gal4* (*ap-gal4*) driver, labelled for TMT (Tomato, red), En (green or white) and DAPI (blue). Magnification in the single channel shows the cell-autonomous downregulation of En protein levels in the dorsal-posterior compartment. Scale bars, 50 µm.

A reduction in the levels of En in P cells (Figure 54B), either by expression of two independent *en^{RNAi}* transgenes or by halving the doses of *en* and *invected* genes by a chromosomal deficiency (in *Df(2)en^{E}/+* individuals), consistently rescued the reduction in the size of the P compartment caused by Dome^{DN} expression (Figure 55A-C). The rescue was larger with the VDRC-RNAi line and milder with the TRiP-RNAi line (two independent and publically available *Drosophila* RNAi collections) or the chromosomal deficiency (compare Figure 55A with 55B,C).

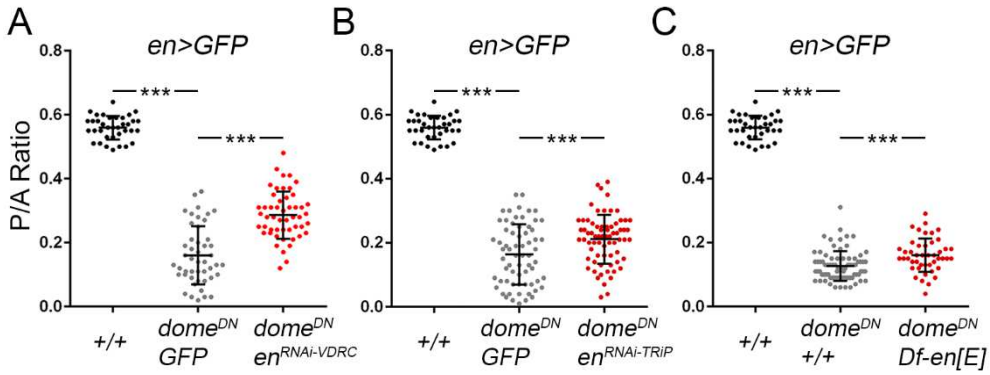


Figure 55. Depletion of En rescues the size of the P compartment. (A-C) Scatter plots showing the size of the P versus the A compartment (P/A ratio) of late third instar wing primordia expressing the indicated transgenes under the control of the *en-gal4* driver. Error bars represent standard deviation. ***p<0,001. Number of wing discs in **A** (n>47), **B** (n>66), **C** (n>45). See also **Tables 9-11.**

Most interesting, the reduction in CycA levels and the amount of cell death observed in JAK/STAT-depleted P compartments were both largely rescued upon expression of *en*^{RNAi} (Figure 56A-C, quantified in 56D). These results indicate that JAK/STAT protects P cells from Engrailed and that the reduced survival and impaired cell cycling of JAK/STAT depleted P-cells are likely the consequence of the negative effect of Engrailed on these processes.

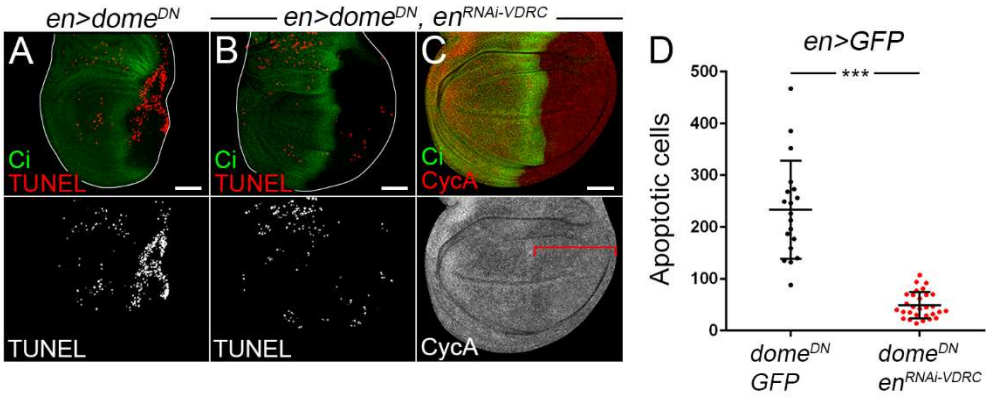


Figure 56. Depletion of En rescues apoptosis and CycA downregulation in P cells. (**A-C**) Wing primordia from third instar larvae expressing the indicated transgenes under the control of the *en-gal4* driver, and labelled to visualize apoptotic cell death by TUNEL staining (red or white, **A, B**), CycA (red or white, **C**) and Ci (green). Red bracket in **C** marks the domain of transgene expression and the rescue in CycA downregulation. Scale bars, 50 µm. (**D**) Scatter plot showing the absolute number of apoptotic cells in the P compartment of late third instar wing primordia from individuals of the indicated genotypes. ***p<0,001. Number of wing discs (n>19). See also **Table 12.**

The *en-gal4* driver used to knockdown *engrailed* function is an enhancer trap construct inserted in the *en* locus that faithfully recapitulates its endogenous expression pattern. Thus, *en-gal4* likely integrates all or most of the regulatory inputs on the *en* gene. Besides, engrailed is subject to multiple modes of regulation in embryogenesis and imaginal discs involving both positive and negative autoregulation by its own En product (Garaulet et al., 2008; Guillén et al., 1995; Heemskerk et al., 1991). Thus, we considered whether the rescue in tissue size, apoptosis and CycA protein levels caused by expression of *en*^{RNAi} might be an indirect consequence of a reduction in

the expression of the *en-gal4* driver, which would lead to lower levels of $Dome^{DN}$ expression and consequently to an apparent rescue of the mentioned parameters. To address this question we drove en^{RNAi} expression under the control of *en-gal4* and monitored the expression levels of this driver. If anything, the levels of this driver increased, monitored by an *UAS-GFP* transgene and antibody to the Gal4 protein (Figure 57A, B, quantified in 57C). This observation is consistent with the reported capacity of En to negatively regulate its own expression in the wing disc that was proposed to be used to finely modulate physiological levels of En protein (Garaulet et al., 2008; Guillén et al., 1995; Simmonds et al., 1995; Tabata et al., 1995).

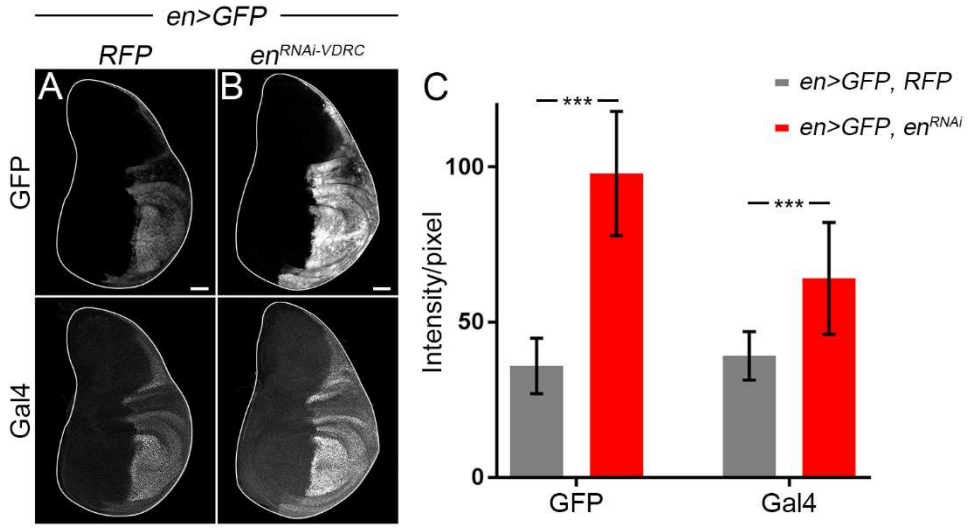


Figure 57. Physiological levels of Engrailed negatively regulate its own expression. (**A, B**) Late third instar wing primordia from larvae of the indicated genotypes labeled to visualize GFP or Gal4 (white) to quantify variations in the strength of the *en-gal4* driver. The white line marks the contour of the discs. All discs were processed and stained in parallel and images were captured with the same confocal settings. Scale bars, 50 µm (**C**) Bar graph showing the average signal intensity/pixel of GFP and Gal4 protein levels in the P compartments of the indicated genotypes. Error bars represent standard deviation. ***p<0,001. Number of wing discs (n>18). See also **Table 13.**

To further reinforce that Engrailed is responsible for the phenotypes observed upon JAK/STAT depletion in the P compartment we tested the capacity of Engrailed to phenocopy the JAK/STAT loss-of-function phenotypes. Temporary-regulated overexpression of En in its domain gave rise to a clear reduction in dIAP1 and CycA protein levels, caused apoptotic cell death and more important, reduced the size of the P compartment (Figure 58A-E, quantification in 58F). All together, these results indicate that JAK/STAT signalling counteracts the negative impact of Engrailed on the cycling and survival of P cells.

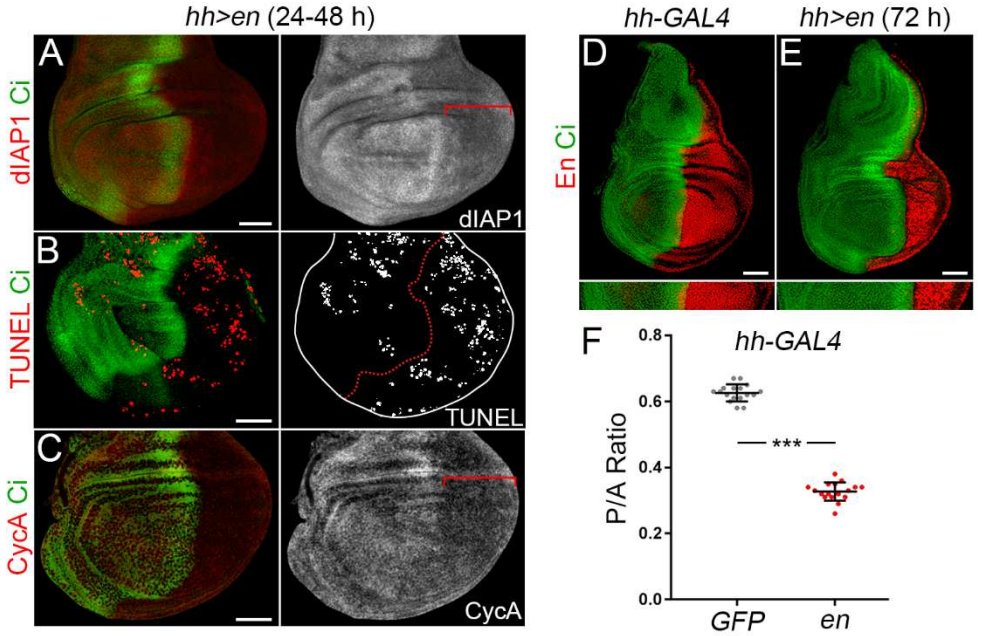


Figure 58. Overexpression of En in the P compartment phenocopies the loss of JAK/STAT in this territory. (**A-E**) Late third instar wing primordia from larvae overexpressing En (**A-C, E**) or GFP (**D**) in the P compartment for 24-48 h (**A-C**) or 72 h (**D, E**) under the control of the *hh-gal4* driver, labelled to visualize dIAP1 protein (red or white, **A**), apoptotic cells by TUNEL (red or white, **B**), CycA protein (red or white, **C**), En (red, **D, E**) and Ci (green) protein. In **A, C,** a red bracket marks the domain of transgene expression where dIAP1 and CycA are downregulated. In **B,** the red dashed line marks the AP boundary. Scale bars 50 µm. (**F**) Scatter plot showing the P/A ratio of wing primordia expressing the indicated transgenes under the control of the *hh-gal4* driver. Error bars represent standard deviation. ***p<0,001. Number of wing discs (n=17). See also **Table 14.**

JAK/STAT promotes the stable localization of the Dpp organizer

Hh from P cells induces the expression of Dpp in A cells abutting the P compartment, and Dpp organizes the growth and patterning of the developing appendage (Affolter and Basler, 2007). We thus analysed whether the strong reduction in the pool of Hh-expressing cells caused by Dome^{DN} expression had any impact on Dpp expression and, consequently, on wing growth. As noted above, two distinct growth phenotypes could be observed in wing discs expressing Dome^{DN} in the *sd-gal4* domain (Figure 42B, C). In most cases, a mild but reproducible growth defect accompanied by a clear size reduction of the P compartment was observed (Figure 42B), and in all these cases the expression of Dpp and its target gene Spalt was maintained (Figure 59B, compare with 59A). However, a certain fraction of wing discs showed a complete loss of the P compartment, accompanied by a strong reduction in the size of the wing pouch (Figure 42C). These features resemble the tissue size defects observed in *dpp* mutant wing discs (Zecca et al., 1995). Consistent with the reduction in the number of Hh-producing cells, the stripe of Dpp expression and its downstream target gene Spalt were lost in these cases (Figure 59C, compare with 59A).

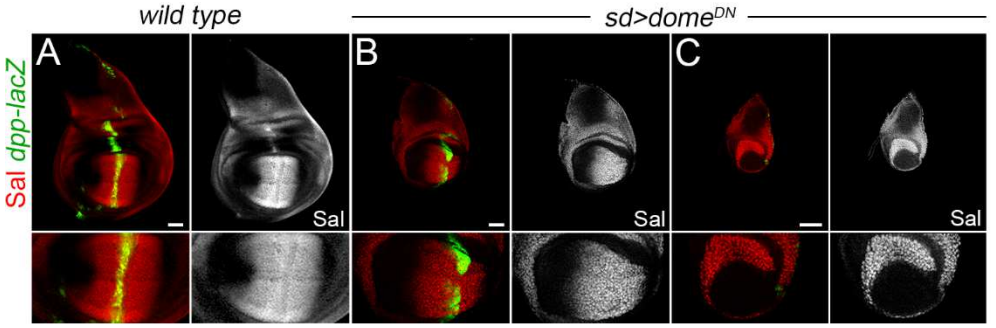


Figure 59. The loss of the P compartment abolishes *dpp* expression at the AP boundary. (**A-C**) Late third instar wing primordia from wild type (**A**) or from larvae expressing Dome^{DN} in the whole wing disc under the control of the sd-gal4 driver (**B, C**), labelled to visualize dpp expression (dpp-lacZ, green) and its target gene Spalt (Sal, red or white). Note in **C** the loss of *dpp-lacZ* and Sal protein accompanied by a strong reduction of the wing pouch (magnification). Scale bars, 50 µm.

These two distinct phenotypes could also be obtained upon expression of Dome^{DN} in the P compartment (Figure 42D and 60A). Remarkably, the fraction of wing discs with loss of Dpp activity, visualized by the expression of Spalt, was clearly reduced upon overexpression of Hh or dIAP1 in the P compartment (compare Figure 60A and B, quantified in 60C). Thus, the pro-survival and mitogenic activity of JAK/STAT signalling in P cells contributes to the maintenance of a pool of Hh-producing cells that induces Dpp expression in nearby A cells to generate a well-sized and fully-functional limb primordia.

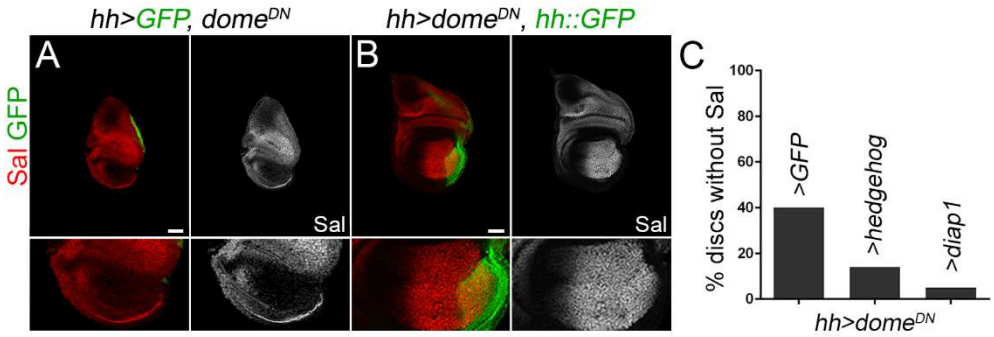


Figure 60. JAK/STAT promotes Dpp expression by maintaining the pool of Hh-expressing cells. (**A, B**) Late third instar wing primordia from larvae expressing Dome^{DN} (**A**) or coexpressing Dome^{DN} and Hh (using a Hh::GFP tagged protein, **B**) in the P compartment under the control of the *hh-gal4* driver labelled to visualize GFP (green, **A**), Hh::GFP protein (green, **B**) and Spalt (Sal, red or white). Scale bars, 50 µm. (**C**) Bar graph plotting the percentage of wing primordia in which Dpp was lost, monitored by the absence of Spalt expression in the wing pouch. Wing primordia are expressing the indicated transgenes under the control of the *hh-gal4* driver. Number of wing discs (n>40). See also **Table 15.**

Interplay between growth and patterning

JAK/STAT restricts Dpp organizing activity to the developing appendage

As development proceeds, high levels of expression and activity of the Upd ligand and the JAK/STAT pathway in the hinge primordium (Figure 61A, B) contribute to its growth (Ayala-Camargo et al., 2013; Hatini et al., 2013; Johnstone et al., 2013). Consistently, expression of Dome^{DN} in the A compartment gave rise to a clear reduction in the anterior hinge size and to the close apposition of the developing appendage and the surrounding body wall or notum (Figure 61C, D, red brackets). Moreover, Dpp is expressed along the AP boundary straddling the wing pouch, the hinge and the body wall, but it exerts its organizing activities only in the wing appendage (Figure 62A).

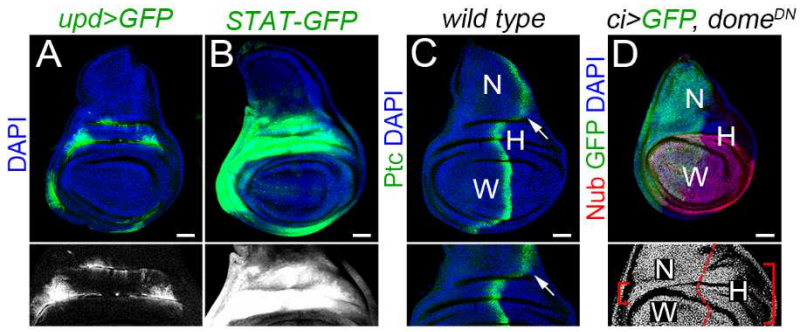


Figure 61. Expression and activity of JAK/STAT and Dpp signalling components in the hinge and body wall. (**A-C**) Late third instar wing primordia from larvae of the indicated genotypes labelled to visualize expression of *upd* (*upd-gal4, UAS-myrGFP,* green or white, **A**), activity of the JAK/STAT pathway (*10xSTAT-GFP*, green or white, **B**) and Ptc (green, **C**) to mark the AP compartment boundary. Magnifications of the hinge region where JAK/STAT signalling is highly active are shown in the lower panels of **A** and **B**. White arrow marks the AP boundary of the body wall in **C**. (**D**) Depletion of JAK/STAT in the A compartment compromises hinge growth. Wing disc is labeled with GFP (green) to mark the A compartment, Nubbin (Nub, red) to visualize the wing pouch and the distal hinge and DAPI (blue). Red brackets in the lower panel show the affected hinge region (compare left and right bracket). Dashed red line marks the AP boundary. Wing (W), notum (N) and hinge (H) territories are marked in **C, D.** Scale bars, 50 µm.

We thus wondered whether the hinge region acts as a fence that contributes to isolating the organizing activity of Dpp to the developing appendage. Interestingly, we noticed that when Dome^{DN} was expressed in the hinge (with *homothorax-gal4* driver, *hth-gal4*), the AP boundary of the body wall became closer to the developing wing (compare Fig 61C and Figure 62B, white arrows). Consequently, this Dpp stripe behaved as a new AP organizer which induced its target gene Spalt in nearby wing cells (compare Figure 62A with 62C, white star) as well as the expansion and concomitant pattern duplications of the endogenous wing (Figure 62D, white star).

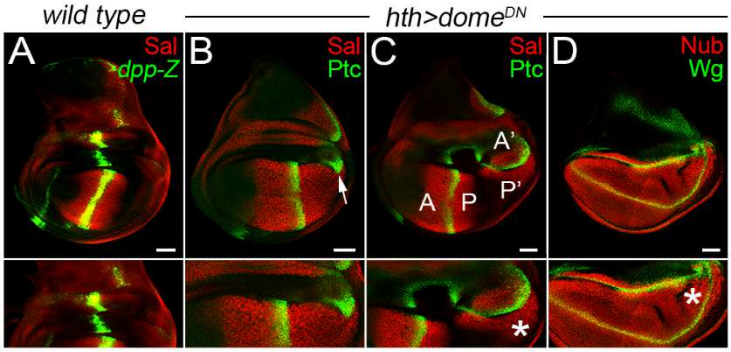


Figure 62. JAK/STAT-mediated hinge growth isolates the body wall and appendage sources of Dpp. (**A-D**) Late third instar wing primordia from *wild type* (**A**) or from larvae expressing Dome^{DN} (**B-D**) in the hinge territory under the control of the *homothorax-gal4* driver (*hth-gal4*). Wing discs are labelled to visualize the Dpp-expressing cells at the AP boundary with *dpp-lacZ* (*dpp-Z*, green, **A**) or Patched (Ptc, green, **B, C**), the wing territory with Nub (red, **D**), Wg (green, **D**) and Dpp activity with Spalt (Sal, red, **A-C**). White arrow in **B** points to the AP boundary of the body wall abutting the wing pouch because of the reduction in hinge size. As a result, the AP boundary organizes the surrounding tissue and induces pattern duplications of the endogenous wing, indicated by the additional A' and P' territories at both sides of the new organizer. Stars in **C** and **D** mark the ectopic expression of Spalt (**C**) and the resulting outgrowth marked by Nub (**D**). Scale bars, 50 µm.

Although all the animals from this experiment eventually died at the pupal stage, we were able to recover a certain number of pharate adults to analyse this phenotype in the adult wings. Consistent with the phenotypes observed

in the wing primordia, we frequently observed pattern duplications in the P compartment of the wing (red stars in Figure 63B, C, compare with 63A). Very often, we observed duplications of the P longitudinal veins L5 and L4 (Figure 63B, C) as well as the presence of A structures such as the anterior wing margin (awm) or anterior hinge elements like the costa (Co) (Figure 63B). Collectively, these results indicate that JAK/STAT signalling contributes, through its growth promoting activity in the hinge region, to isolate the body wall and appendage sources of Dpp, thus restricting the organizing activity of Dpp to the developing appendage.

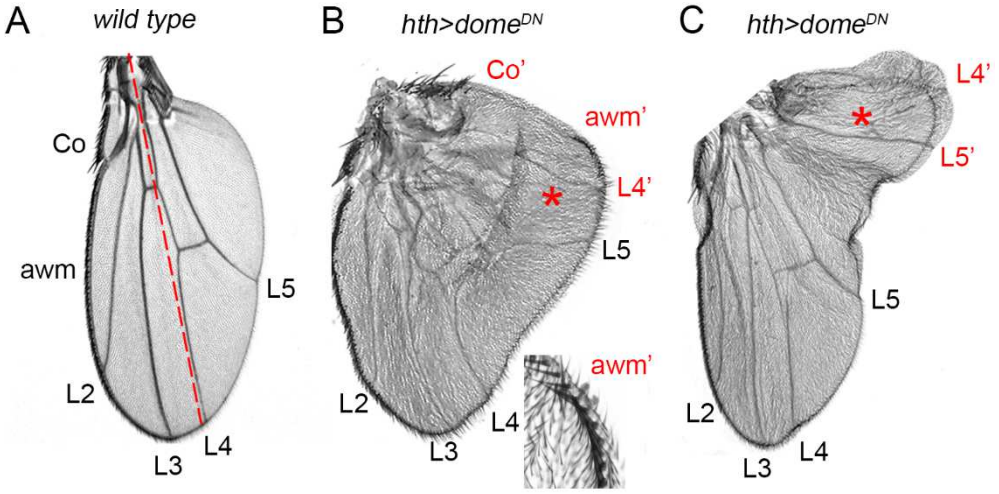


Figure 63. Depletion of JAK/STAT in the hinge generates pattern duplications in the P compartment. Cuticle preparations of adult wings from *wild type* individuals (**A**) or from individuals expressing Dome^{DN} under the control of the *hth-gal4* driver (**B, C**). Red dashed line in **A** marks the approximate AP compartment boundary. L2-L5, longitudinal veins; Co, costa; awm, anterior wing margin. The wings of *hth>dome^{DN}* individuals present pattern duplications of the P structures (eg. L4', L5´, red), and in these duplications A structures (awm' and Co', red) are often observed. Higher magnification of the ectopic awm (awm') is shown in the inset of panel **B.**

Discussion

Morphogens of the Wnt/Wg, Shh/Hh and BMP/Dpp families regulate tissue growth and pattern formation in vertebrate and invertebrate limbs. During this thesis we studied the role of the JAK/STAT signaling pathway in the growth and patterning of the wing primordium. We unravel a fundamental role of the secreted Upd ligand and the JAK/STAT pathway in facilitating the activities of these three morphogens in exerting their fate- and growth-promoting activities in the Drosophila wing primordium

A new role for JAK/STAT in wing fate specification

Targeted inhibition of the JAK/STAT pathway in the whole wing primordium from very early stages results in the total or partial duplication of body wall structures accompanied by the loss or reduction of the wing. The notum duplications are reminiscent of the *wg* mutant phenotype (Sharma and Chopra, 1976), although in these mutants the resulting adults show an "all or none" response without intermediate states such as the partial duplication of the thorax and vestiges of the wing (Morata and Lawrence, 1977). In these hypomorphic mutants there are only two possible outcomes. During second instar, if Wg levels are sufficient to induce wing fate, the resulting adults show no phenotype and display fully-formed wings. In contrast, if Wg levels are too low at this critical developmental stage, the wing fails to specify causing the concomitant and total duplication of body wall structures. The absence of this binary response in our JAK/STAT-deficient wing discs indicates that the mechanism might be different than in the *wg* mutants. Indeed, we show that JAK/STAT does not regulate wing fate cell-autonomously, neither behaves it as Wingless-like molecule nor regulates it the expression of Wg during second instar, since the wing marker Nubbin and the expression of Wg itself are not modulated by JAK/STAT. It is known that overexpression of EGFR or its target gene *araucan* in the distal territory of the early wing primordium can produce either total or partial notum duplications, and in

these cases the wings are lost or reduced (Aldaz et al., 2003; Wang et al., 2000). Interestingly, we observe these two phenotypes in our JAK/STAT depleted wing discs. We found that JAK/STAT has a negative impact on EGFR-regulated genes, acting as a brake, to restrict the notum field to its normal position in the proximal territory and thus ensuring Wg-mediated wing specification. Consistently, the proximal confinement of these EGFR targets, and consequently the notum identity, is impaired in JAK/STAT depleted discs, causing the expansion of the notum to the distal territory taking over most or all of the wing primordium. In fact, reducing the dose of the *Egfr* gene or the whole *Iro-C* complex substantially rescued the frequency of duplicated nota. Therefore, the extent of the notum expansion at the critical moment when the wing has to be specified by Wg, likely dictates whether the notum duplication is complete or partial, and whether this duplication is accompanied or not by a reduced or vestigial wing. An important point that may clarify the differences between the notum duplications observed in the *wg* mutants and in the loss of JAK/STAT is the order of events. In a *wg* mutant, the wing fails to specify first, and as a consequence the distal territory acquires the notum fate as the ground state. In JAK/STAT depleted wing discs, the first event is the expansion of the notum field, and as a secondary effect the wing may or may not be specified.

Regulation of EGFR by JAK/STAT

We found that the EGFR targets *mirror* and *apterous* expand distally in JAK/STAT depleted wing discs. *mirror*, a member of the Iro-C complex contributes to specify the notum fate and apterous determines dorsal identity (Blair, 1993; del Corral et al., 1999; Diaz-Benjumea and Cohen, 1993; Milán et al., 2001). We also show that the initial expression of the EGFR ligand Vn, which is known to depend on transluminal Dpp transmission, is not affected in early JAK/STAT-depleted wing discs (Paul et al., 2013). However, after the

initial induction, *vn* expression is sustained by EGFR as a part of an autoregulatory loop (Paul et al., 2013; Wang et al., 2000). In agreement with JAK/STAT repressing EGFR-regulated genes, *vn* is also expanded in later JAK/STAT depleted second instar wing discs and its expression remains in mature wing primordia. We did not uncover, however, the molecular mechanisms underlying EGFR target gene repression by JAK/STAT signalling in second instar.

The *socs36E* gene is a STAT92E target encoding the SOCS36E protein (Suppressor Of Cytokine Signalling 36E) that forms a negative feedback loop to attenuate JAK/STAT signaling upon pathway activation (Karsten et al., 2002; Stec et al., 2013). In fact, the *10xSTAT-GFP* activity reporter in based on the STAT92E binding sites present in the first intron of *socs36E* (Bach et al., 2007). Moreover, SOCS36E has also been shown to inhibit the EGFR pathway in imaginal tissues (Almudi et al., 2009; Callus and Mathey-Prevot, 2002) as well as to limit EGFR-induced overgrowth in *Drosophila* epithelial transformation models (Herranz et al., 2012). If STAT92E inhibits EGFR activity through SOCS36, the loss of JAK/STAT would reduce SOCS36E levels and cause an EGFR upregulation. However, the crosstalk between JAK/STAT, SOCS36E and EGFR does not seem to be the mechanism, as depletion of SOCS36E in the whole wing primordium from very early stages does not impair wing specification neither causes notum duplications. Moreover, *socs36E* homozygous null mutants are viable and fertile, and only exhibit a mild increase in wing size (Almudi et al., 2009; Herranz et al., 2012). In addition to SOCS36E, SOCS44A has been shown to positively regulate EGFR signaling in the wing (Rawlings et al., 2004). However, this hypothesis seems less possible because *socs44A* has not been identified as a target of STAT92E (Rawlings et al., 2004) and it is not upregulated upon pathway stimulation (Bina et al., 2010).

It is possible that STAT92E inhibits directly each of the EGFR targets here analyzed, but this would require the independent negative regulation of *mirror, apterous* and *vein*. It is more likely that STAT92E confines EGFR-regulated genes and hence the notum identity through the regulation of a single downstream element of the EGFR cascade. For instance, STAT92E might repress (directly or indirectly) the downstream transcription factor of the EGFR pathway, Pointed [Pnt (Rebay, 2002; Scholz et al., 1993)]. In this scenario, JAK/STAT would restrict the availability of Pnt to the proximal regions of the early wing primordium, thus limiting the competence to respond to EGFR only in the proximal cells normally fated to become the notum. If so, downregulation of JAK/STAT signalling at early stages would cause ectopic expression of Pnt in more distal cells, now competent to respond to the Vn ligand coming from neighbouring proximal cells and leading to EGFR target gene expression in a more distal position. As a consequence, Vn would be expressed in these cells in response to the EGFR feedback loop, which would signal distally to recruit more cells into the expanding notum domain. The reiteration of this mechanism based on Pnt derepression and Vn/EGFR autoregulation is likely to result in the progressive recruitment of all or most of the wing disc cells to the notum fate. In favor of this hypothesis, expression of the PntP2 isoform that mediates the positive Vn/EGFR feedback loop starts broad and becomes restricted to the proximal territory in second instar wing discs (Paul et al., 2013). Whether the spatial confinement of PntP2 that limits productive Vn/EGFR signaling to the future body wall relies on JAK/STAT is an open question.

We also show that ectopic expression of the Upd ligand or autonomous activation of the pathway recapitulates the phenotypes associated with the loss of Vn/EGFR, such as the downregulation of *Iro-C* (*mirror*), the reduced notum size as well as ectopic wings emerging from the notum (Austin et al.,

2014; Simcox et al., 1996; Wang et al., 2000; Zecca and Struhl, 2002a, 2002b). We do not observe, however, the loss of *apterous* upon JAK/STAT stimulation, as the apterous-dependent DV boundary formation that bisects the wing into D and V compartments is normal in both the endogenous and ectopic wings. Considering that *apterous* is a low-threshold target of EGFR and that only null *vn* or strong *Egfr* alleles cause the loss of *apterous* expression (Wang et al., 2000), it is likely that ectopic expression of Upd can only moderately inhibit EGFR signaling, impairing notum identity and growth but not the establishment of the D compartment. In fact, *vn* hypomorphs, in which the notum is virtually lost or reduced to a stump, still have a D compartment (Wang et al., 2000; Zecca and Struhl, 2002a).

Non-autonomous effects of JAK/STAT deregulation

The analysis of the behaviour of *stat92E* mutant clones has led to the proposal that JAK/STAT signalling inhibits the formation of ectopic wing fields in the notum. However, stat92E mutant clones do not acquire wing identity cell-autonomously, but they do trigger wing development in the adjacent wild type cells (Hatini et al., 2013). Consistent with this observation, we find that downregulation of JAK/STAT signalling in A cells non-autonomously triggers wing development in the adjacent P notum. indicating that STAT92E normally represses a wing-inducer signal in the A compartment. However, we do not detect any change in the endogenous pattern or levels of Wg in A cells with compromised STAT92E activity, and more important, we do not rescue this non-autonomous effect by knocking down Wg in these cells. This is intriguing since Wg is absolutely required for wing fate specification and sufficient to trigger wing development in the notum when overexpressed (Ng et al., 1996).

We found that induction of ectopic non-autonomous wings depend on Upd. Three independent observations support this hypothesis. First, ectopic expression of Upd or autonomous activation of the pathway are able to bypass EGFR-mediated repression and trigger wing development in the notum. Second, depletion of JAK/STAT in the A compartment and simultaneously trapping the Upd ligand at the source fully supresses the induction of wing structures. And third, inhibition of JAK/STAT signalling results in either ectopic or increased levels of *upd* as well as the failure in the restriction to its five-spot pattern. Therefore, we conclude that JAK/STAT restricts the expression pattern and levels of its own ligand Upd. The negative feedback loop between JAK/STAT and its own ligand prevents high levels of JAK/STAT signalling in proximal territories that would otherwise impair the development of the notum and cause the induction of supernumerary wings. Moreover, it also might contribute to the refinement of the highly resolved expression pattern of *upd* into the characteristic five dots in the hinge and pleura (Johnstone et al., 2013).

It is worth to mention that despite Wg is not ectopically expressed in the JAK/STAT-depleted A compartment and that the ectopic wings depend on Upd, the requirement of Wg to induce wing fate in the notum cannot not be circumvented by Upd and therefore, a source of Wg of unknown origin should be required in collaboration with Upd to generate the ectopic wings. However, we show that Upd missexpression or autonomous activation of the pathway does not induce Wg expression, raising the question of where does Wg come from. One possibility considers that during second instar, the secreted endogenous Wg protein from the distal domain is able to diffuse enough cell diameters and reach the proximal territory. During normal development, the levels of extracellular Wg in the proximal region would be insufficient to induce wing fate because the presumptive body wall is

receiving correct patterning cues and EGFR blocks Wg responsiveness, making this region refractory to any effect of low levels of Wg. Only higher levels of Wg in the proximal domain, such as the obtained by overexpression, would bypass EGFR-mediated wing fate repression and induce ectopic wings emerging from the notum (Ng et al., 1996). Since missexpression of Upd interferes with EGFR-mediated notum development, it is possible that this generates a sensitized proximal region competent to respond to low levels of Wg coming from distal cells. This idea is consistent with the fact that overexpression of a secreted EGFR antagonist also triggers wing development in the notum without inducing ectopic expression of Wg (Wang et al., 2000). The use of standard immunostaining protocols, that highlight the intracellular fraction of Wg at the source, makes difficult to determine the range of Wg diffusion. In this sense, it would be necessary to analyse the extracellular distribution of Wg protein in second instar as well to force its retention to the distal domain as a functional test.

As development proceeds, expression of Wg evolves in a stereotyped pattern in the wing pouch, the hinge and notum. During third instar stage, few hours after the L2-L3 transition, Wg starts to be expressed in a stripe in the notum that runs along the AP axis and is important for the development of the mechanosensory bristles of the notum (García-García et al., 1999). The notal stripe of Wg never induces wing fate in the notum, probably because this region is already committed to the notum fate and in principle, Wg only can do so in a precise time window during second instar when the primordium is in an undetermined state in terms of wing & body wall fate. If missexpression of Upd creates a more permissive region in the notum due to the downregulation of EGFR signalling, it could be possible for the notal Wg stripe to induce wing fate in a group of cells of this sensitized region, even during the beginning of the third instar stage.

A number of genetic manipulations reported in the literature result in notum-to-wing transformations and all of them involve the ectopic expression of Wg or the direct or indirect activation of the Wg pathway (Collins and Treisman, 2000; Grimm and Pflugfelder, 1996; Klein and Arias, 1998; Ng et al., 1996; Pflugfelder et al.; Silver et al., 2007b). Moreover, ectopic wings always emerge from a spot located in the P compartment of the notum, although occasionally, A cells can be recruited into the new developing appendage. The reason for this specificity is unknown but it has to rely on subtle molecular differences between the A and P notum compartments. The D selector gene *apterous* subdivides the entire wing disc into D and V compartments and a stripe of DV boundary cells bisect the disc along the wing pouch, the hinge and the notum. Notch activity induces Wg and Vg expression in DV boundary cells of the wing pouch, but the DV boundary continues in the hinge and notum of both the A and P compartments which express only Vg – and not Wg – in response to Notch signalling. In the A compartment the Notch-Vg combination ends at the presumptive hinge-notum border but in the P compartment the DV boundary and hence Notch-Vg activity continues and reaches almost the end of the notum (Casares and Mann, 2000). Therefore, the P notum has Notch-Vg but the A notum does not. Besides, only in the posterior edge of the notum both the AP and DV organizers get very close, almost in contact. Thus, it seems that this region in the P notum recapitulates some aspects of the endogenous position of the wing during second instar and contains, except Wg, all the elements necessary to trigger wing development and proximo-distal outgrowth. Consequently, when Wg is ectopically provided, this region fulfils all the requirements to initiate the development of a new wing.

Our data indicate that the ectopic wings induced in a non-autonomous manner by RNAi-mediated JAK/STAT downregulation are Upd-dependent

since trapping the ligand at the source rescues this effect. In these experiments, the A compartment cannot respond to Upd (as the pathway is being downregulated) and the levels of the EGFR target genes promoting notum fate are expected to be high, thus inhibiting wing fate specification and preventing the potential recruitment of A cells to the developing ectopic appendage. By contrast, in the nearby P compartment, increased JAK/STAT activity (as a consequence of Upd coming from A cells) results in EGFR target gene downregulation and to the induction of ectopic wings.

Upstream regulators of *upd* during second instar

During second instar, *upd* is expressed in distal cells in a broader domain than *wg*. Based on the nested-like expression pattern of *upd* and *wg*, we hypothesised that *upd* could be a downstream target of Wg signalling, acting as a subordinate gene to execute a subset or complementary functions of Wg beyond the range of action of secreted Wg at this developmental stage. Although it is not clear whether there is a crosstalk between JAK/STAT and Wg signalling in the hinge region of third instar wing primordia (Ayala-Camargo et al., 2013; Rodrigues et al., 2012), both expression of *upd* and STAT92E activity remains normal in *wg* mutant second instar wing discs.

Analysis of *stat92E* mutant clones has revealed a late role of JAK/STAT in repressing the *Iro-C* genes in the hinge of mature wing primordia since these clones, when recovered in the hinge, upregulate both *mirror* and *araucan* (Ayala-Camargo et al., 2013; Hatini et al., 2013). The upregulation of the *Iro-C* genes in *stat92E* mutant clones has been associated with the acquisition of notum-like features in the adult, such as the differentiation of cuticular protrusions decorated with bristles characteristic of the notum (Hatini et al., 2013). Consistent with our results, ectopic activation of the pathway either in clones (Ayala-Camargo et al., 2013) or broadly in the entire

wing disc (Hatini et al., 2013) represses these genes in the notum of mature wing discs. Besides, *Egfr* or *Iro-C* mutant clones are frequently excluded from the notum and displace to the hinge where they acquire hinge-like characteristics in the differentiated adult cuticle (del Corral et al., 1999; Villa-Cuesta et al., 2007). In this case, it is unknown whether the notum-to-hinge transformation of *Egfr/Iro-C* mutant clones is also accompanied by the upregulation of JAK/STAT signalling. Thus, it appears that a mutual antagonism between JAK/STAT and EGFR/Iro-C maintains the hinge-notum border and keeps segregated the notum and hinge developmental fields in maturing third instar wing primordia.

In these sense, we show that JAK/STAT has an earlier and more extensive role in restricting Vn/EGFR targets during second instar and this is required to confine the notum field, allowing the proper specification of the wing. However, EGFR or Iro-C does not appear to negatively regulate STAT92E signalling at this early stage, as overexpression of a constitutively EGF receptor or its target gene *araucan* does not affect the activity of the pathway during second instar. Nonetheless, we do confirm that the development of the notum is largely incompatible with high levels of STAT92E activity in this territory. We have modulated the Wg pathways or the *Iro-C* genes to generate notum duplications without directly manipulating the levels of JAK/STAT signalling. In these two cases, the ectopic body wall develops from the Wg- and Upd-expressing distal territory that is normally fated to become the wing. In this context, we observe the retraction of the *10xSTAT-GFP* reporter from the developing ectopic nota in maturing third instar discs, thereby suggesting that the loss of STAT92E activity from this region is rather a consequence of the loss of the wing and duplication of body wall structures.

At the embryonic stage 15, expression of upd [monitored by an *upd-gal4* enhancer trap (Tsai and Sun, 2004)] in the embryonic wing primordium is low and seems to be mostly restricted to the A compartment (Rodrigues et al., 2012), but in second instar *upd* is expressed in the distal domain of the A and P compartments. This raises the possibility that upd expression could be, at least in the A compartment, inherited from the embryonic ectoderm, perhaps by the activity of Hh coming from adjacent P cells. Thus, it remains an open question the mechanism by which *upd* expression is restricted to the distal domain of the wing primordium in second instar.

A refined model for wing fate specification

The proximo-distal subdivision of the wing primordium into the wing pouch and the body wall takes place early in development during the second larval instar, when the wing disc is very small and contains at most few hundred cells. The current model for wing fate specification proposes that this developmental decision relies on two distinct mechanisms (Rafel and Milán, 2008; Wang et al., 2000). First, the antagonistic expression and activity of the Vn and Wg ligands in opposing domains instruct cells to acquire the body wall and wing fate, respectively. Wg prevents the expression of Vn in the distal domain and induces a set of wing-determining genes (Ng et al., 1996; Wang et al., 2000; Wu and Cohen, 2002). In contrast, Vn, through the EGFR pathway, inhibits the cellular response to Wg and instructs cells to acquire body wall fate [Figure 64 (Wang et al., 2000; Zecca and Struhl, 2002a, 2002b)]. Second, growth promoted by Notch pulls the sources of these two morphogens apart, alleviating the repression of wing fate by Vn/EGFR, and ensuring Wg-mediated appendage specification. If growth is inhibited at this critical stage, the sources of these two ligands remain very close and Wg is unable to induce wing fate due to EGFR-mediated wing fate repression (Rafel and Milán, 2008). Thus, while Wg and Vn play an instructive

role in the segregation of the wing and notum developmental fields, growth promoted by Notch has a permissive role in this process by modulating the timing and range of activity of these two signalling molecules (Dekanty and Milán, 2011; Rafel and Milán, 2008). However, expression of Vn is maintained by a positive feedback loop through the activation of the EGFR pathway (Figure 64). Thus, proximity to other cells generates a "community effect" that serves as a mechanism to sustain Vn expression (Golembo et al., 1999; Paul et al., 2013; Wang et al., 2000). In this sense, it is expected that in the absence of additional repressors or mechanisms of confinement, the signal propagates through a field of cells, resulting in a runaway situation with too many cells becoming part of the community and recruited to the body wall fate. Thus, an autocrine loop that in principle functions to sustain Vn/EGFR – and hence the notum field – in a defined group of proximal cells, can be turned into an amplification loop that colonizes the entire primordium. The results obtained in this thesis suggest that JAK/STAT has a permissive role in the process of wing fate specification, by repressing EGFR-regulated genes and limiting productive Vn/EGFR activity proximally. This restricts the notum field to its normal position in the proximal territory of the early wing primordium, preventing its distal expansion and allowing Wg to correctly trigger wing development (Figure 64).

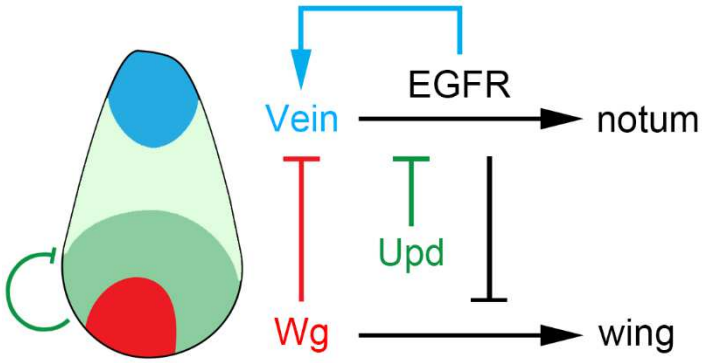


Figure 64. Upd and JAK/STAT signalling in the model for wing fate specification. During second instar the ligands Vein (blue) and Wg (red) are expressed in the proximal and distal territories of the wing primordia, respectively. Vein signals through the EGFR pathway to induce notum fate in the proximal region, while Wg induces wing fate in the distal domain. Vein blocks Wg responsiveness to inhibit wing development in ectopic locations and Wg prevents Vein to be expressed in the distal territory. A feedforward loop between Vein and EGFR sustains Vein/EGFR signalling in the proximal domain. Upd (dark green) is expressed in distal cells in a broader domain than Wg and signals through the JAK/STAT pathway (light green) throughout the wing primordia with lower levels in the proximal region. Low STAT92E activity in the presumptive body wall is required for the proper development of the notum. JAK/STAT represses EGFR target gene expression and limits productive Vn/EGFR autocrine loop proximally. This confines the notum field to the proximal territory, preventing its distal expansion to allow Wg-mediated wing specification.

A cell-autonomous requirement for JAK/STAT in P cells

In the *Drosophila* wing, the biological function of JAK/STAT signalling is best understood by its role in hinge growth and patterning (Ayala-Camargo et al., 2013; Johnstone et al., 2013). Indeed, as development proceeds and the ligand Upd becomes restricted to the hinge and pleura, the *10xSTAT-GFP* activity reporter retracts from the nascent wing pouch and focuses to these regions where it is robustly activated (Bach et al., 2007). Consistently, wings with *stat92E* mutant clones display a near complete lack of hinge structures and are directly attached to the thorax (Ayala-Camargo et al., 2013). However, there is also evidence that JAK/STAT is required for the development of the wing pouch. The adult wing of *Drosophila* is decorated with longitudinal- and cross-veins that are arranged in a stereotyped pattern (Stark et al., 1999), and a number of signalling pathways such as Dpp, Hh, EGFR and Notch are involved in the positioning, patterning and differentiation of veins and intervein territories within the wing pouch (Blair, 2007; De Celis, 2003). Some *hop* or *stat92E* viable alleles show ectopic vein material in the adult wing blade (Rawlings et al., 2004; Yan et al., 1996b), and modulation of JAK/STAT activity causes variable gain or loss of veins along the proximo-distal axis of the wing (Harrison et al., 1995; Rawlings et al., 2004; Stec et al., 2013). These observations indicate that JAK/STAT signalling interacts with other vein-forming genes in the pouch at later stages of larval development. Further evidence that JAK/STAT has a broader and more extensive role besides its reporter activity in the hinge comes from the observation that *hop* mutant animals show a small-disc phenotype, suggesting impaired growth or viability (Mukherjee et al., 2005; Perrimon and Mahowald, 1986). Moreover, JAK/STAT is implicated in the process of cell competition (Rodrigues et al., 2012), a mechanism by which “weaker” but otherwise viable cells are detected and eliminated from mosaic tissues

containing cells with distinct fitness (Amoyel and Bach, 2014; Levayer and Moreno, 2013; Morata and Ripoll, 1975).

In this sense, we observe a mild activation of the *10xSTAT-GFP* reporter in the wing pouch at later stages of development, and confirm that this activity depends on the Upd receptor. While inhibition of the pathway in the A compartment does not cause a noticeable autonomous effect in terms of size, depletion of JAK/STAT in the entire wing or specifically in P cells causes a clear growth defect associated with a strong reduction of P territory. Eventually, the P compartment is completely lost, giving rise to a stronger decrease in the size of the wing pouch, which is now formed only by A cells. Further confirmation of this phenotype comes from the observation that *hop*^{27} mutant wing discs, besides the reported small-disc phenotype (Mukherjee et al., 2005; Perrimon and Mahowald, 1986), discs also show a strong reduction in P compartment. Although the *hop*^{27} mutant allele is described as amorphic (Luo et al., 1999), the P reduction in size is less penetrant than using the Gal4/UAS system for targeted depletion of JAK/STAT signalling components. This is to some extent expected since the maternal gene product deployed in the oocyte is sufficient to rescue embryonic and larval development of zygotic mutants until late larval or pupal stages (Perrimon and Mahowald, 1986). In rare cases, animals complete metamorphosis and die as pharate adults inside the pupal case.

In the leg imaginal disc, JAK/STAT has been shown to negatively regulate Wg and Dpp, restricting them to their normal position and thus promoting the formation of a single proximo-distal axis (Ayala-Camargo et al., 2007). We find that depletion of JAK/STAT in P cells also reduces the size of the P compartment in leg discs but the A compartment seems unaffected when the same RNAi is expressed in A cells. Thereby suggesting that in addition to its reported role in leg patterning, JAK/STAT might also control the size of

the P compartment in the leg. It is possible that JAK/STAT has a prevalent function in the P compartment of *Drosophila* appendages since the size of the P territory of the haltere primordium also depends on JAK/STAT.

The analysis in the size and distribution of clones mutant for a null *stat92E* allele support the results obtained by targeted depletion of JAK/STAT or in hop^{27} mutant wing discs, and further confirm the autonomous and compartment-specific requirement for STAT92E in the P compartment. *stat92E* mutant clones are progressively eliminated from the tissue by cell competition when confronted with wild type cells, but the molecular nature of the low fitness of these mutant cells is not clear and appears to be independent of Myc, Yorkie, Wingless and ribosome biogenesis (Rodrigues et al., 2012). If the growth of the surrounding non-mutant tissue is impaired using the *Minute* technique, this confers a relative growth advantage to the *stat92E* mutant clones, which are now expected grow and colonize large territories of the wing disc. We do see this effect in the case of *stat92E* mutant clones generated in the A compartment, which seem to grow well and extensively populate the A compartment, although they end up covering a significantly smaller portion of this territory (40 %) compared to control clones (52 %). This may reflect either a general defect in cellular growth and/or proliferation or a specific requirement of JAK/STAT in particular regions – such as the hinge – that is not fulfilled by the increased protein biosynthetic capacity conferred by the Minute technique. In the P compartment the situation is different as *stat92E* clones, in spite of being conferred with a relative growth advantage, considerably fail to grow in this territory (21 %) compared with *stat92E* clones in the adjacent A compartment (40 %), or with control clones in the P compartment (59 %). It is important to highlight that even with the *Minute* technique, *stat92E* clones still have a marked growth deficiency in the P compartment, revealing a

cell-autonomous requirement for JAK/STAT that cannot be compensated by conferring these cells a relative growth advantage compared to the surrounding *wild type* cells.

The wing disc appears to possess a size-sensing mechanism that measures global dimensions to arrest growth at a predetermined size, and this size is modulated by external inputs such as nutrient availability and temperature (Edgar, 2006; Mirth and Shingleton, 2012; Shingleton, 2010). This organ-autonomous control of size allows certain flexibility in the number and size of cells that each compartment contains at the end of the growth period while maintaining the final target size and proportions (Neufeld et al., 1998; Weigmann et al., 1997). Thus, the wing disc does not count cells numbers nor cell divisions, but dimensions (Day and Lawrence, 2000). Besides, the wing disc is highly resilient, as it is capable to overcome transient growth perturbations, ablation of fragments or even massive cell death after irradiation without affecting the final adult wing size or proportions (Haynie and Bryant, 1977; Martín and Morata, 2006; Mesquita et al., 2010; Milán et al., 1997). In this sense, JAK/STAT might be persistently required to maintain the size of the P compartment or, in contrast, the requirement might be limited to a defined time window. If the function of JAK/STAT in P cell is only required early but dispensable at later stages, chronic inhibition of JAK/STAT should not cause a strong growth defect since one would expect the wing disc to have enough time to recover during the later stages. However, chronic inhibition of STAT92E activity causes the severe undergrowth of the P compartment or even its complete loss, indicating that the pathway is required at least during late developmental stages. Our temporally and spatially controlled depletion of JAK/STAT indicates that STAT92E activity is required throughout development in P cells. We observe a P reduction already in early L3 (the end of the first half of larval development), and this

phenotype is maintained or even enhanced when we chronically target STAT92E activity until the end of larval development. Interestingly, the reduction in the size of the P compartment observed in young discs is largely rescued when we restore JAK/STAT signalling to endogenous and normal levels during the second half of larval development. Although we have not monitored the cellular and molecular events occurring during the recovery process, it is expected that the growth perturbation caused by JAK/STAT depletion triggers a kind of compensatory proliferation (Mollereau et al., 2013; Perez-Garijo et al., 2004; Ryoo et al., 2004; Smith-Bolton et al., 2009). Thus, during this phase the P compartment would increase its growth rates relative to the A territory to catch up with the A compartment at the end of larval development. It is also possible that the P compartment recovers by just growing at its normal rates and the A compartment slows its growth or event stops growing as it approaches to its final size, thus "waiting" for the perturbed P compartment to reach a final target size (Gokhale et al., 2016; Martín and Morata, 2006; Mesquita et al., 2010). If so, this would require a systemic response to extend the duration of the larval growth period and allow the P compartment to recover while the A territory is growth-arrested (Colombani et al., 2012; Garelli et al., 2012; Katsuyama et al., 2015; Parker and Shingleton, 2011). Although there is not an obvious and generalized developmental delay between experiments and controls neither in chronic nor in temporally-controlled assays, it is possible that heterogeneities at the population level both in growth rates and/or developmental timing permits the operation of both mechanisms.

JAK/STAT promotes the cycling and survival of P cells

We show that the reduction in the size of the P compartment is not a consequence of changes in cell identity as P cells mutant for *stat92E* maintain normal levels of the P-selector Engrailed and consequently respect

the AP compartment boundary. In contrast, in the lineage tracing experiment we do see that a small number of cells from P compartment origin crossed to the A compartment, but this number is very low and does not seem to explain the observed reduction in the size of the P compartment. It is possible that a fraction of these A cells with P origin result from a few founder P cells that violated the compartment boundary and proliferated in their new A territory. Thus, perhaps only a fraction of P-origin cells found in the A compartment genuinely crossed the boundary, while the rest should be their progeny. We found this cellular behaviour very similar to the transgressions of compartment boundaries and cell reprogramming observed during regeneration in imaginal discs (Herrera and Morata, 2014), and this anticipated that reduced survival cues might explain the reduction of the P compartment. We also considered two alterative hypothesis involving cell fate changes in the P compartment: a wing-to-notum transformation and the wing-to-haltere homeotic change. In both cases the molecular markers for wing and haltere identity were unaffected.

JAK/STAT does not autonomously modulate physiological levels of the major organ-intrinsic growth regulators, such as Dpp signalling, Hippo pathway and Myc levels. The case of Dpp is particularly interesting because the width of the Spalt domain does not appear to scale with the reduced size of the P compartment. In general, altering the size of one compartment is accompanied by the expansion or contraction of the Dpp gradient according to the size of the field being patterned, and this ensures that pattern scales to accommodate differences in disc size (Ferreira and Milán, 2015; Teleman and Cohen, 2000). The observation that the Hippo pathway activity and Myc protein are unaffected in P compartments depleted for JAK/STAT is consistent with the fact that *stat92E* mutant cells are outcompeted by winner cells independently of Yorkie and Myc (Rodrigues et al., 2012), and that

stat92E mutant clones are hardly recovered in the P compartment in spite of the growth advantage conferred by the *Minute* technique.

In general, cell death during larval development is very low, except for a moderate increase at the L2-L3 larval molt and at the hinge-notum border during late third instar (Milán et al., 1997). Therefore, the developmental role of apoptosis in wing development is considered to have a minor contribution in terms of size and shape, except for a late function in the refinement of hairs and sensory bristle number and spacing (Koto et al., 2011; Takemura and Adachi-Yamada, 2011). However, we observe high levels of apoptosis in the P compartment of hop^{27} mutant wing discs throughout development as well in targeted inhibition of JAK/STAT in P cells. Indeed, these levels of apoptosis contribute to the reduction in the P compartment since blocking apoptosis by different means substantially rescues the size of this territory. STAT92E has been shown to have a protective role against irradiation-induced apoptosis by regulating dIAP1 (Betz et al., 2008). Consistent with this report, we show that JAK/STAT contributes to maintain endogenous levels of dIAP1 in the P compartment, but A cells do not seem to require STAT92E activity to survive as well as to sustain physiological levels of dIAP1. In agreement with an asymmetric requirement for JAK/STAT in preventing apoptosis, we do not detect high levels of cell death in the A compartment of wing discs entirely mutant for hop^{27}, and we do not observe any noticeable growth defect in the A wing pouch when JAK/STAT is inhibited in this compartment. Although the amount of cell death in the A compartment in hop^{27} mutant discs is low, it seems to be above the basal levels observed in a *wild type* discs. We also observe a slight increase in dying cells in the A compartment while downregulating JAK/STAT activity only in P cells, thereby suggesting a non-autonomous effect. This apparent non-autonomous effect may be explained if posterior-dying cells and

apoptotic corpses, once basally extruded from the epithelium, are free to passively move around the basal plane of the disc. In contrast to this view, it is known that apoptotic cells release a number of signals before dying and there is increasing evidence for a non-apoptotic role of Caspases (Miura, 2012; Perez-Garijo and Steller, 2015). Several non-autonomous effects following Caspase activation have been described, such as apoptosis-induced proliferation [AIP (Fan and Bergmann, 2008)], apoptosis-induced apoptosis [AIA (Mesquita et al., 2010; Milán et al., 1997; Pérez-Garijo et al., 2013)], as well as Caspase-dependent cell cycle arrest (Mesquita et al., 2010). Therefore, it is possible that high levels of apoptosis in the P compartment caused by JAK/STAT downregulation non-autonomously induce apoptotic cell death in the adjacent A compartment, resembling the phenomena of AIA. In favour of this view, blocking Caspase function in P cells rescues the high levels of autonomous cell death as well as the low number of apoptotic cells in the A compartment. However, we cannot exclude that a fraction of these few apoptotic cells in basal planes of the A compartment come from posterior-dying cells.

In addition to its reported protective roles upon tissue stress (Betz et al., 2008; La Fortezza et al., 2016; Verghese and Su, 2016), the JAK/STAT pathway is a positive regulator of proliferation in several *Drosophila* tissues, such as the eye imaginal disc or the hematopoietic organ (Bach et al., 2003; Harrison et al., 1995; Tsai and Sun, 2004), and is involved in a variety of *Drosophila* tumour models (Classen et al., 2009; Herranz et al., 2012; Luo et al., 1995; Tamori et al., 2016; Wu et al., 2010) as well in regeneration (Jiang et al., 2009; Santabárbara-Ruiz et al., 2015). In the wing disc, it has been shown to upregulate CycB upon pathway stimulation (Mukherjee et al., 2005). In this sense, we observe a downregulation in the levels of the G2/M rate-limiting CycA and CycB in the P but not the A compartment upon

targeted inhibition of JAK/STAT, suggesting a cell cycle defect. Indeed, overexpression of CycA largely rescues the size of the P compartment, indicating that impaired cell cycling contributes to the undergrowth of the P compartment. However, it would be necessary to analyse the distribution of cells in the different phases of the cell cycle since the downregulation of mitotic cyclins might reflect either a G1/S or a G2/M defect. For instance, in mature wing primordia, a zone of non-proliferating cells (ZNC) at the DV boundary is arrested in G1, and in these cells CycA is nearly absent (Herranz et al., 2008; Johnston and Edgar, 1998).). In contrast, in *rca1* mutants (Regulator of Cyclin A) both CycA and CycB are prematurely degraded by APC (Anaphase-promoting complex) during G2, and these cells fail to execute mitosis (Grosskortenhaus and Sprenger, 2002). The downregulation of CycB might be consequence of lower levels of CycA, as CycB is prematurely degraded in *cycA* mutant embryos (Dienemann and Sprenger, 2004). Despite mitotic cyclins have partially redundant functions (Knoblich and Lehner, 1993; Lehner and O'Farrell, 1990; Sigrist et al., 1995), CycA is the only essential mitotic cyclin in *Drosophila*, as the presence of CycB is not sufficient to induce mitosis in *cycA* mutants (Jacobs et al., 1998), even after overexpression (Dienemann and Sprenger, 2004). This suggests that a function of CycA can apparently not be fulfilled by CycB, which fits well with our observation that CycB overexpression does not rescue the undergrowth of the P compartment. We have not explored the molecular mechanisms underlying CycA regulation.

Our results indicate that P cells are not fully arrested since blocking apoptosis substantially rescues tissue size, indicating that P cells can proliferate, possibly at slower rates. We cannot rule out that impaired cell cycling may cause apoptosis in some P cells, but it is unlikely that most of the cell death triggered by loss of STAT92E is a consequence of cell cycle

deregulation, as one would expect that overexpression of CycA to fully tissue size. In this sense, visual inspection of basal planes in CycA-overexpressing P compartments confirms the presence of pyknotic cells and cellular debris. We thus favour the model in which STAT92E independently promotes the survival and cycling of the Hh-expressing cell population.

JAK/STAT counteracts the negative effects of Engrailed

We show that the undergrowth of the P compartment caused by JAK/STAT deregulation is due to the presence of the transcriptional repressor Engrailed in P cells, as reducing En levels rescues apoptosis, CycA levels and consequently tissue size. Moreover, overexpression of En in its own domain phenocopies the loss of JAK/STAT in terms of dIAP1 downregulation, apoptosis, reduced CycA levels and more important, a decrease in the size of the P compartment. These results indicate that JAK/STAT promotes the cycling and survival of P cells by counteracting the negative effect of En on these two genes. Interestingly, overexpression of En in P cells considerably reduces the size of the posterior wing pouch and posterior notum, but the posterior hinge – where JAK/STAT activity is highest – is not visibly affected, possibly by enhanced protection of these cells against high levels of En. We have not explored the mechanisms by which dIAP1 and CycA are regulated by STAT92E and En. JAK/STAT has been shown to directly control *diap1* transcription via two conserved STAT92E binding sites (Betz et al., 2008) and cis-regulatory maps of the *Drosophila* genome have identified two En binding sites on the *diap1* locus (Nègre et al., 2011; Roy et al., 2010). Based on these data, STAT92E and En might be directly regulating *diap1* in P cells. However, during normal development physiological levels of dIAP1 do not depend on En, as downregulation of En alone does not increase dIAP1 levels (Figure 65). Therefore, endogenous dIAP1 levels do not result from a balance between positive STAT92E inputs and negative regulation by En.

Interestingly, one of the STAT92E and En binding sites is located in an overlapping fragment at the first *diap1* intron, raising the possibility of a competitive mode of regulation. In this scenario, STAT92E would be normally bound to- and positively regulating *diap1* while preventing En binding and/or *diap1* repression. Thus, depletion of JAK/STAT in P cells would allow En to bind and downregulate *diap1.*

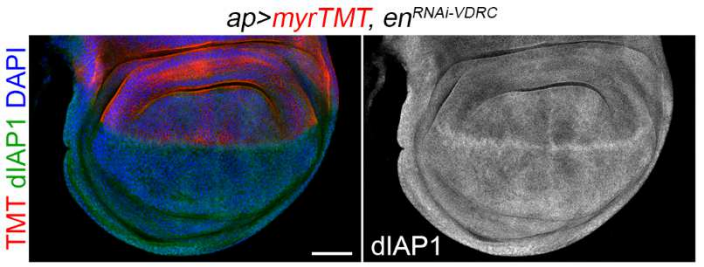


Figure 65. Engrailed does not regulate physiological levels of dIAP1. Late third instar wing disc expressing *en*^{RNAi} in the dorsal compartment under the control of *apterous-gal4* (*ap*), and labelled to visualize dIAP1 protein (green or white), DAPI (blue) and myrTomato to label the domain of transgene expression. Scale bars, 50 µm.

The gene *en* is subject to multiple modes of regulation involving both positive and negative autoregulation as well as epigenetic maintenance through the PcG proteins (Garaulet et al., 2008; Heemskerk et al., 1991; Moazed and O'Farrell, 1992). In the embryo Wg sustains En expression in adjoining cells but later *en* expression becomes independent of this extracellular influence and comes to transiently rely on positive autoregulation (Heemskerk et al., 1991). In the wing imaginal disc, it has been shown that high levels of En interfere with wing development due to the capacity of En to negatively regulate its own expression (Garaulet et al., 2008; Guillén et al., 1995; Tabata et al., 1995). Consistent with this proposal, we observe an increase in the expression levels of the *en-gal4* driver - which is inserted in the *en* locus and behaves as a transcriptional reporter – in *en*^{RNAi} expressing wing discs. The functional significance is uncertain but indicates that the amount of En

transcriptional repressor has to be precisely modulated. In this sense, our data indicates that even endogenous levels of En are potentially deleterious for cellular viability, unless this is prevented by JAK/STAT signalling.

As is it often the case in development, a discrete number of genes is recurrently used to specify cell fate and regulate gene expression in a context dependent manner. We propose that the capacity of En to block cell cycle and promote cell death might be required in another developmental context and that this capacity is specifically suppressed in the developing *Drosophila* limbs by JAK/STAT, and modulated by the negative autoregulation of En. It is interesting to note in this context that En-expressing territories in the embryonic ectoderm are highly enriched in apoptotic cells (Pazdera et al., 1998). Whether this apoptosis plays a biological role and relies on En activity requires further study.

Biological relevance of JAK/STAT in wing growth

Later in development, once the wing field is specified, restricted expression of Dpp at the AP compartment boundary organizes the growth and patterning of the whole developing appendage (Burke and Basler, 1996; de Celis et al., 1996; Martín-Castellanos and Edgar, 2002; Nellen et al., 1996b; Restrepo et al., 2014). Dpp expression is induced in A cells by the activity of Hh coming from P cells which express the En transcriptional repressor (Strigini and Cohen, 1997; Tabata et al., 1995; Zecca et al., 1995). We show that depletion of JAK/STAT in the P compartment or in the entire wing primordia eventually leads to the total loss of the Hh-expressing cell population. Consequently, Dpp expression is abolished at the AP boundary causing a dramatic reduction of the size of the entire wing pouch. Consistently, boosting Hh production in P cells largely decreases the fraction of wing discs that lost Dpp. Collectively, our results indicate that through an

autonomous function in the P compartment, JAK/STAT controls overall organ size by maintaining a pool of Hh-expressing cells sufficient to induce stable Dpp expression at the AP boundary, thus generating a well sized and fully-functional limb primordia (Figure 66).

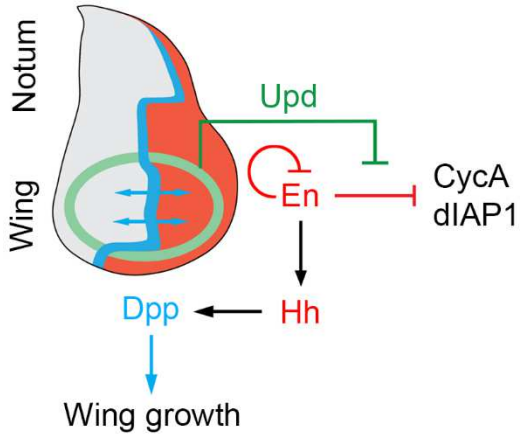


Figure 66. JAK/STAT signalling controls overall organ size. Upd (green) signals through the JAK/STAT and promotes the cycling and survival of P cells (red) by counteracting the negative effects of Engrailed on CycA and dIAP1, thus allowing En-dependent induction of Hh and promoting Dpp-mediated appendage growth (blue). Negative autoregulation of En contributes to buffer the potential deleterious effect of En on cycling and survival.

The development of the wing hinge region, which connects the developing appendage to the surrounding body wall and depends on JAK/STAT activity (Ayala-Camargo et al., 2013; Johnstone et al., 2013), has been previously shown to restrict the Wg organizer and thus delimit the size and position of the developing appendage (Casares and Mann, 2000). Our results support the notion that JAK/STAT and the hinge region contributes to isolate the body wall and appendage sources of Dpp to delimit its organizing activity to the developing appendage. Thus, besides its structural function in the adult, the hinge has an important function in coordinating growth and tissue patterning. Taken together, our results reveal a fundamental role of JAK/STAT in promoting appendage specification and growth through the regulation of

morphogen production and activity, and a role of pro-survival cues and mitotic cyclins in regulating the pool of morphogen-producing cells in a developing organ.

Multicellular organisms exhibit great diversity of body sizes and shapes as well as an ample repertory of appendages and anatomical adaptations. In spite of such diversity, growth control, patterning and morphogenesis appear to be governed by a discrete and conserved number of developmental pathways recurrently used for different purposes (Rokas, 2008a, 2008b). Studies of limb development, both in vertebrates and invertebrates, have been instrumental in the identification of the short- and long-range signaling molecules controlling growth and pattern formation in developing limbs (Affolter and Basler, 2007; Baena-Lopez et al., 2012; Bénazet and Zeller, 2009; Dekanty and Milán, 2011; Restrepo et al., 2014). Morphogens of the BMP/Dpp, Sonic Hedgehog/Hedgehog, and Wnt/Wingless families play a conserved role in promoting growth and fate specification within growing limbs, which emerge as outgrowths perpendicular to the major axes of the developing animal (Capdevila and Belmonte, 2001; Tickle, 1999; Zeller et al., 2009). The striking parallelisms in the molecules and mechanisms underlying limb development in vertebrates and invertebrates have contributed to the proposal that an ancient patterning system is being recurrently used to generate body wall outgrowths (Mercader et al., 1999; Panganiban et al., 1997; Shubin et al., 1997, 2009). Whether the conserved JAK/STAT pathway also plays a developmental role in the specification or growth of vertebrate limbs by regulating morphogen production or activity is a tempting question that remains to be elucidated.

Conclusions

1. Early in development, JAK/STAT ensures proper wing vs notum subdivision.

2. Loss of JAK/STAT early in development impairs wing fate specification and results in the duplication of the notum structures.

3. JAK/STAT represses EGFR target genes and thus restricts body wall identity to the most proximal regions of the wing primordium.

4. Ectopic activation of JAK/STAT in proximal regions generates ectopic wings and phenocopies the loss of EGFR signalling in the body wall.

5. JAK/STAT restricts the expression pattern and levels of its own ligand Upd.

6. JAK/STAT is required throughout development to maintain the size of the posterior compartment of imaginal discs.

7. Apoptosis and impaired cell cycling contribute to the reduction in the size of the posterior compartment.

8. JAK/STAT promotes the cycling and survival of posterior cells through the positive regulation of CycA and dIAP1.

9. JAK/STAT counteracts the negative effects of Engrailed on cell cycling and survival.

10. JAK/STAT controls overall organ size by maintaining the pool of Hh-expressing cells to ensure the stable and localized expression of the Dpp organizer.

11. Growth of the hinge promoted by JAK/STAT isolates the body wall and appendage sources of Dpp to restrict the organizing activity of Dpp to the developing appendage.

Material and Methods

Drosophila strains and genetics

Flies were raised at 25 °C (unless otherwise indicated) in a 12/12 h day/night period 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). Larvae were grown in standard *Drosophila* medium at 25 ºC or 29 ºC to enhance transgene expression. In the case of strong gal4 drivers (eg. *ci-gal4*, *ptc-gal4*, *hth-gal4*, *sd-gal4*) larvae were generally grown at 25ºC to decrease larval and pupal lethality.

Gal4 drivers

The following stocks were obtained from the Bloomington *Drosophila* Stock Center (BDSC) or are described in Flybase: *en-gal4* (BDSC 30564); *hh-gal4; sd-gal4; ci-gal4; upd-gal4* (Tsai and Sun, 2004); *hth-gal4; ptc-gal4.*

Other transcriptional reporter lines

The following stocks were obtained from the Bloomington *Drosophila* Stock Center (BDSC) or are described in Flybase: *10xSTAT-GFP* (BDSC 26197 and 26198); *mirror-lacZ* (BDSC 10880); *apterous-lacZ; vein-lacZ* [(Rafel and Milán, 2008), gift from C. Estella]; *expanded-lacZ; diap1-lacZ* (BDSC 12093); *dpp-lacZ.*

UAS-transgenes

The following stocks were obtained from the Bloomington *Drosophila* Stock Center (BDSC) or are described in Flybase: *UAS-dome*$^{\Delta CYT}$ [(Brown et al., 2001), *UAS-dome*^{DN} in the text]; *UAS-upd* [(Ayala-Camargo et al., 2013), gift from F. Serras]; *UAS-hop::myc* [(Sotillos et al., 2008), gift from J. Castelli-Gair); *UAS-diap1*[(Betz et al., 2008), gift from H. Steller]; *UAS-p35* (BDSC 5072); *UAS-vn::aos* [(Schnepp et al., 1998), gift from A. Simcox];

UAS-Egfr $^{\lambda TOP4.2}$ [(Queenan et al., 1997), gift from G. Jiménez]; *UAS-araucan; UAS-hh::GFP* [(Gradilla et al., 2014), gift from I. Guerrero]; *UAS-en; UAS-sgg* (BDSC 5255); *UAS-notum; UAS-wg; UAS-CycA* (BSDC 6633); *UAS-CycB::HA* [(Dienemann and Sprenger, 2004), gift from E. Martin-Blanco]; *UAS-dcr2* (BDSC 24644 and 24651); ubi-FRT-stop-GFP (BDSC 32250); *UAS-FLP* (BDSC 4539), *UAS-smo*^{5A} (UAS-smo^{DN} in the text, BDSC 23943); *UAS-myristoylated-Tomato* (*UAS-myrT,* BDSC 32222); *UAS-GFP* (BDSC 4775, 6658 and 6874); *UAS-myristoylated-GFP* (UAS-myrGFP BDSC 32196); *UAS-RFP* (BDSC 30556).

The following *UAS-RNAi* stocks were used to knockdown gene function by RNAi-mediated post-transcriptional gene silencing (PTGS), and provided by the BDSC or the Vienna *Drosophila* RNAi Center (VDRC): *UAS-dome*^{RNAi} (VDRC 106071 and BDSC 34618); *UAS-hop*^{RNAi} (BDSC 32966); *UAS-stat92E*^{RNAi} (BDSC 33637 and VDRC 106980); *UAS-dronc*^{RNAi} (VDRC 23033); *UAS-en*^{RNAi} (VDRC 105678 and BDSC 26752); *UAS-wg*^{RNAi} (BDSC 33902).

Following the protocol described in (Green et al., 2014), *UAS-RNAi* strains from the VDRC KK stock collection were routinely tested for the existence of unwanted second site insertions by a diagnostic PCR and cleaned by a genetic recombination scheme. According to the VDRC, the *UAS-dome*^{RNAi} line used in Figure 36 (VDRC 106071) does not target the mRNA encoding for the truncated form of *dome*$^{\Delta CYT}$ (Dome^{DN} in the text).

Mutant alleles and FRT-bearing chromosomes

wg^{CX4} (BDSC 2980); *wg*^{CX3} (BDSC 2977); *Egfr*^{F2} (BDSC 2768); *iro*^{EGP7} [(Andreu et al., 2012), gift from S. Campuzano); *hop*^{27} (BDSC 8493); *FRT82B stat92E*^{85c9} [(Rodrigues et al., 2012), gift from F. Serras]; *FRT82B stat92E*^{85c9}

ubiRFP [(Schroeder et al., 2013), gift from G. Halder); *FRT82B M(3)95A2 ubiGFP; FRT82B arm-lacZ; FRT82B; Df(2)en^{E}* (a deficiency that covers both the *engrailed* and *invected* genes, BDSC 2216).

Mosaic analysis and lineage tracing

Loss of function clones for the *stat92E^{85c9}* allele were generated in the following genotypes:

(1) *hs-FLP; FRT82B stat92E^{85c9}/FRT82B M(3)95A2 ubiGFP* (Minute + clones)

(2) *hs-FLP; FRT82B stat92E^{85c9}/FRT82B arm-lacZ* (twin/clone analysis)

(3) *upd-gal4, UAS-myrGFP/hs-FLP;; FRT82B stat92E^{85c9} ubiRFP/FRT82B*

Flies were allowed to lay eggs for 4 h at 25 ºC in 55 mm petri dishes with standard fly medium. Hatched larvae were synchronized at early first instar and allowed to grow at 25 ºC in standard fly medium. 16 h later (40 h after egg laying, AEL), larvae were heat-shocked at 38 ºC for 1 h, and wing discs were dissected ~100 h after clone induction for the Minute clones (1), ~85 h after clone induction for the twin/clone analysis (2), and 24-48 h after clone induction to monitor *upd* expression in stat92E mutant cells (3). Lineage tracing of the P compartment was generated as described in (Evans et al., 2009) using the following genotype: *UAS-FLP/ubi-FRT-stop-FRT-GFP; UAS-dome$^{\Delta CYT}$/hh-GAL4.*

Temporal and regional control of target gene expression

Transgene expression was temporally controlled in the following experiments:

(1) Ectopic expression of Upd. To monitor *mirror-lacZ* expression, flies were allowed to lay eggs at 25 ºC overnight and *ptc-gal4, UAS-GFP/UAS-upd;*

mirror-lacZ/tub-gal80^{TS} larvae were raised at 29 ºC until dissection in second or third instar larval stages. To visualize ectopic wings emerging from the notum, larvae were maintained at 18 ºC, shifted to 29 ºC in late second instar (5 days after egg laying, AEL) and dissected at late third instar stages.

(2) Overexpression of Shaggy. Flies were allowed to lay eggs at 18 ºC overnight, *ptc-gal4, UAS-GFP/ UAS-sgg; tub-gal80^{TS}/+* larvae were initially grown at 18 ºC to bypass early larval lethality, shifted to 29 ºC in early second instar stage (4 days after egg laying, AEL) and wing discs were dissected at late third instar stages.

(3) Temporal depletion of JAK/STAT in the P compartment. Flies were allowed to lay eggs at 25 ºC overnight, control (*UAS-GFP/+; hh-gal4, tub-gal80^{TS}/+*) and experimental (*hh-gal4, tub-gal80^{TS}/UAS-dome^{DN}*) larvae were transferred to 29 ºC, and wing discs were dissected at day 3 and 5 AEL (at early and late third instar stages, respectively). To address the capacity of the wing disc to recover P compartment size, experimental (*hh-gal4, tub-gal80^{TS}/UAS-dome^{DN}*) larvae were grown at 29 ºC, shifted to 18 ºC at day 3 AEL (at early third instar), and kept at this temperature for 4 days until wing disc dissection (at late third instar).

(4) Overexpression of Engrailed. Flies were allowed to lay eggs at 18 ºC overnight, and *hh-gal4, tub-gal80^{TS}/UAS-en* larvae were shifted to 29ºC in early second (4 days AEL) or mid third instar (7 days AEL), and wing discs were dissected 72 h or 24-48 h later, respectively.

Histochemistry

Antibodies

Mouse anti-Wg (1:10-50) (4D4, DSHB); goat anti-Hth (1:50) (sc-26187, Santa Cruz Biotechnology); mouse anti-Nub (1:10) (gift from S. Cohen); rabbit anti-Nub (1:600) (gift from X. Yang); mouse anti-βgal (1:50) (40-1a, DSHB); mouse anti-En (1:5) (4D9, DSHB); rat anti-Ci (1:10) (2A1, DSHB); mouse anti-Ptc (1:50) (Apa1, DSHB); mouse anti-CycA (1:50) (A12, DSHB); mouse anti-CycB (1:50) (F2F4, DSHB); rabbit anti-CycE (1:100) (sc-481, Santa Cruz Biotechnology); mouse anti-Diap1 (1:200) (gift from B. Hay); rabbit anti-Tsh (1:600) (gift from S. Cohen); rabbit anti-Sal (1:500) (gift of R. Barrio), guinea pig anti-dMyc (1:1000) (gift from G. Morata); rabbit anti-Gal4 (1:100) (sc-577, Santa Cruz Biotechnology); sheep anti-Dig-AP (1:2000) (11093274910, Roche Diagnostics); Secondary antibodies labelled with Cy2, Cy3, Cy5 and Alexa 647 fluorophores were obtained from Jackson ImmunoResearch and diluted (1:400) in PBT.

Immunostaining

Larvae were dissected in cold PBS, fixed for 20 minutes in 4% paraformaldehyde in PBS, rinsed 3 times during 15 minutes with PBT (PBS + 0,2% Triton-X), and blocked for 1 hour in BBT (PBT + BSA 0,2% + 250 mM NaCl) in a nutating mixer. Primary antibodies were diluted in PBT in a final volume of 50 µl and incubated overnight at 4 °C. After incubation, samples were rinsed 4 times during 15 minutes with PBT, and stained with DAPI and fluorophore-conjugated secondary antibodies diluted in PBT for 2 hours at room temperature in a nutating mixer. After secondary antibody incubation, samples were rinsed 4 times during 15 minutes in PBT with a final 5 minutes rinse in PBS to eliminate the excess of detergent. Samples were stored and mounted in glycerol-based mounting medium

[40 ml Glycerol + 5 ml PBS 10X + 400 µl N-propyl-gallate (50% m/v in ethanol)]. Images were captured using a Leica TCS SP2 or SP5 confocal microscopes and analyzed with the FIJI software for image processing (Schindelin et al., 2012). Images were mounted into figures using Adobe Photoshop CS5.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a method for detecting double-stranded, low molecular weight DNA fragments as well as single strand breaks, by labeling the terminal end of nucleic acids. TUNEL assay was adapted from (Wang et al., 1999) with the In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics).

In situ hybridization

Detection of *upd* mRNA was performed by the NBT/BCIP enzymatic amplification reaction, which produces a chromogenic staining, following the protocol described in (Milán et al., 1996). The Dig-labeled antisense RNA probes were generated and kindly provided by F. Serras (Santabárbara-Ruiz et al., 2015).

Measurements and statistical analysis

For each independent experiment, a control using the same number of UAS-transgenes was raised in parallel. For each experiment and control, the corresponding mean and standard deviation was calculated, and a two tailed unpaired *t*-test assuming equal variances was carried out in Microsoft Excel, *p<0.05; **p<0.01; ***p<0.001. p-value < 0,05 was considered

statistically significant for all analyses. Graphical representations of data were done using GraphPad Prism version 6.07.

Quantification of tissue size

(1) Quantification of the wing discs P/A ratio in JAK/STAT depleted discs: flies were allowed to lay eggs at 25 °C overnight, resulting larvae of the genotype *en-gal4, UAS-GFP; UAS-dome^{DN}* were transferred to 29°C and wing discs were dissected in late third instar stages. The size of the A compartment and of the whole wing primordium were measured using Fiji Software and the size of the P compartment was obtained by subtracting the A compartment size from total wing disc size. The number of wing discs analysed for each experiment are indicated in the corresponding figure legends. Since the P/A ratio of late third instar wing discs is largely constant, the same dataset (n=36) of the genotype *en-gal4, UAS-GFP/+* was used as a *wild type* control.

(2) Quantification of the wing discs P/A ratio in wing discs overexpressing Engrailed: overexpression of UAS-en was temporally controlled to bypass embryonic lethality. Flies were allowed to lay eggs at 18 °C overnight, and *hh-gal4, tub-gal80^{TS}/UAS-en* larvae were shifted to 29°C in mid third instar (7 days AEL). Wing discs were dissected 72 h later and the P/A ratio was measured as described above (1). Since the P/A ratio of late third instar wing discs is largely constant, the same dataset (n=17) of the genotype *UAS-GFP/+; hh-gal4, tub-gal80^{TS}/+* was used as a *wild type* control.

(3) Clonal area measurements: the Fiji Software, was used to measure the size of the A compartment and of the whole wing primordium. The size of the P compartment was obtained by subtracting the A compartment size from total wing disc size. The clonal area that covers each compartment was obtained by measuring the area devoid of GFP expression in each domain

with a macro for the Fiji Software provided by the Advanced Digital Microscope Facility at the IRB Barcelona. The clone area/compartment area ratios were calculated.

Quantification of fluorescent signal intensity

Control (n=18) and experimental (n=23) wing discs were fixed and stained together to avoid variability between discs. Samples were imaged under identical settings using a Leica SP5 confocal microscope. Confocal conditions were adjusted to minimize saturated pixels with maximal intensity. To quantify GFP and Gal4 expression levels in the posterior compartment, average signal intensity per pixel was obtained from raw images using the histogram function of Fiji Software. The corresponding mean and standard deviation was calculated, and a two tailed unpaired *t*-test assuming equal variances was carried out in Microsoft Excel. ***p<0.001. Graphical representations of data were done using GraphPad Prism version 6.07.

Quantification of cell death

Images from basal planes were considered for the determination of the number of cells labeled by TUNEL in the P compartment, and absolute numbers of apoptotic cells were quantified with the Fiji Software. All genotypes were analyzed in parallel. The corresponding standard deviation was calculated, and a t-test analysis was carried out. *p<0.05; **p<0.01; ***p<0.001. Graphical representations of data were done using GraphPad Prism version 6.07.

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Supporting Tables

% Duplicated nota			
Genotype	n heminota	Duplicated heminota	% Duplicated nota
sd>dcr2; dome^{RNAi}; +/+	102	81	79,4
sd>dcr2; dome^{RNAi}; wg^{CX4}/+	106	97	91,5
sd>dcr2; dome^{RNAi}; Egfr^{F2}/+	197	77	39,1
sd>dcr2; dome^{RNAi}; lro^{EGP7}/+	180	5	2,8

Table 1. Quantifications regarding Figure X. Percentage of duplicated nota of the indicated genotypes. Absolute number of total (sample size) and duplicated heminota for each experiment are indicated.

P/A Ratio				
Genotype	n	Average	Std Dev	p-value
tub-Gal80^{TS}, hh>GFP (eL3) @29ºC	23	0,40	0,06	-
tub-Gal80^{TS}, hh>dome^{DN} (eL3) @29ºC	26	0,13	0,06	$4,5 \times 10^{-21}$
tub-Gal80^{TS}, hh>GFP (wL3) @29ºC	17	0,63	0,03	-
tub-Gal80^{TS}, hh>dome^{DN} (wL3) @29ºC	34	0,21	0,09	$9,2 \times 10^{-23}$
tub-Gal80^{TS}, hh>dome^{DN} (wL3) @29ºC + 4days@18ºC	42	0,50	0,06	$5,1 \times 10^{-12}$

Table 2. Quantifications regarding Figure X. Average values of the ratio between Posterior and Anterior compartment size with their corresponding standard deviation. Sample size (n) for each experiment is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

Clone/Compartment (% area) ± Std Dev					
Genotype	n	Anterior	p-value	Posterior	p-value
FRT Minute (+)	45	52 ± 16	-	59 ± 21	-
FRT Minute (+) stat92E^{85c9}	102	40 ± 13	$5{,}4 \times 10^{-6}$	21 ± 17	$4{,}2 \times 10^{-22}$

Table 3. Quantifications regarding Figure X. Average values of the percentage of clone area per compartment area with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance. The differences between Anterior and Posterior in the control clones were not significant (p=0,06) while in the *stat93E* mutant clones the percentage of clone area in the P compartment was significantly lower than in the A compartment ($p=5{,}5 \times 10^{-15}$).

P/A Ratio				
Genotype	n	Average	Std Dev	p-value
en>GFP	36	0,56	0,04	-
en>2xGFP, dome^{DN}	41	0,13	0,07	$2{,}5 \times 10^{-48}$
en>GFP, dome^{DN}, diap1	30	0,34	0,05	$1{,}1 \times 10^{-23}$

Table 4. Quantifications regarding Figure X. Average values of the ratio between Posterior and Anterior compartment size with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

P/A Ratio				
Genotype	n	Average	Std Dev	p-value
en>GFP	36	0,56	0,04	-
en>2xGFP, dome^{DN}	87	0,15	0,06	$8{,}4 \times 10^{-36}$
en>GFP, dome^{DN}, dronc^{RNAi}	45	0,28	0,07	$3{,}9 \times 10^{-20}$

Table 5. Quantifications regarding Figure X. Average values of the ratio between Posterior and Anterior compartment size with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

P/A Ratio				
Genotype	n	Average	Std Dev	p-value
en>GFP	36	0,56	0,04	-
en>GFP, dome^{DN}	43	0,20	0,09	2,9 x 10^{-36}
en>p35, dome^{DN}	28	0,36	0,07	7,2 x 10^{-12}

Table 6. Quantifications regarding Figure X. Average values of the ratio between Posterior and Anterior compartment size with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

P/A Ratio				
Genotype	n	Average	Std Dev	p-value
en>GFP	36	0,56	0,04	-
en>2xGFP, dome^{DN}	66	0,16	0,09	1,5 x 10^{-43}
en>GFP, dome^{DN}, cycA	92	0,29	0,08	1,5 x 10^{-15}

Table 7. Quantifications regarding Figure X. Average values of the ratio between Posterior and Anterior compartment size with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

P/A Ratio				
Genotype	n	Average	Std Dev	p-value
en>GFP	36	0,56	0,04	-
en>2xGFP, dome^{DN}	47	0,16	0,09	2,1 x 10^{-11}
en>GFP, dome^{DN}, cycB	25	0,16	0,06	0,92

Table 8. Quantifications regarding Figure X. Average values of the ratio between Posterior and Anterior compartment size with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

P/A Ratio				
Genotype	n	Average	Std Dev	p-value
en>GFP	36	0,56	0,04	-
en>2xGFP, dome^{DN}	47	0,16	0,09	1,1 x 10^{-39}
en>GFP, dome^{DN}, en$^{RNAi\text{-}VDRC}$	51	0,29	0,07	3,9 x 10^{-10}

Table 9. Quantifications regarding Figure X. Average values of the ratio between Posterior and Anterior compartment size with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

P/A Ratio				
Genotype	n	Average	Std Dev	p-value
en>GFP	36	0,56	0,04	-
en>2xGFP, dome^{DN}	66	0,16	0,09	1,5 x 10^{-43}
en>GFP, dome^{DN}, en$^{RNAi\text{-}TRiP}$	70	0,21	0,08	0,0015

Table 10. Quantifications regarding Figure X. Average values of the ratio between Posterior and Anterior compartment size with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

P/A Ratio				
Genotype	n	Average	Std Dev	p-value
en>GFP	36	0,56	0,04	-
en>GFP, dome^{DN}, +/+	78	0,13	0,05	2,1 x 10^{-77}
en>GFP, dome^{DN}, Df-en[E]/+	45	0,16	0,05	0,0004

Table 11. Quantifications regarding Figure X. Average values of the ratio between Posterior and Anterior compartment size with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

Apoptotic cells (a.n.)				
Genotype	n	Average	Std Dev	p-value
en>2xGFP, dome^{DN}	19	233	94	-
en>GFP, dome^{DN}, en$^{RNAi\text{-}VDRC}$	29	49	25	$3{,}7 \times 10^{-13}$

Table 12. Quantifications regarding Figure X. Average values of the absolute number of apoptotic cells in the Posterior compartment with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

GFP Intensity/Pixel				
Genotype	n	Average	Std Dev	p-value
en>GFP, RFP	18	35,93	8,93	-
en>GFP, en$^{RNAi\text{-}VDRC}$	23	97,86	19,88	$5{,}9 \times 10^{-15}$
Gal4 Intensity/Pixel				
Genotype	n	Average	Std Dev	p-value
en>GFP, RFP	18	39,14	7,79	-
en>GFP, en$^{RNAi\text{-}VDRC}$	23	64,13	18,27	$3{,}3 \times 10^{-6}$

Table 13. Quantifications regarding Figure X. Average values of signal intensity per pixel in the Posterior compartment with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

P/A Ratio				
Genotype	n	Average	Std Dev	p-value
tub-Gal80^{TS}, hh>GFP	17	0,63	0,03	-
tub-Gal80^{TS}, hh>en	17	0,33	0,03	$2{,}7 \times 10^{-26}$

Table 14. Quantifications regarding Figure X. Average values of the ratio between Posterior and Anterior compartment size with their corresponding standard deviation. Sample size (n) for each genotype is indicated. A *t*-test was carried out to calculate the p-value as a measurement of the statistical significance.

% Discs without Spalt in the wing pouch			
Genotype	n	Wing discs without Spalt	% Wing discs without Spalt
hh>GFP, dome^{DN}	53	21	40
hh>dome^{DN}, hh::GFP	43	6	14
hh>dome^{DN}, diap1	42	2	5

Table 15. Quantifications regarding Figure X. Percentage of wing discs without Spalt expression in the wing pouch of the indicated genotypes. Absolute number of total wing discs (sample size) and wing discs without Spalt for each experiment are indicated.

Abbreviations

A: Anterior

as-cs: achaete-scute

AEL: After Egg Laying

AP: Anterior-Posterior

Ap: Apterous

ara: araucan

BMP: Bone Morphogenetic Protein

Brk: Brinker

BX-C: Bithorax Complex

caup: caupolican

CDK: Cyclin-dependent kinases

Ci: Cubitus Interuptus

CycA: Cyclin A

CycB: Cyclin B

CycE: Cyclin E

D: Dorsal

Dad: Daughters against dpp

Diap1: Death-associated inhibitor of apoptosis 1

DNA: Deoxyribonucleic Acid

Dome: Domeless

Dpp: Decapentaplegic

Dll: Distalless

DP: Disc Proper

dsRNA: double strand ribonucleic acid

DV: Dorsal-Ventral

EGFR: Epidermal Growth Factor Receptor

eL3: early third instar

En: Engrailed

Ex: Expanded

GFP: Green Fluorescent Protein

H: Hinge

Hh: Hedgehog

Hop: Hopscotch

Hth: Homothorax

Iro-C: Iroquois Complex

JAK: Janus Kinase

L1: First larval stage

L2: Second larval stage

lL2: late second instar

L3: Third larval stage

MAD: Mothers against decapentaplegic

mL2: mid second instar

mL3: mid third instar

mRNA: Messenger Ribonucleic Acid

mirr: mirror

N: Notum

Nub: Nubbin

Omb: Optomotor-blind

P: Posterior

PcG: Polycomb Group

PD: Proximo-distal

PE: Peripodial Epithelia

PM: Peripodial Membrane

PntP2: Pointed-P2

Ptc: Patched

RNAi: RNA interference

RFP: Red Fluorescent Protein

Sal: Spalt

Sd: Scalloped

Sens: Senseless

Sgg: Shaggy

Shh: Sonic Hedgehog

STAT: Signal Transducer and Activator of Transcription

Stg: String

TMT: Myristoylated-Tomato

TrxG: Trithorax-group

Tsh: Teashirt

UAS: Upstream Activation Sequence

Ubx: Ultrabithorax

Upd: Unpaired

V: Ventral

Vg: Vestigial

Vn: Vein

W: wing

wL3: wondering third instar

Wg: Wingless