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TITOLO TESI

Wing Shape Evolution:
A Role for Cell Competition in Shaping the Proximal Distal Axis of *Drosophila* Wing.

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GENERAL INTRODUCTION

Drosophila melanogaster (Fruit fly) has from nearly a century a fundamental role in the understanding of the basis of genetic mechanisms, due to the relative semplicity of its genome, to the short life cycle, to the abundance of the progeny and to the great quantity of genetic markers of its body wall.

From the pioneering studies of Thomas Hunt Morgan and the members of his laboratory, the fruit fly has early become the most characterized model organism for genetic studies.

The development of mutagenesis techniques by nobel prizes Christianne Nüsslein-Volhard and Eric Wieschaus has permitted the isolation and characterization of a large number of genes involved in each step of the early development.

Antonio Garcia-Bellido, John Merriam and Peter Bryant, by clonal analisys techniques have characterized the basis of the genetic programs involved in the development of the adult appendages.

With the development of molecular biology techniques and by the genome sequentations, this animal represents today an excellent model to understand the genetic mechanisms at the basis of almost all metazoan development.

The fruit fly is an holometabolous organism, characterized by a life cycle that starts from an embryonal stage, made inside the egg, followed by three larval instars (L1, L2, L3), during which the larva increases its dimensions. At the end of the third larval instar, at the pupal stage, a catastrophic metamorphosys occurs characterized by the histolysis of the larval tissues and by imaginal tissues differentiation, responsible for the adult appendages definition.

At the end of this stage the adult insect (also called *imago*) ecloses.

The life cycle of a wild type organism is about ten days at 25°C.

OOGENESIS

The oogenesis is the process that drives the fomation of the oocyte in the ovary.

Each ovary is composed by 10-17 ovarioles, in wich the most apical portion is called germary, that is responsible for the creation of pro-oocyte cells. They migrate basally in a region called vitellary, where they reach the maturation before the release into the oviduct.

The oogenesis begins in the germary, in which a germinal stem cell will divide, generating another stem cell and a cytoblast that in turn originates the pro-oocyte and the nurse cells.

The oocyte and the nurse cells are surrounded by somatic cells called follicular cells; these three kinds of cells together build the egg chamber (Carpenter, 1975).

The *Drosophila* oocyte development is subdivided in 14 stages (King,1970).

At the beginning of stage 8 the vitellum accumulation occurs in the oocyte, that rapidly increases at the expenses of the nurse cells; as a result at the end of oogenesis the egg chamber is entirely occupied by the oocyte.

EMBRYOGENESIS

Drosophila embryo develops immediatly after the egg laying (AEL), and it is characterized by a superficial meroblastic segmentation.

The mature oocyte is quiescent at the metaphase of the first mitotic division of the meiosis until fertilization. After that, male and female pro-nuclei perform a sincronous division followed by fusion, that generate the zygote.

The first 12 rounds of cell division are syncytial and lead to the syncytial blastoderm formation; at the end of this stage a large number of nuclei migrate to the embryo surface (Foe and Alberts, 1983), with the formation of the polar cells, successively there is the cellularization of the nuclei and the beginning of the blastodermal stage with the creation of the somatic and germinal cell lines (the second derived by the polar cells), 6.3h AEL (Glover 1991).

This is a key step because while the early stages are governed by the maternal genome, from this moment the development is driven by the zygotic genome.

During the successive phases of gastrulation, major migratorial events (medioventral furrow and cefalic furrow) occur, generating mesodermal structures and the head region. During this stage, mesoderm is subdivided in splancnopleura, responsible for visceral muscles, and somatopleura, from wich all the other mesodermal structures originate (muscles, circulatorial system, fat bodies and somatic components of the gonads).

Nervous system is constituted by a series of elements distributed longitudinally to the embryo body.

Successively to the formation of the germinative "stria", there is the invagination of the polar cells and other structures, and the formation of the tracheas.

The last morphological event is head involution (Fullilove and Jacobson, 1978). The mascellar, mandibular and labial segments of the larval head and the cells of the imaginal discs that are external invaginate inside the embryo, and this process is followed by an external cuticular deposition and by the differentiation of internal organs (Wieschaus and Nüsslein-Volhard, 1986).

LARVAL AND PUPAL DEVELOPMENT

After 23h AEL the L1 larva emerges; the three larval instars are spaced out by moults and followed by metamorphosis.

Moults and metamorphosis are governed by ecdyson peaks, a steroid hormon produced by the ring gland.

The larva is characterized by two cellular types: larval cells, that are polyploid, and imaginal cells that are diploid.

Imaginal cells segregate precociously from the surrounding larval cells, forming small cell groups at 9-10h AEL and are organized in two fundamental groups, imaginal discs and abdominal histoblasts.

Imaginal discs begin an intense proliferative activity from the second larval instar until pupariation, while abdominal histoblasts proliferate later, during the pupal stage. At this moment the majority of larval cells are eliminated and substituted by imaginal cells that originate the integument and the adult appendages.

Imaginal discs originate the structures of the head, thorax external appendages, genitalia and adult muscles. The histoblats originate the abdomen structures at the exception of the 8th segment that derivates from the genital imaginal disc.

During the metamorphosis three important steps occur:

1.elongation

2.eversion of the imaginal structures

3.enlargement and fusion of the imaginal structures, with the formation of a continuum epithelium (Fristrom and Fristrom, 1993).

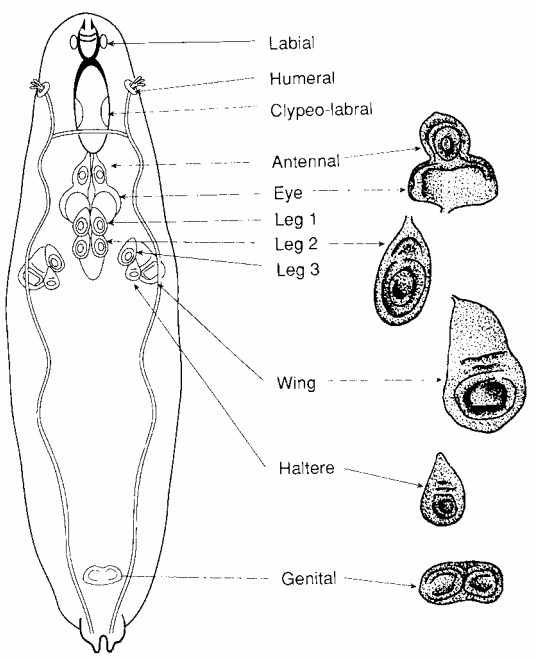


Figure 1 – Localization of the imaginal discs in the larva at the L3 instar (Bate e Martinez-Arias, 1991).

Wing imaginal disc structure, specification and development

Each imaginal disc at the end of larval development is constituted by a pseudostratified columnar epithelial tissue, that represents the actual imaginal disc, and by a squamous epithelium that forms the peripodial membrane.

The first one originates the integument and the appendices, the second one originates the epithelial veil that welds the structures derived from different imaginal discs.

Each imaginal disc is located in a precise position inside the larval body.

One interesting feature of the imaginal disc is the possibility to transplant it in adult abdomens, in which they can survive, metamorphose and differentiate (Schubinger et al, 1969; Simcox et al., 1989).

Fragments of imaginal discs can regenerate the entire disc structure and experiments in this direction have allowed the characterization of "fate maps" of regeneration (Bryant, 1978).

The wing imaginal disc is an excellent model for the elucidation of organogenesis and proliferation mechanisms; at the end of L3 it is subdivided in a series of folds. The centrifugal regions of the imaginal disc originate the thorax structures, the notum and the pleura. The middle region originates the hinge region while the central region is the presumptive territory that makes the wing lamina.

Imaginal tissues are virtually two-dimensional structures, but they originate adult appendages with three axes (AP, DV, PD); this is due to a mechanism of eversion during methamorposis, in which the most central regions of the disc originate the distal structures (wing from distal to proximal), while the external regions originate the most proximal structures of the appendage.

During the embryogenesis the action of the segment-polarity genes generate parasegmental restriction boundaries and positional information for the specification of the imaginal discs primordia (Choen, 1990).

The patterned expression of the selector gene *engrailed* is present in the anterior region of the parasegmental compartment and in the posterior region of the wing imaginal disc, suggesting a derivation from two different parasegments (Kornberg, 1981a, 1981b; Kornberg et al., 1985; DiNardo et al., 1985; Ingham, 1985)

Imaginal discs primordia are constituted by 20 founder cells, identified by the expression of specific markers.

The direct source for the genesis of positional signals for the founder cells is Wingless. Without this signal, no imaginal structures arise.

Escargot (Esg) is a marker of all the primordial imaginal cells and it is indispensable for the diploid maintainment.

Several indications exist that wing imaginal disc originate ventrally and represent a branch of the second leg imaginal discs (Cohen,1997). The activation of differential selector genes separates the two structures early making them

become independent, and the imaginal disc grows dorsally, while legs are ventral appendages.

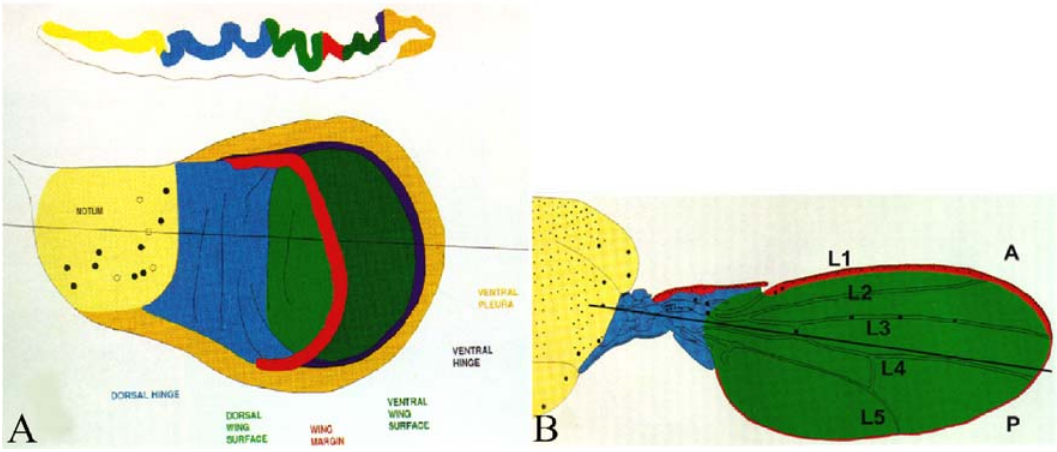


Figure 2 – Schematic representation of the wing imaginal disc at L3 instar (A) and of the adult wing (B). Different colors in the figure A indicate the presumptive territories of the structures visualized in B (from Bate and Martinez-Arias, 1991).

The wing

The wing is composed of a plate of epithelial bi-stratified cells in which a ventral and a dorsal surface are present.

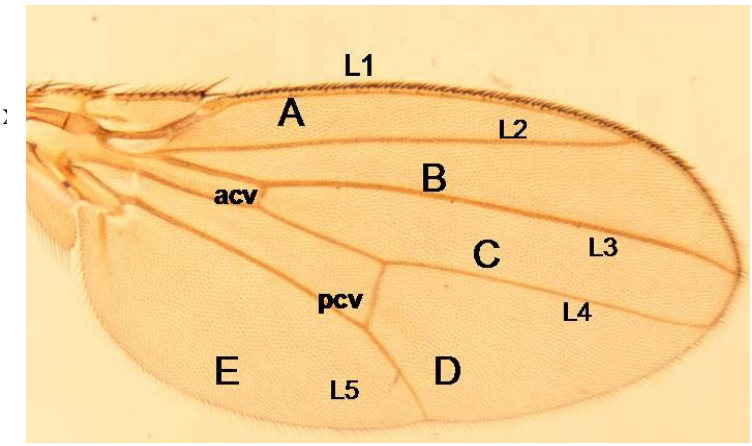


Figure 3- Vein and Intervein Territories of *Drosophila* wing,
Longitudinal veins are five and are called L1-5; there are also two cross-veins, the anterior (acv) and posterior (pcv) cross-veins. Intervein compartments are also five and named A-E.

The proximal region is called the hinge, and it connects the wing to the thorax, dorsally (*notum*) and ventrally (*pleura*) respectively.

The wing lamina is called wing blade, and in the anterior border it is provided with three series of sensorial bristles. The wing blade is constituted by two types of cells: vein and intervein.

Veins are constituted by alive epithelial cells that form tubular structures that confere stiffness to the wing and accomodate tracheas and neuronal sheafs.

Intervein cells are dead and chitinized, and are characterized by the differentiation of a single trichome for each cell.

DEVELOPMENTAL IMAGINAL DISC PROGRAM: AXES AND BOUNDARIES

Transplantation assays demonstrated that developmental patterning is not determined by external influences but by a disc-specific intrinsic developmental program. Originally defined in the wing disc by Antonio Garcia-Bellido in 1973, the compartments are the basic components of the *Drosophila* body plan. In genetic terms they are parts of the body that originate from the same cell lineage. From the beginning of the embryogenesis, as a consequence of the pair rule gene expression and segmental differentiation of embryo, the primordia of the imaginal cells of the wing contain two separate cell lineages, which form the anterior and posterior compartments.

This is a consequence of the patterned activation of the homeobox gene *engrailed* (en), which segregates anterior and posterior compartment cells. Antonio Garcia-Bellido in 1973 showed by clonal analysis the existence of a restriction boundary between the anterior and posterior regions of the wing imaginal disc that clones of cells can't cross during proliferative stages.

The discovery of compartments surely plays a pivotal role in the developmental genetics of the past 35 years and the use of mitotic analysis is yet today the war horse of developmental geneticists.

The existence of restriction boundaries in the *Drosophila* wing imaginal disc allowed to elaborate a series of developmental models about the genetic program

involved in morphogenesis. The Entelechia model by Antonio Garcia-Bellido proposes that the restriction boundaries are the organizers of developmental programs responsible for the achievement of correct size and shape of the organ, by the activation of specific hypotesized genes called “martial genes”. The expression level of those genes is gradual and decreases from the boundary. Cells respond to martial genes information and stop dividing when reach the limiting quantity of signal. (Garcia-Bellido and De Celis, 1992; Garcia-Bellido and Garcia-Bellido, 1998).

The other current model is the morphogen one, in which diffusible factors generated along the restriction boundary (Dpp or Wingless for example), generate positional cues that cells detect as the quantity of factor received. A different distance from the boundary is responsible for diverse abundances in factor caption and this gradient drives cell proliferation, allocation and survival (Wolpert, 1969; 1971).

After the characterization of the AP restriction boundary, other boundaries where discovered. The DV boundary, formed by the activity of Notch and Wingless and the less canonical proximal-distal boundary that separates the wing blade from the hinge and the hinge from the thorax.

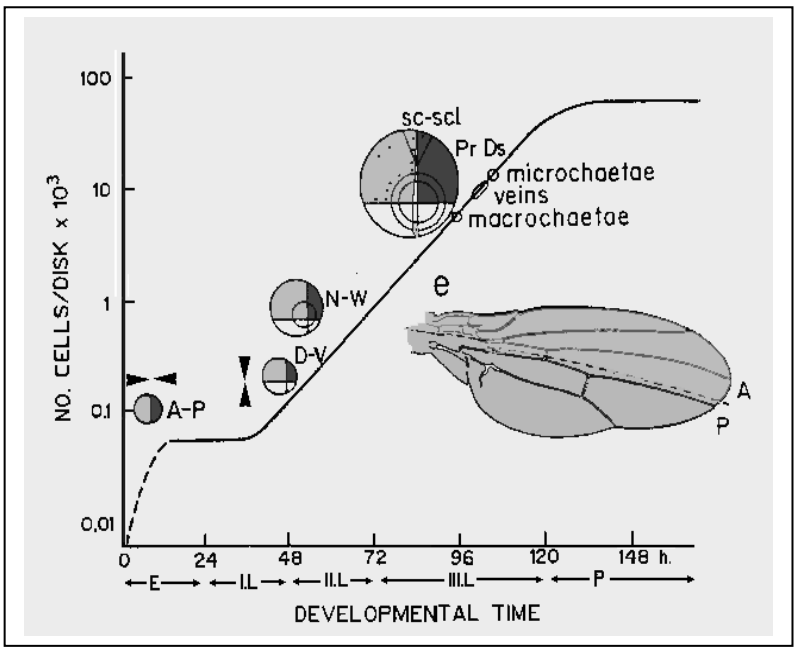


Figure 4 – Clonal restrictions and developmental times (from Garcia-Bellido and Mari-Beffa, 991).

Anterior-Poserior Determination

One of the first events in the imaginal disc development is the formation of the Anterior Posterior polarity, as a consequence of the activation of the gene *engrailed*. *engrailed* is expressed in all the posterior compartment and its protein induces the expression of *hedgehog* (*hh*) in the whole posterior compartment (Tabata and Kornberg TB, 1994; Zecca, Basler, Struhl G, 1995).

Hh protein is a morphogen that spreads in a line of cells along the anterior-posterior border where it induces the expression of another morphogen, Dpp (Tabata and Kornberg TB, 1994; Zecca, Basler, Struhl G, 1995).

This process is the basis of a complex regulative pathway, that leads to the activation and the repression of a large number of genes complexes. The final result is the definition of the anterior-posterior axis and the definition of positional information that leads to the creation of the anterior-posterior boundary and the specification of the vein and intervein regions.

Dorsal-Ventral Determination

Dorsal-Ventral polarity is the second axis of polarity to form in a temporal succession. The definition of this axis occurs at the beginning of the second larval instar (Garcia-Bellido, 1973).

The dorsal-ventral restriction border originates through the action of the selector gene *apterous*, that is expressed only in the dorsal compartment (Diaz-Benjumea and Cohen, 1993; Blair, 1993).

Ap protein activates *fringe* in the dorsal compartment (Irvine and Wieschaus, 1994), a secretion factor that induces the activation of Serrate (Kim et al.,1995), a Notch ligand (Rebay et al.,1991), and inhibits the expression of Delta, another Notch ligand (de Celis et al., 1996; Milan and Cohen, 2000).

Fringe is a glycosil transferase that modifies Notch receptor, inhibiting its affinity for Serrate but increasing its affinity for Delta, that in turn increases the affinity for Notch.

The final picture is that dorsally Serrate is inhibited by the activity of Fringe, while ventrally Delta can't activate Notch.

The only region where Serrate and Delta can both activate Notch is at the boundary, where Apterous is expressed in two cell lines (de Celis, 1996).

Notch activates the expression of *wingless*, *cut* and *vestigial* and, as a consequence, the DV restriction boundary is formed and the wing blade presumptive territory begins to be defined.

Proximal-Distal Determination

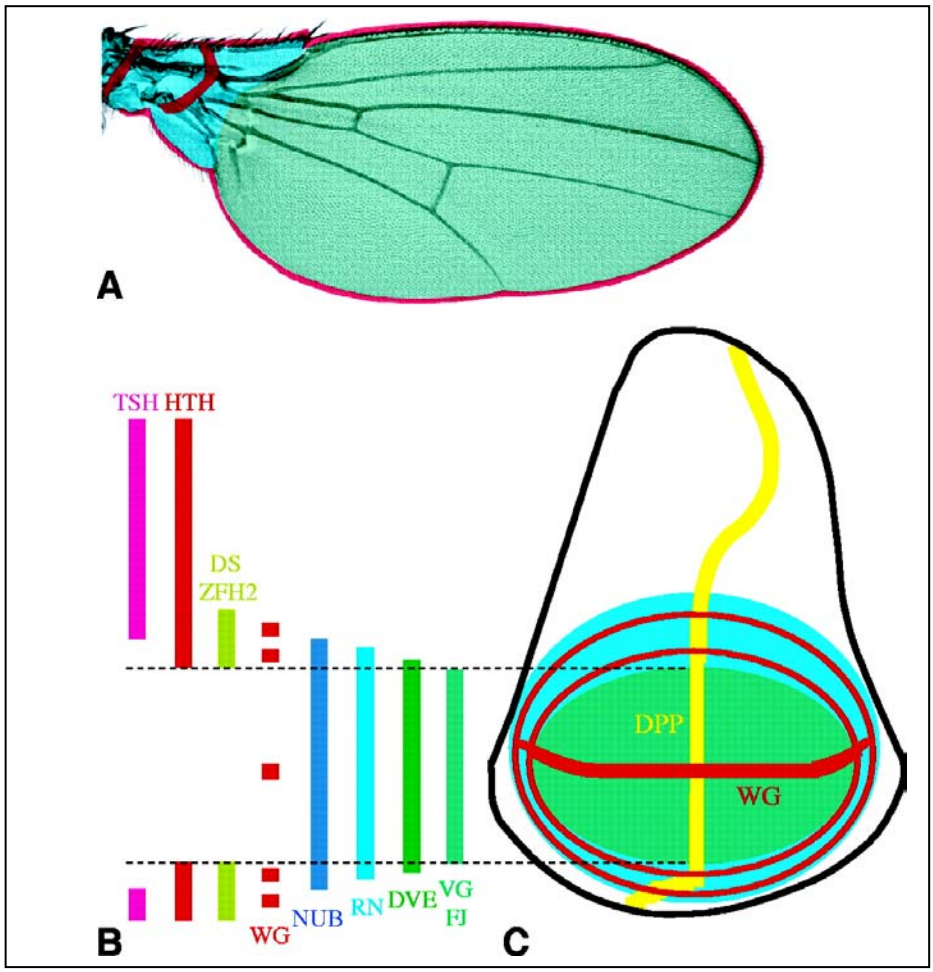


Figure 5 - Genes involved in Proximal Distal Axis formation show a circular patterning (Cho et al. 2004)

While much is known about the development of the patterning mechanisms that specify the A/P and D/V axes, very little is known about the determination of the P/D. The formation of the three structures of this axis (notum, hinge, wing

blade) is due to the sequential activation-repression of a moltitude of selector genes, in response to the formation of the AP and DV axes (*engrailed*-AP axis and *apterous*-D/V axis) and to the generation of morphogen activity at the boundary of the two axes (Dpp at the AP border and Wg at the DV border).

The correct wing patterning requires the activity of Wg morphogen; in fact the reduction of Wg function causes a complete loss of wing structures and notum duplication (Morata and Lawrence 1977).

Wg expression begins in a ventral-anterior wedge of the early second instar wing disc and is extremely dynamic throughout the larval stages (Couso 1993; Ng 1996). At this stage, after the formation of AP and DV axes, another subdivision occurs in the formation of the proximal-distal axis that subdivides the wing disc in the Notum, Hinge and Blade regions (Klein, 2001). The activity of Wingless promotes the repression of *teashirt* homeobox gene that produces the imaginal body wall formation, and *vg* expression (Wu and Cohen, 2002).

At the second instar Wg activity is repressed in the Dorsal and Posterior compartments by the activity of EGFR (Baonza, 2000), the EGFR activity promotes the specification of the notum identity in the proximal region by the activation of the *iroquois* complex (Iro-C) genes (Zecca and Struhl, 2002), ectopic expression of EGFR at this stage causes a notal duplication similar to the Wg loss (Wang SH, Simcox A, Campbell G: 2000). During the second instar, the combined activities of Wg and Notch signalling pathways induce the expression of the nuclear protein Vestigial (Vg), wich is essential for the wing blade development. Vg is firstly just along the D/V boundary by the activation of the so called boundary enhancer (vg^{BE}; Couso 1995; Kim 1996: Klein and Martinez Arias 1998, 1999; Neumann and Cohen, 1997; Williams et al, 1991, 1994).

In the early third instar, N, Wg and Vg act together to activate a second enhancer, the vg quadrant enhancer (vg^{QE}), that induces the *vg* expression across the wing pouch (Klein and Martinez Arias, 1998). Later the vg^{QE} is regulated in a dosage-dependent manner by the activity of Wg and Dpp signaling pathways (Kim 1996; Klein and Mrtinez Arias, 1998).

Vestigial is a transcription factor (see also cap1) that acts in a complex with the product of the *scalloped* gene (*sd*). *vg* loss of function prevents the development of the wing blade (Delanoue 2004, van de bor 1999) while its

overexpression may promote cell proliferation (Delanoue et al. , 2004; Halder et al., 1998; Kim et al., 1996; Simmonds et al., 1998; Baena-Lopez et al., 2003).

The integration of the signal derivating by Dpp and Wg-N produces a graded activity of Vg along the proximal-distal axis, and this distribution can trigger local cell interactions between neighboring cells, globally modulating the growth and shape of the wing blade (Baena-Lopez, 2006), so generating positional information.

Wg is also required for the correct development of the proximal region of the wing (Neumann and Cohen, 1996; Witworth and Russell, 2003), where Vg is not expressed. This leads to the activation of the homeobox gene *homothorax* (*hth*) by Wg activity. Here Hth plays a dual role, to limit the size of developing distal wing, by repression of target genes, and to upregulate Wg expression (Azpiazu and Morata, 2000; Casares and Mann, 2000).

A key step in the distal identity formation is the extreme dynamics of pattern expression of Wg, that change during the third instar; in particular Vg acts in the activation of Wingless expression in a ring of surrounding cells expressing the Sd-Vg complex (Liu et al., 2000; del Álamo Rodriguez et al., 2004) .

At the end of the third larval instar Vg is expressed in the distal part of the wing while Wg is expressed in the proximal region of the wing in two concentric rings, one mediated by the Vg-Sd activity and another due to the activation of an enhancer called *spad-flag* (Neumann and Cohen, 1996).

In particular, Wg activity in the proximal region promotes the initial expression of *zfh2* homeodomain containing gene, a proximal determinant, and the *homothorax* expression (Witworth and Russell, 2002; Azpiazu and Morata, 2000); the function of Wg is fundamental for the identity, proliferation and survival of the proximal region of the wing (Neumann and Cohen, 1996; Johnston, 2002).

Recapitulating, at the first larval instar there is the subdivision of the wing region in the notal *versus* wing structures due to the repression of *teashirt* in the distal region by Wingless; successivley, during the second instar, the hinge region is defined by the repression of *teashirt* and by the activation of *homothorax* by Wingless, and wing identity results from the activation of the selector gene *vestigial* (due to the integration between Dpp and Wg-N signaling pathways).

The events that drive the proximal-distal axis formation in the wing blade region are poorly understood. There are a series of mutant conditions that lead to a reduction of specific regions along this axis: *rotund* (*rn*) (Kerridge and Thomas-Cavallin, 1998) mutation leads to the reduction, mutations in *fat* (*ft*), *dachsous* (*ds*), *approximated* (*app*) and *four jointed* (*fj*) interfere with the development of medial and proximal regions of the wing (Lindsley and Zimm, 1992; Garoia et al., 2002-2004). *nubbin* is responsible for a reduction of the distal regions of the wing (Cifuentes and Garcia-Bellido, 1997).

Clonal analysis experiments have failed to detect restriction boundaries between proximal and distal regions of the wing blade, nevertheless the selector gene *nubbin* appears to have an important role in the development along this axis. At the end of L3 *nub* is expressed in the region of the wing blade (Cifuentes and Garcia-Bellido, 1997).

Clonal analysis has revealed that *nubbin* mutant clones, even if small, may affect the entire P/D axis pattern when arising in the proximal region of the wing; on the contrary distal clones do not (Ng and Garcia-Bellido, 1998), suggesting the existence of a proximal-distal organizing center in the proximal region.

In the determination of proximal and distal elements of the wing, Wingless and Vestigial act in the regulation of *homothorax* and *teashirt*. The interaction between Wg and Vg expression is important for the differentiation of wing blade structures, while the action of Wingless alone is important in the hinge definition (Klein and Martinez-Arias, 1998).

Wg appears to have a dual role. While in the distal region of the wing it contributes in the wing blade formation, in the proximal region Wg and Teashirt act together in repressing Vg and therefore the expansion of wing blade territory (Casares and Mann, 2000).

In the hinge and proximal wing structures fomationm, *dachsous* has been seen to be determinant, and appears to act as a Wg modulator. *ds* LOF mutants often show notum duplication instead of the wing blade, similarly to the phenotype commonly observed in Wg LOF mutants (Rodriguez, 2004).

CELLULAR DYNAMICS INVOLVED IN GROWTH AND SHAPE CONTROL IN THE IMAGINAL WING DISC.

Cell proliferation

The wing imaginal disc represents an outstanding model in the understanding of proliferative mechanisms involved in organognesis. This structure is indeed characterized by a rapid growth that, from few initial cells (20-30), in almost 120 hours allows to reach a final number of 50.000 cells, with a proliferative rate of about 8.5 hours/cell division.

The proliferation is intercalar and exponential in almost all the wing pouch (Garcia-Bellido, 1965; Resino et al., 2002). Imaginal disc cells show a sinchrony of divisions and growth preferentially in the proximal to distal direction (Milan et al.,1996).

Final shape and dimension are genetically programmed, but the mechanisms are poorly know. The postulated models that try to explain the spatial control of the proliferation inside the discs are the morphogen model, proposed by Peter Lawrence, and the Entelechia model proposed by Antonio Garcia-Bellido.

According to the first model, cell proliferation follows the signals dictated by diffusible morphogens originated along the restriction boundaries; in the second model, direct interactions between cells mediated by adhesion molecules are involved in which Fat is a possible candidate (Le Cuit and Le Goff, 2007). Still today proofs in favour or in discussion of these two model are provided, and none can be discarted in favour of the other.

Mutations in cell cycle components have an effect on cell growth without affecting final size and shape of the disc. Weigman and collaborators (1997) stopped mitotic divisions specifically in the anterior compartment of the wing disc inactivating the cdc2 kinase, a mitosis promoter. The result was that imaginal disc with normal dimensions and shape grew in which the anterior compartment was composed of fewer but larger cells.

Neufeld and collaborators (1998) induced mophogenetic clones overexpressing cell cycle regulators: cycE and CDC25 or the gene E2F. In this case mitotic rate is increased, producing more cells of reduced size, preserving the compartment size and shape.

These results show that genes that affect cell cycle can affect cell number or cell size without affecting the final size and shape of the disc.

Experiments on the *Minute* mutation allowed to discover the phenomenon of *cell competition* (discussed more in detail bellow) an important mechanism of compensation involved in the homeostasis of organs.

The insulin pathway (Inr) is a cell autonomous process involved in cell growth control. Manipulations at different levels of this pathway can affect both cell proliferation and cell growth. Loss of function mutations of the Inr gene lead to a delayed development with a smaller final dimension of the fly, less and smaller cells (Böhni et al., 1999).

Mutations of the gene S6K, involved downstream in the Insulin pathway, lead to smaller individuals in which the reduction is only due to a smaller cells.

Other mutations are of interest in cell cycle control, for example *ras1*, *cycD/cdk4* and *myc* that are discussed in detail in this thesis.

Finally, transmembrane cadherins and cytoplasmic associated catenins hare the fulcrum of adherens junctions, and are responsible for cell shape, because they are a link between cell membrane and cytoskeleton.

Classical cadherin mutations are often associated with cancer onset, and Fat and Dachsous, two protocadherins, are involved in proliferation control.

Spatial control of proliferation. Oriented cell division and cellular reallocation.

Clonal analysis has highlighted that cell divisions in imaginal discs are intercalar (Garcia-Bellido, 1994), this means that proliferation is not generated in specific regions to follow migratory events, but it is homogeneous inside almost all the disc.

This process is cell autonomous and small fragments of imaginal disc can regenerate an entire compartment (Briant, 1975). Despite that, a series of non cell autonomous interactions between mutant and wild type cells in mosaics show effects of accomodation, demonstrating that cell proliferation is the result of collaboration between neighboring cells (Resino, 2004).

Imaginal disc cells appear organized in small groups, not clonally related, that during the disc growth proliferate sincronically; these groups are not fixed and often change during development (Milan et al., 1996).

Cell-cell contacts are fundamental in those processes, and coordinate the growth of this monostratified epithelium. Cell junctions are indeed docking structures for a multitude of tumor suppressor genes (TSG).

Clonal analysis has permitted to understand the lineage of single marked cells during development, finding that clone shape is related to organ shape (Garcia-Bellido, 1994; Resino et al., 2002; Dolan et al. 1998).

In the wing, clones of cells show an elongated shape, and grow prevalently in the proximal-distal direction (Resino, 2002). In 2005 Luis Alberto Baena-Lopez showed that this situation was largely determinated by the orientation of mitotic divisions (OCD), and by the fact that after dividing, cells maintain their position (Baena-Lopez et al.,2005). Cells of the wing blade proliferate in PD direction with the exception of the wing margin in which cells proliferate in DV direction.

Mutants that affect OCD also affect clone shape that appears rounded, and the entire organ shape, that appears affected in the proximal-distal direction.

Genes involved in this process are often involved in planar cell polarity (PCP), but the two processes appear indipendent (matakatsu, Strutt, lawrence).

Also in this process *fat* and *dachsous* are implicated, underlining the great pleiotropy of these genes and the importance of cell adhesion in communication among cells.

ft and *ds* mitotic clones show rounded shape instead of the wild type elongated shape, and grow prevalently in the proximal wing regions while the wild type twin grow preferentially in the distal direction (Garoia et al., 2000-2004).

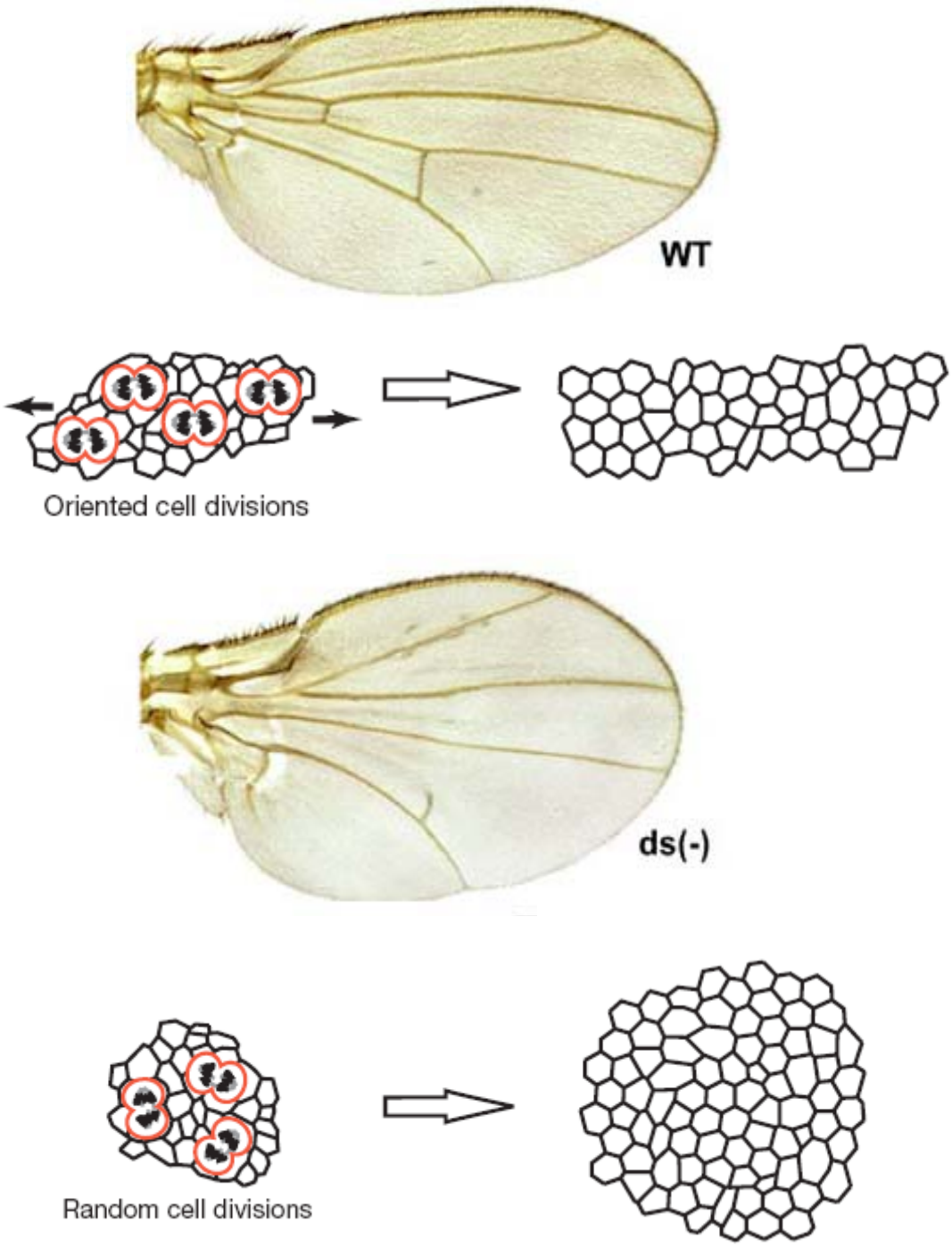


Figure 6 – Oriented Cell Division (OCD) vs Random Cell Division in the definition of organ shape

Cell competition

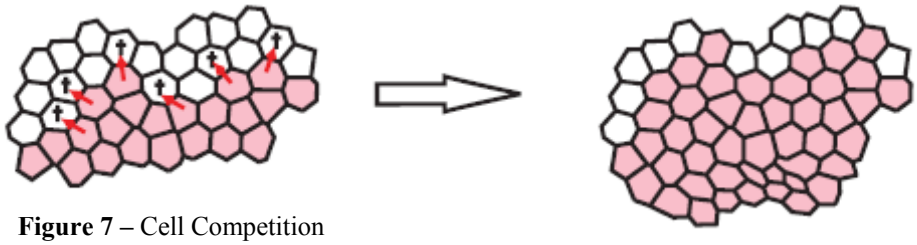


Figure 7 – Cell Competition

Cell competition was discovered in imaginal discs over 30 years ago (Morata and Ripoll, 1975), in which cells with a lower dividing rate, although viable, were eliminated by flanking faster dividing cells.

The *Minute* mutation is subjected to cell competition. Homozygous cells (M/M) die but heterozygous (M/M+) are viable, but with a lower dividing rate. When M/M+ cells are flanked by wild type cells (by mitotic recombination), *Minute* heterozygous cells are eliminated through apoptosis and the process is called "Cell competition" (Cytagon).

There are more than 60 *Minute loci* in the *Drosophila* genome, each showing, if mutated, growth delay due to some defects in ribosomal proteins or activity. Mutations at the *diminutive locus* (*dmyc*) of the homologous human oncogene *c-myc,* has also been shown to trigger cell competition (for review see Moreno, 2008).

Clones of epithelial cells carring a *dmyc* hypomorfic condition are outcompeted and die if flanked by a *wt* twin. By contrast, the same cells are viable when allocated within cells with the same hypomorphic condition.

Many other genes have been shown to regulate cell competition, in some cases genes regulating *dmyc* activity. Those genes include the homologues of the retinoblastoma family (Rbf), of the E2F family of transcription factors, of the Ras family of proto-oncogenes. Members of the decapentaplegic (Dpp) signaling pathway such as the transcription factor Brinker (Brk) or the Dpp receptor Thickveins (Tkv) might also trigger cell competition, and it is not clear if

components of the insulin pathway are also involved (De la Cova, 2004; Moreno and Basler, 2004).

It is possible that cell competition may act through different mechanisms and does not use a universal pathway, but this is not currently known.

Interestingly, outcompeted cells seem to have a decreased activation of survival signaling pathways, in particular the Dpp pathway (Moreno 2004).

Moreno, Morata and Basler have formulated the ligand-capture hypothesis, which proposes that cells compete for the active uptake of extracellular survival and growth factors, and winner cells may inhibit or limit the loser cells in the uptake of survival factors, eventually causing their death.

However this mechanism is not clear, and evidence suggests that ligands cells compete for are not in a limiting supply, for example Dpp (moreno 2007).

Cell competition is not a passive mechanism in which winner cells induce loser to suicide, and cell culture experiments suggest that soluble factors are involved secreted by both winner and loser populations of cells (Johnston 2007).

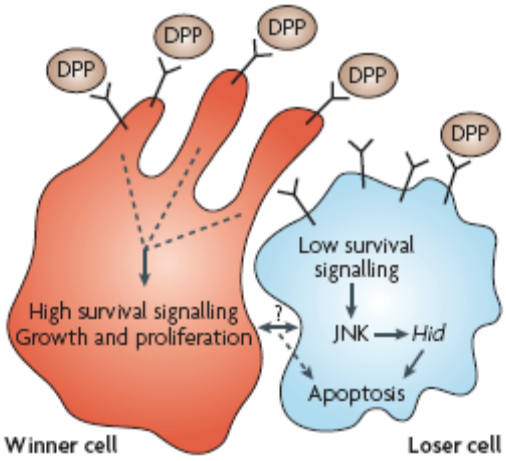


Figure 8 – The ligand capture model (Moreno 2007)

Cell competition apoptosis is mediated by Jun N-terminal kinase (JNK) and the proapoptotic genes *hid* (also called Wrinkled) and *rpr* (Johnston 2004), and winner cells appear to amplify this death signals through the activation of a mechanism called “engulfment” (Li and Backer, 2007). Cell competition in wing

imaginal disc cannot occur without engulfment and it has been proposed that with this mechanism winner cells can take the position of losing cells by phagocytosis.

Genes involved in engulfment are known (*draper*, *WASp*, *PSR*) but mechanisms through which winner cells activate its machinery remain to be determined.

Notably, cell competition appears to act within a small range of 5-8 cells from the *dmyc* overexpressing cells, but is incapable to cross compartment borders.

Laura Johnston (2004-2007) and Eduardo Moreno (2002, 2004, 2007) showed that cell competition is relevant both to evolution and cancer onset.

The evolutive hypothesis of Laura Johnston is that cell competition may act like a mechanism intrinsic to the imaginal disc or possibly to another tissue involved in the maintainment of developmental homeostasis, conferring a flexibility important to the elimination of misspecified or growth-impaired cells without affecting the final developmental architecture. This is supported by the fact that *dmyc* overexpression leads to cell competition through the substitution of the surrounding wild type tissues, without affecting the final dimension and shape of the organ (2004).

Recently Nicholas Baker has demonstrated, by genetic experiments and imunofluorescence, that *fat* and other genes of the Hippo pathway protect cells from competition (Baker 2006).

Proliferation, oriented cell division and cell competition appear to converge on *fat* and *dachsous* genes, that may play a fundamental role in orchestrating two fundamental mechanisms in organogenesis: the control of organ size and shape.

fat (*ft*), *dachsous* (*ds*) and *four jointed* (*fj*)

Recessive mutations of *fat* tumor suppressor gene leads to hyperplastic extragrowth and morphogenetic defects.

fat gene encodes for a very large transmembrane protein, member of the protocadherins and is constituted by a lot of protein domains: an extracellular portion with 34 cadherin homology domains, 4 EGF-like and 2 LaminiG domains, a transmembrane domain and a cytoplasmic domain (Mahoney et al.,1991).

Cadherins are transmembrane glycoproteins Ca^{2+} dependent that mediate cell adhesion prevalently by homophylic interactions (Tomschy et al., 1996).

This mechanism is essential for the organization of almost all methazoan tissue development and integrity (Hyafil et al., 1981; Gumbiner, 1996).

Cadherins and protocadherins can inhibit cell proliferation acting on cycline dependent inhibitor kinases, interacting with different signaling pathways as the EGFr (Levenberg et al., 1999; Hermiston et al., 1996; Garoia et al., 2004), by cytoskeleton stabilization (Chausovsky et al., 2000) and by planar cell polarity (PCP) control (Muller, 2000; Woods et al., 1997).

Fat also interacts with the Wingless pathway in more than one manner: mutants for *fat* can release more Armadillo (β-catenin) into the cytoplasm (Greaves et al., 1999), and *fat* appears to regulate glycoproteins levels that mediate Wingless spreading (Baena-Lopez, personal communication).

The interaction between Fat and Wingless plays an important role in the proximal to distal axis organization (Cho et al., 2004-2006), Fat can inhibit Wg expression in certain locations in developing wings, and the increased Wg expression in *fat* mutants partially accounts for the hyperplastic growth that occurs in the wing, that can be suppressed by loss of Wg, but only in the proximal region of the wing with a former role in the proximal-distal signaling (Cho et al., 2006).

Viable hypomorphic alleles like ft^1 show anterior to posterior reduction of abdominal structures, larger thorax and a proximal to distal reduction of all the appendages with the presence of planar cell polarity defects (Lindsley and Zimm, 1992).

Alleles like *fat*^{8} or *fat* Gull revertant (*ft*$^{G\text{-}rv}$) (Bryant et al., 1998b) are lethal recessive with a hyperplastic extragrowth of larval imaginal discs, in particular in the proximal region of the wing imaginal disc that shows extra folds but maintain a monostratified structure.

Cell dimensions are generally smaller than the control and during the differentiation show strongest cuticolar posing, probably due to cytoskeleton defects (Garoia et al., 2000).

In *fat*^{8} mutant pupariation is delayed of 3.2 days at 25°C, and the final cell number is about 122.000 against the 50.000 of the wild type (Briant et al.,1988b).

ft^{Gull} is an anthimorphic allele, that in heterozygosis can resemble the viable phenotypes of the other recessive alleles (Mahoney et al.,1991) and can be rescued by the lethal recessive allele *ft*$^{G\text{-}rv}$.

ft is involved into the proximal-distal axis shapening of the appendage development and this role appears to be related to that of another protocadherin, Dachsous, and to a Golgi transmemembrane protein, Four jointed (Villano and Katz, 1995).

ds encodes for a protocadherin similar to Fat, constituted by 27 cadherin domains, a transmembrane domain and a cytoplasmatic domain (Clark et al, 1995). *ds* viable mutants show strong similarities to *ft* viable mutants and the suppression of *ft*^{Gull} phenotype by *ds*^{1} viable allele has suggested a possible interaction (Mohr, 1929).

Heterophyilic interactions between those proteins have been demonstrated (Matakatsu and Blair, 2004), and are singular in cadherins that prevalently interact in a homophylic manner. Ft-Ds interaction is important for cell adesion, for the activation of different signaling pathways, probably involving also the Hippo pathway (Choo et al., 2006, my data) and for the regulation of the planar cell polarity (PCP) in the eye, in the wing (Matakatsu and Blair, 2004-2006) and in the abdomen (Laurence 2007), by the *frizzled* (*fz*) regulation and by direct binding with the co-repressor Atrophin (Fanto et al.,2003).

Remarkably, Dachsous is the only cadherin expressed in a particular pattern in the developing wing, with a preference for the proximal cells representative of the hinge (Clark et al.,1995); low level of *dachsous* are also present in more distal regions with a decrescent intensity from proximal to distal (Strutt and Strutt, 2002; Ma et al., 2003).

Garoia et colleagues (2000) have demonstrated that *fat* clones are allocated prevalently in the proximal region of the wing. *fat* cells grow prevalently in proximal direction, differently from the *wt* cells that grow prevalently in distal direction.

Also the majority of growth defects are localized prevalently in the proximal regions.

ft clones are rounded and planar cell polarity inside the clones is strongly perturbed. Cells tend to be located at the clonal boundary, losing the normal proximal-distal grow direction; this is prevalently due to a loss in oriented cell division direction (Baena-Lopez et al. 2005) responsible for strong defects in the entire organ.

Fat is an EGFR antagonist (Garoia et al. 2004); double clones for fat^{18} and EGFR pathway mutants are smaller and tend to be distributed all along the proximal-distal wing region (Garoia 2000, 2004), and this is an indication that *fat* participates in the differentiation *vs* proliferation program of the imaginal discs.

fat clones fail to grow in a *diminutive* (*dm*) background (Garoia et al., 2004), suggesting a role in cell competition (demonstrated in this thesis).

Fat is the first transmembrane protein recently connected to the Hippo pathway (Bennett and Harvey, 2006; Cho, E. et al., 2006; Silva, E. et al., 2006; Willecke et al., 2006; Tyler et al., 2007) and a dramatic suppression of *fat* mutant overgrowth phenotype can be obtained by removal of just a single copy of *yki*. Thus Fat signaling is highly sensitive to Yki dosage.

fat mutants increase the levels of Cyc-E and dIAP1, like all the genes of the hippo pathway.

Probably the link between Ft and the Hippo pathway is represented by Expanded, but some data indicate that they act in a independent manner (Feng J. and Irvine K.D., 2007).

Fat and Expanded strongly co-localize at apical junctions, but attempts to detect a direct interaction between these two proteins have not yet been successful.

Expanded is absent from the membrane in *fat* mutants. It seems reasonable to suggest that the ability of *fat* to recruit Ex to the membrane enables Ex to signal

efficently to Hippo and Wts, potentiating the hippo signalling (Yin and Pan, 2007).

How Fat receive signals and how it exerts its receptor property is an unknow matter; the only known protein that is suspected to interact extracellularly with Fat is Ds (Matakatsu and Blair, 2006). Cells that express Fat preferentially adhere to cells that express Ds (Matakatsu and Blair, 2004), and the expression of both Ft and Ds is required for the intracellular positioning of proteins that are involved in planar cell polarity as well as proximal-distal patterning.

Dachsous is involved in the specification of the proximal-distal axis in the early wing imaginal disc. At the present it is the only known cadherin in *Drosophila* that shows a spatially restricted pattern of expression in wing imaginal discs from early stages onwards, and it is considered one of the earliest specific markers for the hinge territory (Rodriguez, 2004).

The pattern of expression of Ds is relevant for the control in the proximal-distal axis shape of the wing (see results).

Four jointed (Fj) is a type 2 transmembrane protein localized in the Golgi membrane. It has a specific distal pattern of expression in the wing disc, and *fj* mutants do not to affect the proximal region of wing disc (Villano and Katz, 1995; Brodsky and Steller, 1996). The pattern of expression of *ds* is complementar to that of *four jointed*, and this has been shown to be important for the polarization of the eye in PCP (Zeidler, Perrimon and Strutt, 1999) defects. The transcription of *four jointed* is repressed by Fat activity via the Hippo pathway (Choo et al., 2006), suggesting that Fat is activated by a proximal source, possibly Dachsous.

In a model proposed by Cho and Irvine (2004), *fat*, *dachsous* and *four jointed* act together in regulating the proximal-distal axis development, acting on Wingless and Rotund activity, mediated by Dachs and the Hippo pathway (Cho et al., 2006).

Hippo pathway

The size of developing organs is controlled by cell growth, cell proliferation and apoptosis, and the Hippo pathway has recently been described to be involved in those processes.

An important function of this pathway is to limit imaginal disc size, and loss of function of those genes leads to hyperplastic overgrowth (due to cell growth and proliferation), defects in apoptosis or cell competition and in the oriented cell divisions and Planar Cell Polarity (PCP).

This genetic pathway has been proposed to act like a "size checkpoint" operating in a tissue autonomous manner, sensing the total mass of the organ, rather than the size.

Genes owing to the Hippo pathway can be divided in "core genes" and "accessory genes", the signal from the core converges on the co-transcriptional factor Yorkie.

Hippo pathway core members

Salvador (Sav)

It is a scaffold protein containing a WW domain that can interact with WTS, and a C-terminal SARAH domain that mediates the bond to Hippo (in competition with RASSF). The primary function of Sav is to facilitate the close association of Wts and Hpo kinases, and/or to recruit Hpo to its site of activation (Tapon et al., 2002).

Warts and Hippo

They are both serine/threonine kinases that belong to the nuclear NDR and sterile-20 kinase families, respectively.

Hippo phosporylates Warts, *Drosophila* inhibitor of apoptosis 1 (dIAP1) and Salvador, whereas Warts phosporylates the co-transcriptional factor Yorkie (Harvey and Tapon, 2007). The founding member of this pathway is Warts (*wts*; also known like large tumour suppressor or *lats*), a gene coding for a kinase

similar to the human Dystrophin. Mutations in this gene result in dramatic outgrowths in the epithelial tissues, however cells do not lose their identity and the tumoral behaviour is hyperplastic (Justice et al., 1995; Xu et al., 1995).

The phosporylation of Salvador by Hpo activity leads to a physical contact between Hpo and Wts mediated by Sav scaffold activity.

The successive phosphorylation of Wts mediated by Hpo activates the pathway by the inhibition of the co-activator Yki.

Yorkie

Yki, wich was identified in a yeast two-hybrid screen for interactors of Wts (Huang et al., 2005), is an oncogene that regulates the transcription of genes involved in cell proliferation and apoptosis.

It is the homologue of the human YAP that has been reported to bind and regulate the activity of various transcriptional regulators, including p73, p53BP2 and several TEAD/TEF-type transcription factors. A point of interest in my thesis, successively discussed, is the very recent finding that Yki can bind the TEAD/TEF protein Scalopped, the transcriptional factor also complexed with Vestigial, the selector gene of the wing (Wu S. et al., 2008; Goulev Y. et al, 2008).

Dephosphorylated forms of Yki go into the nucleus where they activate a series of target genes; the sequential activation of the Hpo pathway drives to the phosphorylation of Yki by Wts. This process leads to the functional repression of Yki through its sequestration in the cytoplasm (Huang et al., 2005).

Yki phosphorylated form binds to 14-3-3 protein, *via* a specific motif. 14-3-3 is a protein that leads to the shuttling to the cytoplasm of phosphorylated proteins (Dong et al., 2007).

Activated forms of Yki control the transcription of several genes implicated in cell cycle, like Cyc-E, or in apoptosis repression, like dIAP1, or both, like the miRNA *bantam*. In this thesis I expose how Yki leads also to the transcriptional activation of the oncogene *dmyc*, involved in cell cycle regulation, apoptosis and cell competition.

YAP activity is influenced both in *in vivo* and *in vitro* assays, where the cytoplasm sequestration is dependent by the cell density status. This finding leads

to the hypothesis that also *in vitro* cell-cell contact interactions stimulate the activation of the Hippo pathway, mediating the growth inhibition by confluency, in this model, Fat and Dachsous are two possible surface receptors (Bin Zao et al, 2007).

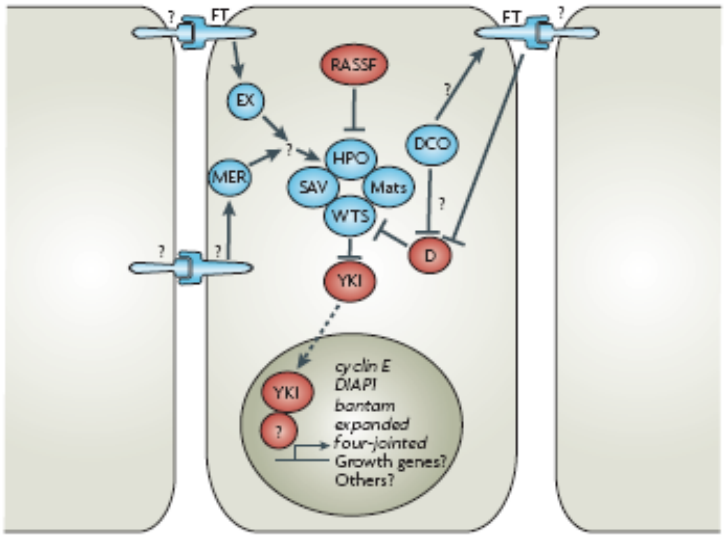


Figure 8 – The Hippo Pathway (Harvey and Tapon, 2007)

Hippo pathway accessorial members

More than ten proteins have been implicated so far as components of the Hippo signaling pathway in *Drosophila*, from transmembrane proteins to nuclear transcription factors.

Expanded (Ex) and Merlin (Mer).

Ex and Mer are part of the 4.1 family of proteins. Mammalian members of this protein family such as Ezrin, Radixin and Moesin are thought to relay signals from cell surface receptors to the cytoskeleton. The mammalian orthologue of *Drosophila* MER is the tumour-suppressor protein NF2 (Mc Clatchey et al., 2003).

Phosphorylated forms of Ex and Mer activate the Hippo pathway .

Discs overgrown/ Double Time (Dco)

It is a highly conserved protein kinase orthologue of mammalian Casein Kinase 1ε (CK1ε). Dco is a pleiotropic protein involved in a wide series of processes, and it is important for the control of Hedgehog signaling, the Wnt-Wingless (Wg) signaling, circadian rhythms, planar cell polarity and organ size (Kloss et al., 1998; Zilian et al., 1999).

Dachs (D)

It is an unconventional myosin that is predicted to function as a motor protein, or a scaffold molecule. Genetic epistasis experiments place *dachs* upstream of *warts* and downstresam of *fat* and *dco* (Choo 2004-2006). Mutations in *dachs* can suppress excessive growth and proliferation in a tissue that lacks either *ft* and *dco*. Dachs can complex with Wts *in vitro*, but the mechanism by which it suppresses the Hippo pathway is unclear (Choo 2006).

dRASSF

It shares homologies with many human proteins and antagonizes the Hippo pathway by competing with Salvador for Hippo (Polesello 2006). RASSF proteins have several functional domains: a Ras-association domain, which mediates binding to Ras and other small GTPases; a SARAH domain, which homotypically or heterotypically binds to other SARAH domain proteins. In addition they have a C1 or LIM domain that mediate binding to membrane (Schell and Hofman, 2003).

Hippo pathway downstream target genes

Yki activation has been shown to phenocopy *hpo*, *wts* and *sav* loss of function mutations, whereas *yki* loss of function clones have opposite effects and do not survive (Huang et al., 2005).

Strongly candidate target genes for *yki* exist, but no direct targets have been formally defined.

DIAP1 and *cycE* were the first discovered targets of this pathway, where mRNA for these genes is increased in mutant clones for *sav*, *hpo*, *fat* and other pathway members (Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003; Huang et al. 2005).

DIAP1 (for *Drosophila* Inhibitor of Apoptosis1) has the function to inactivate caspases activity and to prevent apoptotic processes (for a review see Montell, 2006). In *yki* loss of function clones, DIAP1 overexpression permits cells to survive (Thompson and Cohen, 2006).

Cyc E is a cell cycle effector whose mutations limits the G1-S progression in epithelial tissues and it is often overexpressed in neoplastic human cells (Hwang et al., 2005).

bantam (*ban*) miRNA was identified as a component of the Hpo pathway owing to phenotypic similarities (Thompson and Cohen, 2006). Bantam was identified as a growth promoter and apoptosis inhibitor (Brennecke et al., 2003). The only described target of *ban* is the IAP inhibitor *hid* (Brennecke et al., 2003). Genetic experiments of epistasis place *ban* downstream of *yki*, and *yki* activity is sufficient to trigger *ban* sensor in imaginal tissues, and *bantam* overexpression in *yki* loss of function clones makes them survive and accounts for the 70% of *yki* phenotype (Thompson and Cohen, 2006).

Although the transcriptional inhibition of *bantam*, *cyclin E* and DIAP1 by Hippo signaling in flies provide an appealing explaination for the overgrowths resulting from a loss of function in Hpo pathway genes, the contribution of these factors to the overgrowth has not been rigorously tested and they appear not to be sufficient to induce the hyperproliferative phenotype.

In particular there is a lack of information regarding how Hpo pathway regulates cell growth, or better how Hpo pathway can supply the strong resource accumulation needed to support the strong proliferation rate.

Several components of the Hpo pathway are regulated by a feedback loop, in particular *four jointed* appears to be positively regulated by *yki* and is also increased in cells in which the Hpo pathway is inhibited as in *fat* loss of function clones (Cho et al., 2006).

myc (*diminutive* in *Drosophila*)

Myc is a member of a family of transcription factors of the basic-helix-loop-helix-leucine zipper (BHLH-LZ) class, involved in several fundamental processes of cellular biology: cell growth, cell division and survival.

Myc acts together with a binding partner called Max and with a repressor called Mad/Mxi/Mnt, this network is evolutionarily conserved in almost all metazoan phyla (Gallant P, 2006). Interestingly, although *C. elegans* has functional Max and Mnt orthologs, Myc is apparently absent from its genome (Yuan J et al., 1998), also if it has recently been identified a protein named MML1 for "Myc and Mondo-like 1" that forms a complex with Max and functions prevalently in cell migration (Pickett et al., 2007). In humans three Myc proteins are known (c-, N-, L-) with different patterns of development; and *Drosophila* has one homologue for each member of the network: dMyc, Max and Mnt. This absence of redundance, added to the advanced genetic tools available, makes *Drosophila* the best choice for studying the Myc family members.

The first *myc* mutant was identified as a spontaneous mutation in *Drosophila* in the 30s by Eleanor Nichols-Skoog and Calvin Bridges. They called it *diminutive* (*dm*) for its small body size.

dm was then characterized like a mutation in the *Drosophila myc* gene (*dmyc*) in 1996, by Peter Gallant in the laboratory of Prof. Eisemann (Gallant et al., 1996). Afterwards, all the the findings related to this gene are relatively recent and a lot of aspects related to the network of regulation of this gene are still unclear.

dMyc heterodimerizes with the partner dMax and binds as a complex a DNA sequence called E-box (CACGTG) (Gallant et al.,1996). The protein dMnt (Mad) is a functional antagonist of dMyc for the binding to dMax.

Within the N-terminus of c-Myc there are two short motifs that are highly conserved among vertebrates, known as Myc-box I (MBI) and Myc-box II (MBII) (Grandori et al., 2000). In *Drosophila* the MBI is not conserved but anyway dMyc can rescue c-Myc defective cells in a transactivation assay in human cell culture (Gallant et al., 1996); can transform rat embryo fibroblasts when expressed along with human Ras^{V12} (Schreiber-Agus et al., 1997) and can rescue growth defects in mouse fibroblast derived from *c-myc* conditional knock-outs (Trumpp et al. 2001).

In *Drosophila*, some variations in *dmyc* transcript length have been reported, but only one form of dMyc protein has been isolated (Benassayag et al. 2005).

dMnt was identified in a two-hybrid screen as a dMax interacting protein (Loo et al., 2005). In transactivation assays dMnt/dMax heterodimerize and repress transcription from canonical E-box sequences (Loo et al., 2005).

Three variants exist of dMnt as a result of differential splicings, that appear necessary in cell-size regulation. *dmnt* mutants are viable with defects opposed to *dmyc*, they are characterized by a large body size for an effect in cell volume, but they have a shortened life span (Loo et al., 2005).

dmax is poorly known and the characterization of mutant forms is actually on course (Peter Gallant, personal comunication). It was found that dMax bound to a large number (365) of genes not bound by dMnt or dMyc, and it is possible that the mechanism of action of dMax does not only involve the presence of the E-boxes (Orian et al., 2003).

As expected, flies lacking *dmax* share some phenotypes with *dmyc* mutants. Surprisingly, however, these defects are much less severe than those of *dmyc* mutants, demostrating a dMax function independent from dMyc.

All *dmyc* mutations deeply affect growth processes, null mutant individuals fail to grow and die early during the second larval instar (Pierce et al., 2004).

Hypomorphic alleles are lethal at progressively later stages of development, depending on severity (Pierce et al., 2004, Maines et al., 2004).

In animals bearing weak alleles like dm^{1} and $dmyc^{P0}$, development is delayed and yelds to smaller flies due to smaller cells (Johnston et al., 1999).

Animals carrying the stronger $dmyc^{P1}$ allele also show a significant reduction in cell number. The reduction of dimension is interesting because the allometric proportions are not affected (Johnston et al.,1999). Proliferating mutant *dmyc* imaginal cells are markedly reduced in size and spend a lot of time of the cell cycle in the G1 phase; this cells are also smaller in G2 and M phases (Johnston et al., 1999). Conversely, overexpression of *dmyc* increases cell growth and when it is overexpressed throughout the animal the size of the fly is increased by nearly 30% (Johnston et al., 1999).

Both flies and mice carrying *myc* mutations are small in size, but the basis for this effect appears to be different. Like *dmyc* mutant flies, *c-myc* null mice

generated with conventional “knock-out” techniques die at early stages (Davis et al. 1993).

Conditional floxed *c-myc* tissues avoid lethality and are smaller (Trumpp et al., 2001). In mouse the smaller size of tissues appears to be due solely to cell death, while in *Drosophila* the reduction of body size appears to be mainly due to cell size.

dMyc is required for an efficent transition from G1 onto S phases, *dmyc* mutations stall cells in G1 and its overexpression accelerates this phase (Pierce et al., 2004; Maines et al., 2004; Johnston et al., 1999).

In endoreplicating cells the role of *dmyc* is not clear but several indications shows a competition between *dmyc* and the PI3K adaptor p60 of the insuline pathway (Johnston et al., 2006).

In *Drosophila* imaginal cells the regulators of the G1/S and G2/M transitions are Cyclin E and Cdc25 phosphatase, String, respectively (Neufeld et al.,1998). Overexpression of dMyc increases Cyc-E levels and accelerates the G1/S transition (Johnston et al.,1999; Prober and Edgar, 2000) prevalently by post-transcriptional regulation. dMyc also regulates the levels of E2F/RB complex increasing both the mRNA and protein levels.

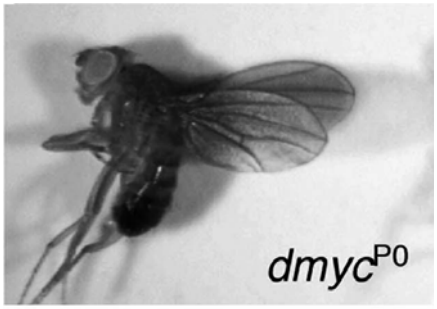
G2 regulation appears independent from *dmyc* activity (de la Cova and Johnston, 2006).

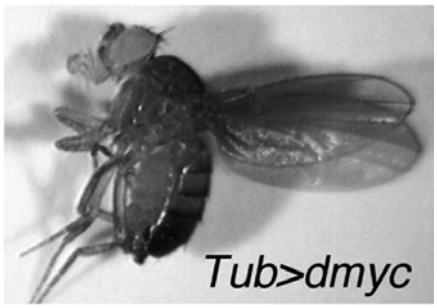
Interestingly, for developmental and evolutive aspects *dmyc* expression is regulated by at least two of the major developmental signaling pathways that regulate patterning in *Drosophila*, Wingless and Dpp. This suggests that *dmyc* is involved not merely in size control but it contributes to the definition of body size and proportions, and possibly in the maintainment of a correct homeostasis; evidence of that emerged in the works made by Claire de la Cova and Laura Johnston in 2002 and 2004, confirmed by Eduardo Moreno and Conrad Basler in 2004.

Myc is important for almost two developmental processes: patterned cell cycle arrest and cell competition. Developing wing cells exit cell cycle at the end of development, but specific cells at the Dorsal-Ventral (DV) boundary arrest more than a day earlier than the rest as part of the neural differentiation program and form the zone of non-proliferating cells (ZNC) (Johnston and Edgar, 1998).

Those cells are arrested in G1 phase by the activity of Rbf. Overexpression of CycE is sufficient to prevent this arrest by repressing Rb. Since dMyc is a strong regulator of CycE expression, this activity must be inhibited in the ZNC. During the later phases of development *dmyc* is repressed in the ZNC by the activity of Wg, by the expression of a *dmyc* repressor called Halfpint (Hfp).

The activity of *dmyc* in cell competition is a very recent finding (2004) and is also discussed in chapt... . The mechanism by which *myc* triggers cell competition is not completely clear, there are two models, one proposed by Basler and Moreno that involves growth factors deprivation (such as Dpp, Wg and EGF). This model is supported by a report in which some *Minute* cells are deficient in responding to Dpp (Moreno and Basler, 2004). However Claire de la Cova and Laura Johnston have shown that *dmyc* expression does not alter the levels of Dpp and Wg response in neighboring cells and have proposed a model of ligand-capture (de la Cova and Johnston, 2006-2007), in which physical contacts between cells is not necessary, but cells can sense dMyc levels of neighboring cells and this activates an apoptotic program that triggers the elimination of cells in which dMyc levels are lower.





Interestingly, *Minute* mutants and *dmyc* mutants show defects in ribosomal biogenesis.

Experiments in mouse blastocyst have demonstrated that cell competition involving riboproteins also occurs in mice (Oliver et al., 2004), this opens the perspective that this mecanism is evolutionarily conserved, and can be an important process in the definition of size and shape of organs in response to patterning signals.

HOW DEVELOPMENT CANT CONTRIBUTE TO EVOLUTIVE TEMATICS: *EVOLUTION AND DEVELOPMENT (EVO-DEVO)*

Evolutionary Developmental Biology (Evo-Devo or Devo-Evo) is a relatively new discipline, or more correctly a new multidisciplinar approach, that investigates the developmental mechanisms involved in the phenotypic evolutive changes of the organisms.

The main questions investigated by this discipline are:

How did development originate?

How did the evolution repertoire evolve?

How are developmental processes modified by evolution?

To answer that, Evo-Devo exploits developmental genetics tools to address evolutive thematics, in particular developmental genetics can be useful to understand how development can influence phenotypic variation, how development can contribute in phenotypic novelty and how development affects the final organization of phenotypes.

The relevance of developmental mechanisms in the understanding of evolutive thematics arose immediately after the publication of "*On the Origin of Species*" by Charles Darwin (1859), in wich Darwin underlines the importance of Embriology in providing convincing evidence of the evolutionary processes, sensing the importance of "growth correlations" between organisms of different species that during the early steps of development showed strong similarities; this perception was lately recapitulated and formalized by Ernst Haeckel in 1916, who showed how developmental processes proceed in a homologous manner in different organisms, demonstrating a common lineage for all animal species.

Thomas Huxley himself, a great supporter of Darwin theories, in the text "*Man's Place in the Nature*" (1863) largely referred to the embryological data of the age to bring humans into the animal kingdom and to irrefutably demonstrate the theory of evolution.

Although Darwin and Huxley considered with greath insight the development as the key of the evolution, during the first half of the XX century evolution and development advanced as separate disciplines, and when the first one focalized on categorization and relationships between different species (phylogenesis), the study of development focalized on the understanding of the origin of the single

individual and of its components (onthogenesis) to establish relationships among different body districts.

The famous cartesian coordinates of D'Arcy Wentworth Thompson published in the *trattato* "*On growth and Form*" in 1917, put the bases of the study of allometries and underlined how animal forms follow mathematical and physical laws to be generated.

The visual approach of Thompson has influenced many generations of biologists to come.

In 1932 Julian Huxley published "*Problems of Relative Growth*" and rised the question of how different organs and body districts relate with the final dimensions of the organism.

Despite those fundamental contributions, also after the "darwinian neosynthesis", in wich several current opinions in biology were refuted, from paleonthology to classic morphology, from embryology to genetic mendelism, rised in the first half of the XX century, development was not sufficiently considered in evolutive terms.

With the findings of mechanisms involved in genetic regulation, the DNA sequentiation and gene cloning, in other words with the impact of the molecular biology in the study of biological questions, emerged the importance of developmental mechanisms in the understanding of evolutive processes.

The Evo-Devo concept was attributed to Stephen J. Gould, who in 1977 published "*Onthogenesis and Phylogenesis*", in which he enlighted the importance of heterocrony as a mechanism of evolutive variation but the most convincing evidence of the importance of development in evolutive thematics came from the discovery of homeotic genes by Lewis in 1978 and Ghering in 1985 who highlighted the existence of specific genetic programs in the creation of the various body structures.

Gould analyzed the Evo-Devo perspective from paleonthological data, Lewis and Ghering from developmental genetics, thus one of the most relevant features of Evo-Devo is the multidisciplinar approach. Evo-Devo can be considered a small synthesis inside the big darwinian neo-synthesis.

The mechanisms of development are characterized to be hierarchical, and are involved in a huge series of genetical and epigenetical processes, such as the epigenetic maternal control of the zygote, cell-cell interactions that drive most of

cell dynamics (differentiation, proliferation, apoptosis and migration), functional interactions tissue- or organ-specific involved in the control of organ shape and dimensions.

Understanding how the environment interacts with the developmental processes and how the enviromental changes affect the evolution of phenotypes is of fundamental importance in the Evo-Devo perspective.

The study of those tematics is undertaken at many levels: among individuals of the same species, among individuals of different species or among populations or species and enviroment.

In the review essay "Evolution at Two Levels: On Genes and Form" (PLoS Biology, 2005), Sean B. Carroll exposes the importance of regulative and non-coding sequences in the evolution of animal, and possibly vegetal forms, recapitulating the most important steps that have built the so called "regulatory thinking". One of the most important aspects in Evo-Devo is how and where evolutive processes affect genomes to build different forms in different species. This question was arised more than 30 years ago, when first molecular evidence had shown that different species with different forms are characterized by a great similarity among homologous proteins.

Almost 50 years ago, the first sequences of proteins from different species were determined, and this opened the doors to study the evolution with a molecular perspective (Crick, 1958). This had revealed that different species show strong homologies in protein sequences (Zuckerkand and Pauling, 1965) and raised the question if the functional relevance is related in some little changes of sequences (Kimura, 1969).

The existence of "controller genes" derived by the characterization of the first operon model had rised the question of the importance of regulatory elements in evolution, and the possibility that the evolution act preferibly on a small sub-set of regulatory proteins that control the expression of a large number of proteins, instead that on the entire genome (Zukerkandl, 1964).

In the 1970, Ohno focused on the importance of gene redundancy in allowing mutations that can impart new functions to protein. He marked the concept that allelic mutations of already existing genes can't account to major changes in evolution, and that the duplications of regulatory *loci* is necessary to induce an evolutive jump.

This was a good intuition, but the thought of Ohno was principally focused on the relevance of coding sequences, excluding the control and non-coding regions.

In a vast series of comparative studies, principally focused in the investigations of relationship of genetic and molecular differences between human and apes, Mary-Claire King and Allan Wilson (1975, science) concluded that human and chimps are not so similar at the molecular level. They found a very high level of discrepancy between protein sequences and anatomical or ecological and behavioural differences that occur between our species and one of the most close to us.

This led to the conclusion that the most important change at the molecular level that can explain evolutive changes is at the level of regulatory circuits.

Pleiotropy and Evolution

Pleiotropy occurs when a genetic mutation at the level of a single *locus* affects multiple phenotypics traits. In general, mutations with greater pleiotropic effects will have more deleterious effects on organismal fitness; perhaps it is difficult that this kind of mutations may act as evolutive source, more probably, the evolution engine is alimented by a series of mutations with less widespread effects.

At the level of single genes many types of mutations can occur, with a different level of pleiotropic effects.

An excellent example is represented by the trancription factors, that often act in different tissues or at different times and levels of induction. A single mutation in the coding regions of this kind of genes may affect all the genes that these proteins regulate in all the tissues in which they are expressed during development.

Conversely, a single mutation at the level of cis-regulatory elements represented by a promoter, an enhancer or a splicing site, may affect the function of that protein only in a specific body district or at a specific time of development, modulating proteins activity at different levels, so reducing pleiotropics effects.

In this manner it is possible to affect only restricted protein networks locally defined, with less pleiotropics effect that may conduct at new phenotypic variants.

In this context, mechanisms of gene duplication may be interesting for a lot of aspects, the genetic redundance may contribute to the onset of new genetic variance, reducing the pleiotropic effect of a new mutation that occurs at the level of a protein that can be substituted in its function by another redundant protein.

In this way it is also possible to generate specific variants of the same protein, with small differences and specializations during different steps of development or in different tissues.

Stuctural proteins that participate in shaping body wall, or that affect cell allocation and the orientation of cell divisions may have functional aspects only if they are modulated in regulatory sites that coordinate the time, the pattern and the level of expression in the various body districts.

It is possible this way to remodel the architecture of a single structure of an organism without changing the structure of the protein that probably would produce deleterious effects if affecting all the body wall.

The presence of developmental compartments enables differential control of gene transcription in different body parts, and the existence of splicing mechanisms permit the creation of different isoforms of the same protein enabling a high level of tissue or cell-type specificity.

Those concepts are the “last frontier” in the Evo-Devo perspective and are so powerful to explicate the enormous variability of the animal forms, compared with the relatively small number of existing genes.

Selector Genes

The selector gene concept was first articulated by Antonio-Garcia Bellido in 1975 (ref) to describe a class of genes that governs fates of groups of cells.

This term is now used to describe the function of genes that specify cell, tissue, organ, as well as regional identity in animals.

Selector genes are transcription factors of particular evolutive relevance for the formation and identity of cell types, organs, and other body parts.

Selector genes perform diverse roles during development; removing their activities consequently produces a wide variety of defects, more generally the loss of identity of the interested cells.

In *Drosophila melanogaster* selector genes are intensely analyzed through the emerging concept that there are different classes of selector genes that act at different levels.

The first class of selector genes are the region specific genes (most known as homeotic genes), involved in the identity of an entire segment of the body, an example is Ubx that controls the identity of the T3 segment in which the second pair of wings is repressed and a pair of halteres form instead.

The second class are the compartment-specific genes, that control the identity of compartments in each segment. Two such genes are known in *Drosophila*: *engrailed* and *apterous*.

Field-specific selector genes are the third class, which controls the identity of specific fields inside a region, for example the product of the *vestigial* gene controls the identity of the wing inside the wing imaginal disc that comprises other structures, like hinge or thorax (see after).

Other two classes are distinguished, the organ- and the cell-type specific genes.

The existence of selector genes leads to the idea that there exists an underlying ground plan upon which region-specific selector proteins operate (Carroll 2003).

Regulatory Architecture

Selector genes are transcriptional regulators, and act in most cases in combination with specific co-factors (for example *vestigial* needs the co-factor *scalloped*). The specificity of a transcription factor is due to specific enhancer sequences that activate a specific gene. The consequence is that a single gene can be controlled by more than one enhancer and can be activated by different combinations of selector genes, giving specific modularities in different contexts.

For this reason selector genes act in synergy and in a combinated manner, to provide cells with a specific identity. As an example, the posterior haltere in the fly requires three selector genes: the field-selector *vestigial* to specify the appendage identity (wing and haltere), the region-selector Ubx to specify Haltere instead of wing, and the compartment-specific *engrailed* to specify posterior identity.

Combinatorial control can be also a switch in the activation/repression of genes; for example *spalt* is a wing-specific gene in wich a single enhancer appears to be activated in the wing by the Vestigial-Scalloped complex and repressed in the haltere by Ubx and Hox selector proteins (Carroll, 2003).

THESIS OUTLINE

Drosophila wing is an excellent model for studying shape evolution, especially in the study of the minimal shape variations occurring in natural population.

Gene functions involved in the development of this tract are not sufficently understood, and how cell dynamics dictate organ shape variations are in large part an unknow matter.

Proximal-Distal axis of *Drosophila* wing is affected by several mutations, in particular in the genes *fat*, *dachsous* and *four jointed*, and very recently they have been associated with the same genetic cascade called Hippo pathway.

Some findings have also shown that this pathway is involved in cell competition, but the mechanisms that lead to this phenomenon are poorly understood.

With the aim to enlight how *ft* and *dachsous* lead to cell competition, we have focalized the first part of this thesis in the research of a possible involvement of *dmyc*, the best characterized gene that leads to cell competition, in the Hippo pathway.

To do that we have performes a series of genetic interaction experiments among *dmyc* and genes involved in shaping the proximal-distal axis of the wing.

In the second part of this thesis we have posed our attention in highlighting how those genes affect cellular dynamics of proliferation and allocation acting during wing development.

In particular we have focalized our work on the phenotypes produced modulating the expression patterns of *fat*, *dachsous* and *four jointed*, suggesting a possible relevance of the expression pattern variation in the explaination of evolutive changes in shape.

This analysis was performed by morphometrical methods.

GENERAL MATERIAL AND METHODS

Nomenclature, Breeding Conditions and Used Stocks

In this thesis we used mutant conditions and transgenic lines here described.

For the nomenclature of the mutations and balancer chromosomes we follow the rules described by Lynsdley and Zimm, 1992.

All the crosses were performed at 25°C, the growing medium is composed by: H_2O, agar, corn meal, yeast and glucose in the right proportions. To avoid mould infection we have added the antifungine Nipagine.

Sp (Sternopleural) Dominant mutation on the 2° chromosome showing an increase in sternopleural bristles.

y^1 *(yellow)* Recessive amorph mutation on the X chromosome that leads to a yellow coloration of the body wall, and brown pigmentation of the boral apparatus.

w^{1118} *(white)* Recessive amorph mutation on the X chromosome that leads to a white color of the eye.

ft^1 Viable recessive spontaneous mutation, flies show reduction in proximal distal axis of wing and leg, and the abdomen results shortened and broadened (Bryaant et al. 1988).

ft^{Grv} Lethal recessive mutation provoked by an insertion, in the 33th cadherin domain, of the trasposon 412, and by a deletion and inversion of 2kb in 5' direction from the transposon insertion (Mahoney et al., 1991).

GAL4-lines:

MS1096-GAL4 Construct generated on the coding sequence of the GAL4 transactivator under the control of regulative sequences of the *Beadex* gene, lcated on the X chromosome (Capdevila and Guerrero, 1994).

apterous-GAL4 Construct generated on the coding sequence of the GAL4 transactivator under the control of regulative sequences of the *apterous* gene, located on the second chromosome.

engrailed-GAL Construct generated on the coding sequence of the GAL4 transactivator under the control of regulative sequences of the *engrailed* gene, located on the second chromosome.

np4678-GAL4 enhancer trap construct located on the second chromosome (GETDB).

dpp-GAL4 Construct generated on the coding sequence of the GAL4 transactivator under the control of regulative sequences of *dpp blk*, inserted on the third chromosome (Wilder and Perrimon, 1995).

np4657-GAL4 Enhancer trap construct located on the third chromosome (GETDB).

UAS-lines:

UAS-yki Construct on the third chromosome (Huang, 2005)

UAS-vg Construct on the second chromosome (kindly provided by Baena-Lopez)

UAS-ft Construct on the second chromosome (Matakatsu and Blair, 2004)

UAS-ds Construct on the third chromosome (Matakatsu and Blair, 2004)

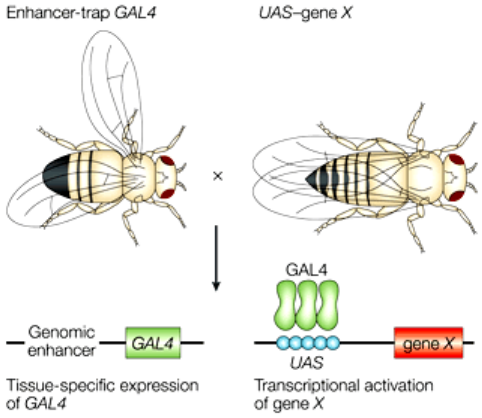
UAS-fj Construct on the third chromosome (Zeidler et al., 1999)

UAS-dmyc RNAi Construct on second chromosome (Vienna RNAi Center n 2947)

UAS-vg RNAi Construct on third chromosome (kindly provided by Baena-Lopez)

Genetic Methods

UAS-Gal4 system (Brand and Perrimon, 1993)



The UAS-Gal4 is a system that allows, in the fly, to study the effects of the ectopical expression of a gene of interest.

Gal4 is a powerful transcriptional activator of *Saccharomyces cerevisiae*, that recognizes and binds to specifical regulatory sequences called UAS (Upstream Activating Sequences).

In *Drosophila* it is possible the construction of two separate transgenic lines.

A first line contains the Gal4 sequence under the control of a regulative element of a *Drosophila*'s gene, that can induce the expression in specific moments of development and in specific body districts. The second line contains a construct in which the cDNA sequence of a gene is posed downstream one or more UAS elements.

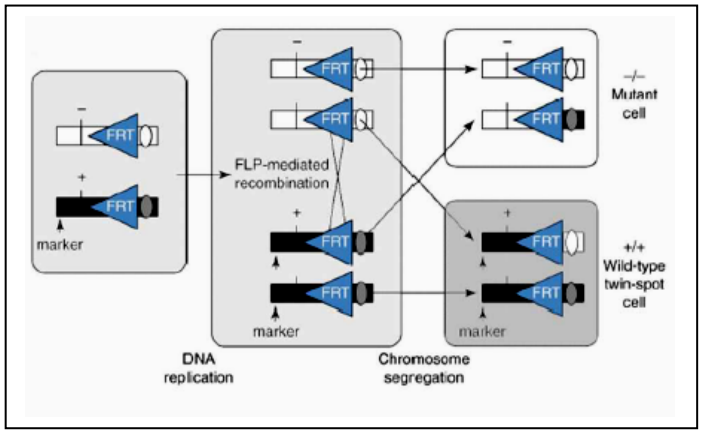
By crossing those 2 lines it is possible to originate progeny containing both constructs, this allows the expression of Gal4 transactivator, that in turn activates the gene posed under the UAS control in a pattern specific manner (Brand and Perrimon, 1993).

A large series of promoters exists stored in stock centers (the most important is the Bloomington Stock Center) that can drive the Gal4 in

defined types of cells or tissues; it is also possible to drive Gal4 expression by a heat shock promoter, that permits a temporal control of induction.

The use of UAS-hairpin constructs permit to silence post transcriptionally the expression of determinated genes (UAS-RNAi).

FLP-FRT (Xu and Rubin, 1993)



In *Drosophila* exists also the to exploit the yeast binary sistem FRT-Flp, that permits a site specific recombination.

The Flippase (Flp) recombinase induces recombination at the level of specific sequences called FRT (Flippase Recognition Targets) (Golic and Linquist, 1989).

This system can be used for generating genetic mosaics very useful to study loss of function mutations.

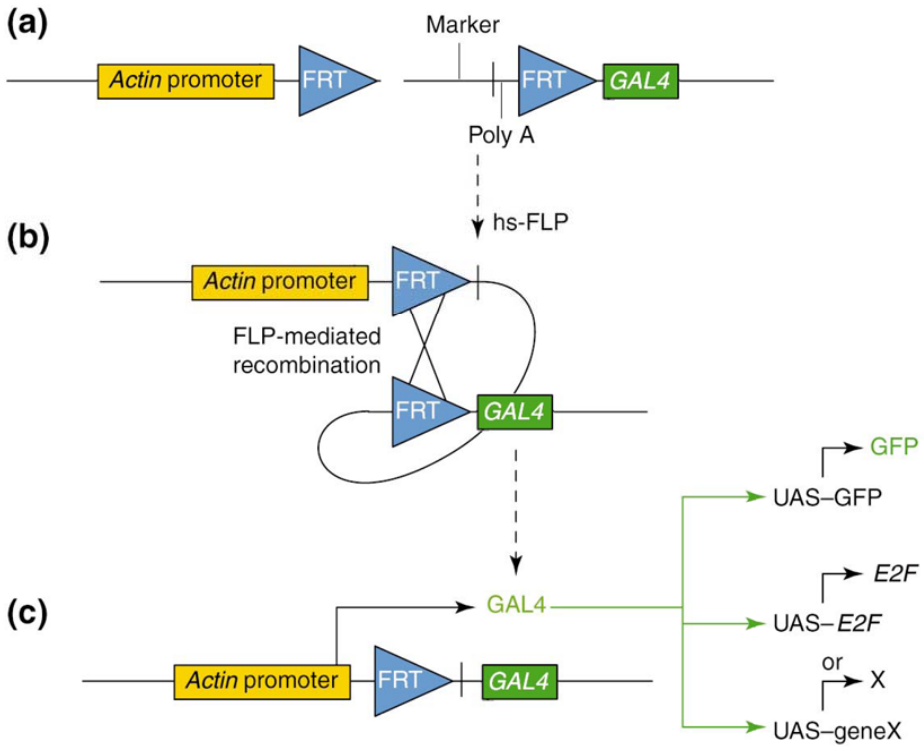
Stock are available with the FRT sequence posed in pericentromeric region in all the chromosome arms, this allows the exchange of the entire chromosome arm by mitotic recombination.

In heterozigous mutants for a gene of interest, it is possible to induce mitotic recombination by the activation of the Flp, as a result it is possible to obtain a homozygous clone for the mutation and a wild type twin clone (Xu and Rubin, 1993).

The possibility to generate homozygous cell clones is very useful in the study of lethal recessive conditions and to investigate cell specific interactions.

The Flp activity can be regulated through a heat shock promoter, that functions transferring individuals at 37°C for several minutes, leading to clones generation only at determined stages of development.

Flp - out (Neufeld, 1998)



The FLP-Out technique permits the overexpression of UAS controlled sequences in a clonal manner.

This is possible because The FLP-Out combines the UAS-GAL4 system to activate the expression of a UAS-line, and the Flp-FRT system to define how and where to activate the GAL4 expression.

A FLP-Out construct consists of a constitutive promoter (i.e. actin or tubulin) followed by an FRT sequence, a marker with a polyA terminator, a second FRT sequence and a Gal4 sequence.

The Flippase expression in this case can induce the DNA excision between the two FRT sites, and the approach of the constitutive promoter to the Gal4 sequences leading to his expression.

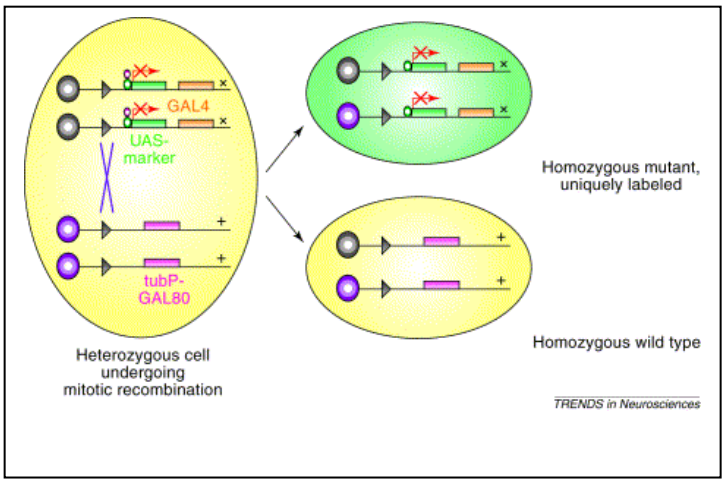
The Gal4 activity permits the expression of all the downstream UAS sequences present in the line, and often a FLP-Out line has inserted in its genome almost a cell marker under the UAS control (UAS-GFP or UAS-LacZ).

In *dmyc* rescue assay performed in this thesis (see results) we have used a particular FLP-Out line, in wich instead of the marker with the polyA terminator of a classical FLP-Out, dmyc cDNA is present.

This permits the expression of *dmyc* in an ubiquitary manner and the recombination event leads to *dmyc* cDNA excision and to GAL4 activation.

This generates a competitive condition and only the overexpression of UAS-lines that confere competitive ability can permit the survival of clones (Moreno and Basler, 2004).

MARCM system (Tee and Luo, 1999)



The MARCM system (Mosaic Analysis with a Repressible Cell Marker) (Tee and Luo, 1999) allows one to label homozygous mutant cells uniquely in mosaic tissues, which is essential for performing mosaic analysis. To achieve this, the yeast GAL80 protein was introduced into the GAL4-UAS binary expression system in *Drosophila* (Brand and Perrimon, 1993). The MARCM system initially contains cells that are heterozygous for a transgene encoding the GAL80 protein, which inhibits the activity of the transcription factor GAL4.Following FLP/FRT-mediated mitotic recombination, the GAL80 transgene is removed from one of the daughter cells, thus allowing expression of a GAL4-driven reporter gene specifically in this daughter cell and its progeny (see figure). If there is a mutation located on the chromosome arm *in trans* to the chromosome arm containing the GAL80 transgene, the uniquely labeled GAL80-negative ($GAL80^{-}$) cells should be homozygous for this mutation. Therefore, one can specifically label the homozygous mutant cells in a mosaic tissue using the MARCM system.

In particular the MARCM system can be usefull in the study of genetic interactions for its ability to generate LOF clones for recessive mutations in which it is possible to overexpress another gene posed under the control of a UAS element.

Immunostaining

step 1- Larvae were dissected in PBS, and the anterior section containing imaginal discs was fixed in 4% PFA in PBS for 20 minutes.

After three washinsg in PBS for ten minutes, larvae sections were permeabilized in 0.1% Triton x-100 in PBS (0.1% PBT) for three times X ten minutes.

Successively larvae were posed in PBT and BSA (3%) three times for ten minutes to reduce aspecific activity of antibodies.

Primary antibody was posed in PBT and BSA with specific concentration for an incubation time from 2 hours to over night.

step 2- A series of three washes with PBT for ten minutes followed by a series of washes in PBT and BSA was performed for eliminate the primary antibody in excess.

The secondary antibody was posed in PBT and BSA (3%) for a time of incubation of 2.5 hours at RT.

After a series of four washes in PBT, imaginal discs were dissected and mounted in Fluormount.

To mark nuclei we used the DNA-binding molecule DAPI.

Primary antibodies utilized:

dMyc: mouse, monoclonal, 1:5 (Paola Bellosta).

Dachsous: rabbit, polyclonal, 1:100 (David Strutt).

cleaved caspase 3: rabbit, polyclonal, K9661S, Cell Signaling Technologies.

dIAP1: monoclonal, mouse, 1:200 (Bruce Hay).

Vestigial: monoclonal, mouse, 1:100 (Sean B.Carroll).

βGAL: polyclonal, rabbit, 1:200

Secondary antibodies utilized:

Alexa fluor 555, a-mouse, 1:200
Alexa fluor 488, a-mouse, 1:200
SantaCruz Laboratoires, a-rabbit (TRITC), 1:50
SantaCruz Laboratoires, a-rabbit (FITC), 1 :50

X Gal Staining

To induce the expression of the β-galactosidase we have used a UAS-lacZ line kindly provided by the Bloomington Stock Center (University of Indiana – stock number 3955).

Larvae were dissected in PBS, and the anterior section containing wing imaginal discs was fixed in 4% PFA in PBS for 20 minutes.

Successively larvae were washed in PBS for three times for 5 minutes and incubated at 37°C in staining solution (5mM K4[FeII(CN)6], 5mM K3[FeIII(CN)6], 0.3% Triton x-100, 0.2% x-gal in dimetyilformammide) until the appareance of a blue precipitate.

Pupal wings were extracted from pupae and posed in water to allow their extension, due to an osmotical effect on wing cells.

After the wing expansion, the same protocol of fixation and staining of the imaginal discs was followed.

***in situ* hybridization**

The *dmyc* cDNA construct was kindly provided by Paola Bellosta & Laura Johnston (Jhonston et al., 1999).

The *in situ* hybridization was performed following Gerald Rubin protocol published on the web site:

http://www.bioprotocol.com/protocolstools/protocol.jhtml;jsessionid=BCONUP42AWET3R3FQLMSFEWHUWBNQIV0?id=p16

CHAPTER 1

MYC AND YKI

***dmyc* is a target of *dachsous* and *fat* tumor suppressors genes**

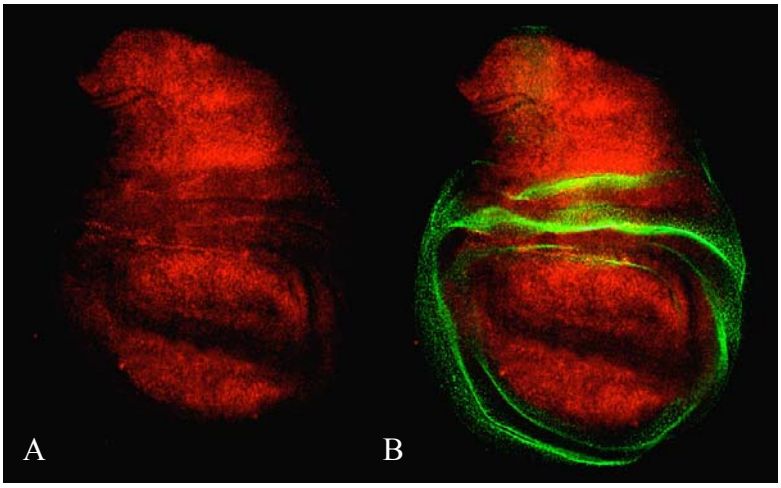


Fig.1 – dMyc and Dachsous show a complementar expression pattern
Double Immunostaining for dMyc and Dachsous in wing imaginal disc from L3 larvae.
A. in red dMyc expression.
B. merged image, in green Dachsous expression pattern.
Image were collected through epifluorescence mycroscopy

dMyc protein is not uniformly expressed during development, in particular in L3 instar wing imaginal disc dMyc shows a defined pattern of expression (Fig.1A).

dMyc appears expressed prevalently in the wing pouch territory and in the notum region and is completely repressed in the DV border, in a zone also called Zone of Nonproliferating Cells (ZNC), by the action of the Wingless/Wnt signal (Duman-Scheel et al., 2004).,

The hinge region presents a strong decrease of dMyc levels, and this is probably due to the action of the Wnt pathway; Dachsous protocadherin, involved in Wnt signal and probably direct activator of *fat* and the Hpo pathway, is strongly expressed in the hinge region (fig.1B in green) and shows a complementar signal with respect to dMyc expression pattern (fig.1B in red).

This observation suggested the hypothesis that *ds* is involved in *dmyc* repression, accounting for the overgrowth phenotypes displayed in Hpo pathway defects, not sufficently explained by the target actually identified.

To demonstrate that *ds* effectively regulates *dmyc* we have performed a clonal analysis using the FLP/FRT system with the null condition ds^{d36}.

The figure 2 shows a dMyc immunostaining in *ds*^{d36} mitotic clones. In figure 2B dMyc results strongly upregulated in *ds* clones, in particular in the hinge and notum regions, where *ds* is normally expressed. In the distal region of the wing pouch, dMyc expression appears slightly affected, and this is explainable by the fact that Dachsous in this region is present at low levels, and probably its activity in cell growth and proliferation is reduced.

Following to the finding that dmyc is a novel *ds* interactor, we have tested if also *ft*, a putative interactor of *ds*, is involved in dMyc protein regulation. The figures 3A and B show mitotic clones for *fat*$^{G\text{-}rv}$ (a strong hypomorph allele) stained for dMyc. *fat* appears to affect dMyc expression in the wing pouch, but surprisingly, does not affect dMyc expression in the hinge region. *fat* appears to regulate dMyc prevalently in the proximal wing region, and seems to be particularly active in the proximal dorsal region of the wing (fig 3, yellow ribbon). This data underline that dmyc is regulated by *ds* and *ft*, and rise the question of how dmyc can be regulated by those genes. A possible explaination was searched in the Hpo pathway, and furter experiments focalize on *yorkie*, the downstream target activator of this novel signal cascade.

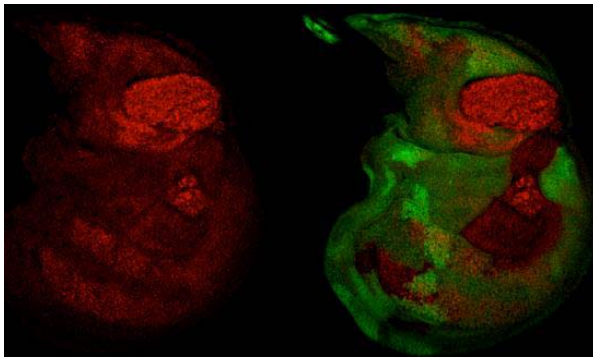


Fig.2 – dMyc is upregulated in ds clones.
A. Imunostaining for dMyc (in red).
B. merged image with *dachsous* clones marked by loss of GFP signal. dMyc results overexpressed in notum, hinge and proximal wing pouch.
Image were collected through a confocal microscope.

Genotypes:
hsFLP; Ubi-GFP, FRT40A X *ds*^{D36}, FRT40A
Clones were induced 48hrs AEL by 20minutes of heat shock at 37°C and allowed to grow until the end of L3 (wandering stage)

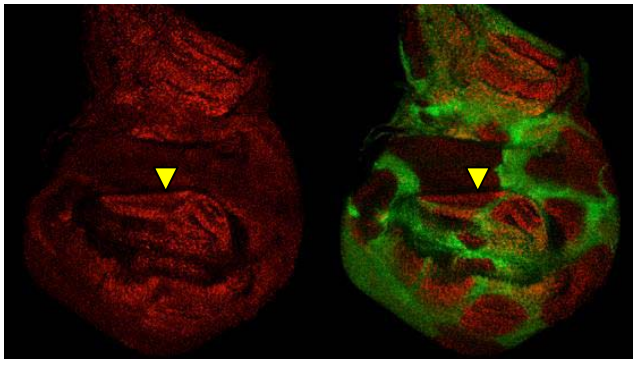


Fig.3 – dMyc is upregulated in ft clones
A. Immunostaining for dMyc (in red).
B. merged image with *fat* clones marked by loss of GFP signal. dMyc results overexpressed in the wing pouch, but not in the hinge region.
In fig.3 image were collected through a confocal microscope.

Genotypes:
hsFLP; Ubi-GFP, FRT40A X *ft*^{Grev}, FRT40A
Clones where induced 48hrs AEL by 20minutes of heat shock at 37°C and allowed to grow until the end of L3 (wandering stage)

Yki is a *dmyc* activator

To evaluate if the Hpo pathway is involved in dMyc oncoprotein repression, we have extended the mitotic analysis to *yki*. We have induced *yki* GOF clones through a Flp-out system highlighting a strong increase of dMyc level (fig. 4) in *yki* overexpressing cells. The hyperactivation is clearly visible in all the regions of the wing disc, indicating an independence from patterning. Indeed, *yki* ectopic expression increases dMyc levels also in the Zone of Non proliferating Cells (ZNC) where *dmyc* is strongly repressed by the *Wnt* pathway (Duman-Scheel et al., 2004).

This demonstrates that Hpo pathway is involved in *dmyc* regulation and strongly suggests that also *ft* and *ds* act in the regulation of the levels of dMyc through this pathway.

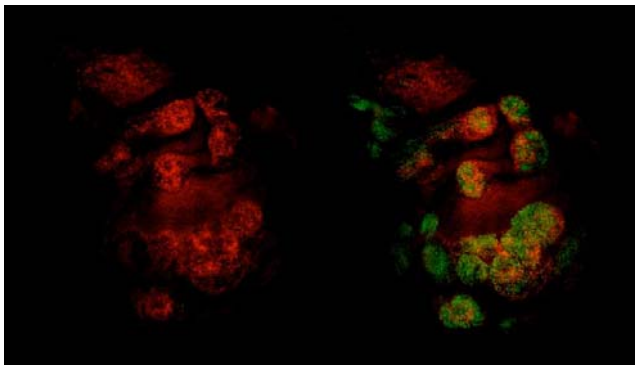


Fig.4 – FlpOut clones for UAS-Yki.
A. dMy staining.
B. Merged image with Yki GOF clones (in green), dMyc results overexpressed in all the clones
Images were collected through epifluorescence microscopy.

Genotypes:
yw, hs-FLP; act,FRT,y^+,FRT,GAL4, UAS-GFP X UAS-yki (on third chromosome)
Heat shock was induced 60 hrs AEL for ten minutes at 37°C.

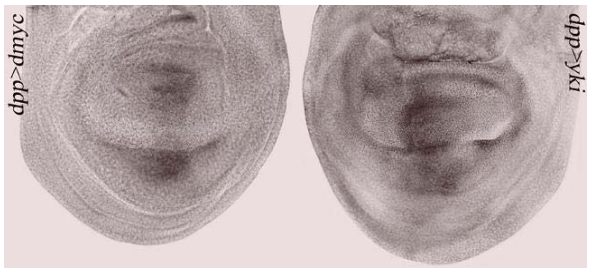


Fig.5 – *in situ* hybridization with *dmyc* RNA probe.
A. Control with UAS-*dmyc* X dpp-Gal4 (expressed in the AP border of the wing); *dmyc* probe signal is increased in the *dpp* territory.
B. UAS-*yki* X dpp-Gal4, *dmyc* probe signal is increased in the dpp territory (the area is larger than the control for the hyperproliferative effect of *yki* overexpression).

Yki is a transcriptional co-activator, and possibly the regulation of *dmyc* is at the transcriptional level. To test this hypothesis we have performed an *in situ* hybridization assay on wing imaginal disc and a luciferase assay in S2 cultured cells.

The *in situ* hybridization assay was performed on wing imaginal discs overexpressing the UAS-yki line with a dpp-Gal4 driver (expressed along the AP border of the wing), detecting an increase of *dmyc* RNA level in *yki* overexpressing cells (fig.5B), suggesting a transcriptional regulation of *dmyc* by Yki.

dmyc repression affect yki activity

To test how *yki*-induced overproliferation is affected by dMyc levels, we have induced *yki* GOF clones, *wt* clones, *dmyc* clones (using a UAS-dmycRNAi construct) and *yki* GOF clones simultaneously interfered for *dmyc*. In this experiment, clones were all induced at 60 hours after egg laying (AEL) and allowed to grow for the same time, imaginal discs where dissected at 96 hrs AEL.

As it can be seen in Fig.7, we found that loss of *dmyc* strongly reduces *yki* clonal proliferation (n= 15-35, 35%, two tailed t-test $p>0.002$).

These data show that *yki* requires dMyc to overgrow and the observation that *yki* clones trimmed for *dmyc* mantain their rounded shape indicates that *dmyc* is not responsible for the morphology of *yki* clones.

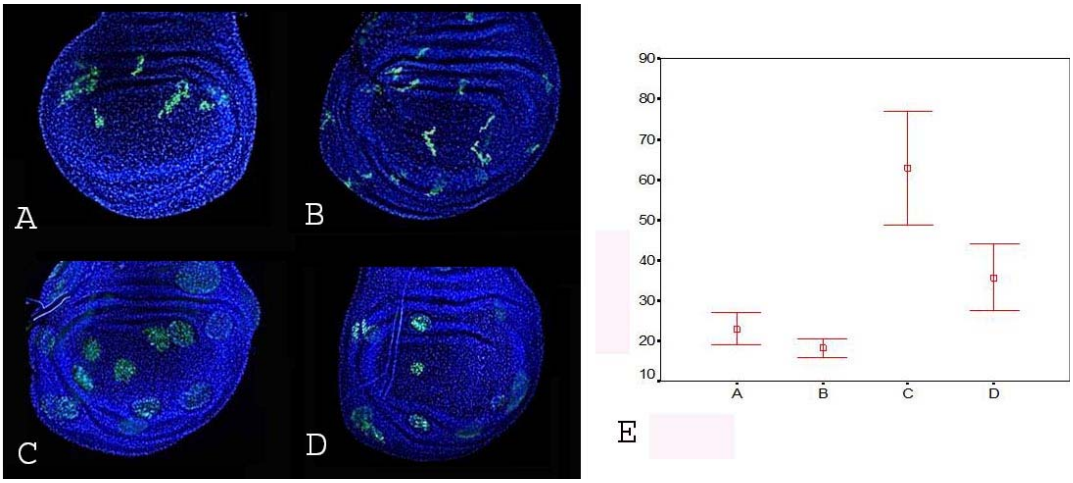


Fig.7. *dmyc* RNAi can rescue *yki* overexpression phenotype.
Marked with GFP:

A. wt FLP-Out clones; n=31;
B. *dmycRNAi* FLP-Out clones; n=35;
C. UAS-*yki* FLP-Out clones; n=19;
D. UAS-*dmyc RNAi*; UAS-*yki* FLP-Out clones; n=15;
E. Mean of nuclei inside each clone.

Clones were induced simultaneously at 60hrs. AEL through a heat shock of 5 minutes at 37°C (time of induction was short to avoid clonal confluency).
dmyc-RNAi line was provided by the Vienna *Drosophila* RNAi Center (stock number 4657).

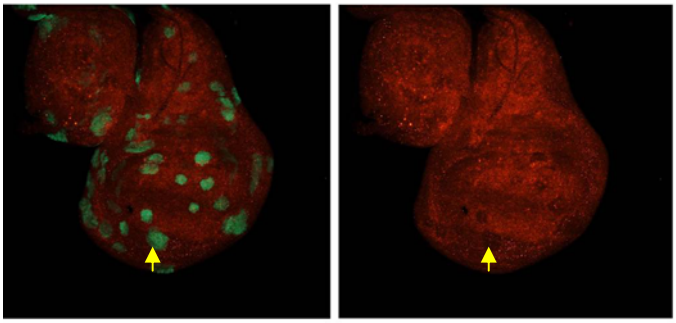


Fig.8. dMyc expression is effectively reduced in UAS-yki; Uas-dmyc FLP-Out clones

A. UAS-yki; UAS-dmyc RNAi FLP-Out clones (GFP).
B. Immunostaining with α-dMyc antibody (in red).

dMyc levels are reduced inside the clones (yellow arrowheads)

Yki ectopical expression transforms cells into supercompetitors

Some evidence reported in previous works showed a possible involvement of *yki* in cell competition (Huang et al., 2005). The fact that *ft* cells are protected from cell competition, as well as other mutants of the Hpo pathway upstream *yki*, rises the question if this property is due to *yki* activation.

In *yki* overexpressing cells the apoptosis is indeed localized prevalently in the *wt* cells surrounding the clone (Huang et al., 2005) rather than in the mutant cells within the clone.

To demonstrate that *yki* is effectively a super-competitor we have evaluated the ability of *yki*-overexpressing cells to reduce the surrounding *wt* territory (see figure 9). We have overexpressed *yki* through a MARCM modified system in which we introduced an Ubi-GFP construct in the chromosomal arm 2R. This way we were able to distinguish the heterozygous background (light green) from *wt* twins (black). The results (see figure 9) show that the overproliferating *yki* clones eliminate the sorrounding wild type cells. The clones were induced at 60 hours AEL (+-12hr) and larvae were dissected after 96 or 120hrs AEL. At 96 hours AEL (fig.9B), *yki* GOF clones were significatively larger than *wt* twins; futhermore, we observed strong cell death in *wt* cells flanking *yki* clones while lower levels of cell death were detected inside the clones. At 120 hours AEL (Fig.9C) most of the *wt* twins were eliminated by the *yki* cells, and an increasing level of apoptosis was detected within the *yki* clones. The control experiment shows that *wt* and twin clones grow in a similar manner, and very low levels of apoptosis were detected (Fig.1C). Thus, the results of this experiments strongly suggest that *yki* overexpression provides cells with a competitive ability.

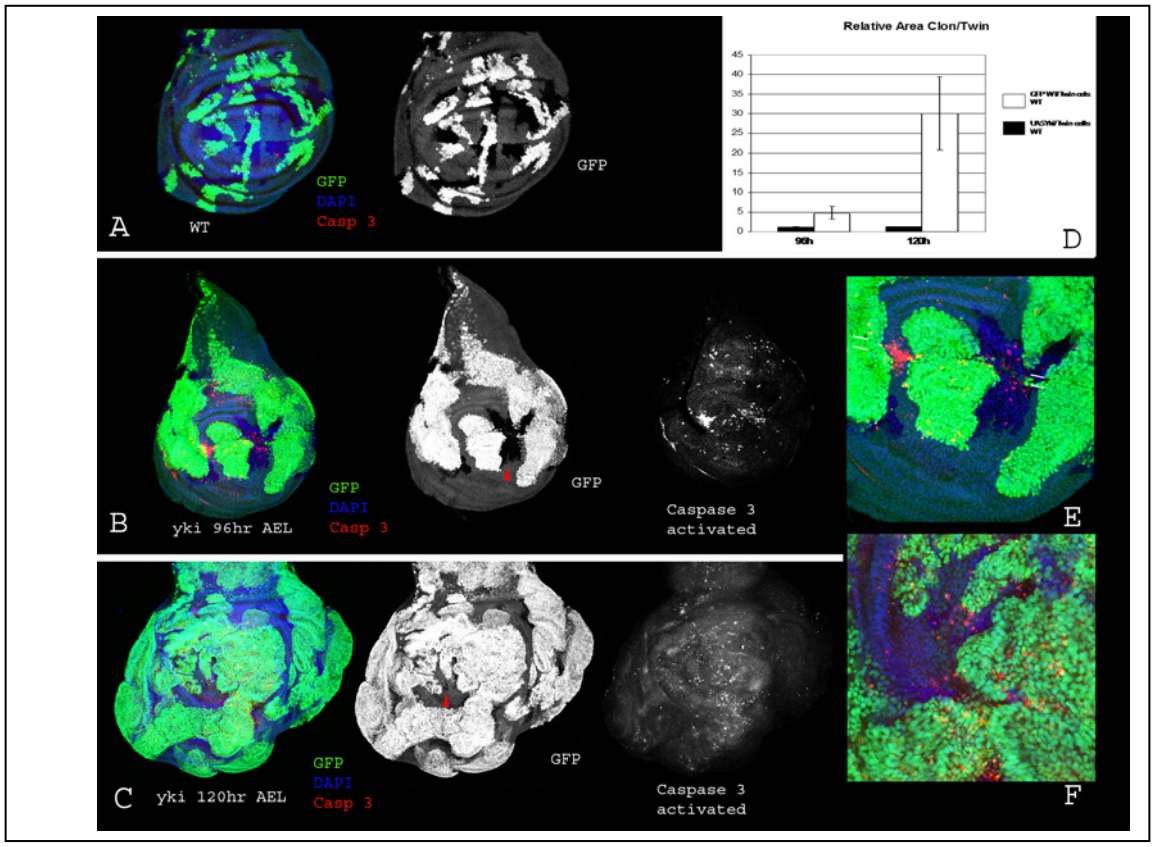


Fig.9. Yki clones grow faster and eliminate surrounding wt territories.

This is demonstrated by the elimination of twin cells and by the activation of caspase 3 in cells at the border of the clones.

Yki clones were generated by the MARCM system, following this cross:

Ubi-GFP, FRT42a; UAS yki X MARCM; FRT42A, tub gal80; UAS-GFP

yki clones were marked by GFP, twin clones were marked by loss of Ubi-GFP construct (dark).

(A) wt clones induced 60hr AEL grow similarly to the twin, low level of activated caspase 3 was detected.

(B) Yki clones induced 60hr AEL dissected at 96hr AEL: clone size is bigger than wt twin, high level of activated caspase 3 in wt territories.

(C) Yki clones induced 60hr AEL, dissected 120hr AEL: almost all wt twin were eliminated, yki clones hyperproliferate, high level of activated caspase 3 inside and outside clonal territories

(D) Quantification of the relation between wt and yki clonal size at 96 and 120 hr AEL.

(E) High magnification detail of Fig.B that underline high level of activated caspase 3 signal in wt territories.

(F) High magnification detail of Fig.C that underline high level of activated caspase 3 signal in wt and yki clone territories.

yki and compartmentalization

Cell competition is a non autonomous phenomenon affecting cells surrounding a competitive source within a range of five-eight cells, involving the existence of soluble molecules (De la Cova et al., 2004 and 2007).

To investigate the competitive ability of *yki*, we have ectopically expressed it in the posterior compartment (through *engrailed*-GAL4 driver) and in dorsal compartment (through *apterous*-GAL4 driver).

In the first case, posterior compartment resulted strongly increased with resprect to the anterior compartment. To understand if this phenotype involved also apoptosis in the anterior compartment, we have performed an immunostaining for the activated caspase 3.

The result in figure 10 shows that anterior cells were not outcompeted, this is highlighted by the low level of apoptosis in this compartment and according to what reported in literature, competitive activity appears to be incapable to cross the AP restriction boundary.

A lot of apoptosis level was displayed in the posterior compartment, as a result of *yki* overexpression, confirming that *yki* expressed at high levels shows cytotoxic properties.

Surprisingly, *yki* overexpression in the dorsal compartment provoked a different result, in fact in this condition the ventral compartment, where *yki* was not upregulated, was strongly outcompeted and eliminated by apoptosis (see fig.11B.).

There are two possible explainations for this dramatic competitive effect: it is possible that *yki* begins to be overexpressed before the boundary formation, in fact the DV border forms lately in time with respect to the AP border, but the formation of this compartment directly depends on the activity of *apterous*. It is also possible that *yki* signal can cross this border, in fact *yki* overespressing clones show a *dmyc* upregulation also at level of the ZNC (See fig. 4), bypassing the restriction signal generated along the DV boundary by Wingless.

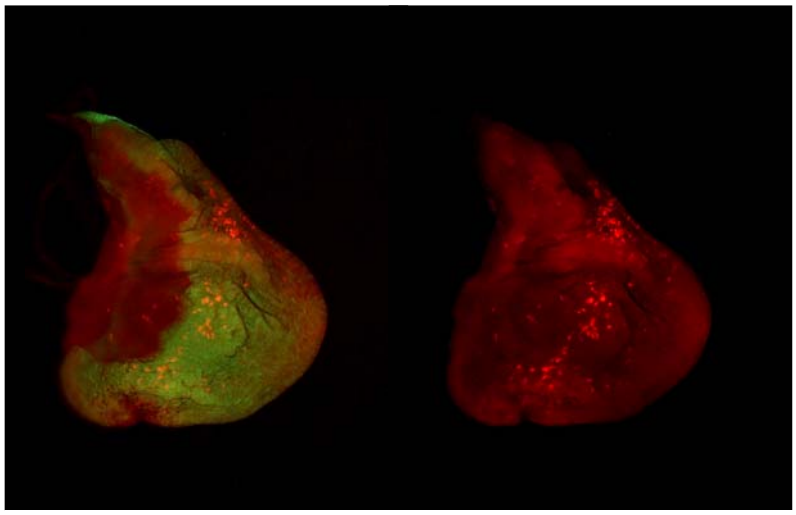


Fig.10. *yki*-induced cell competition fails to cross AP restriction boundary

A. *en*-gal4, UAS-GFP X UAS-*yki*
   L3 wing imaginal discs.
B. Immunostaining for activated caspase 3

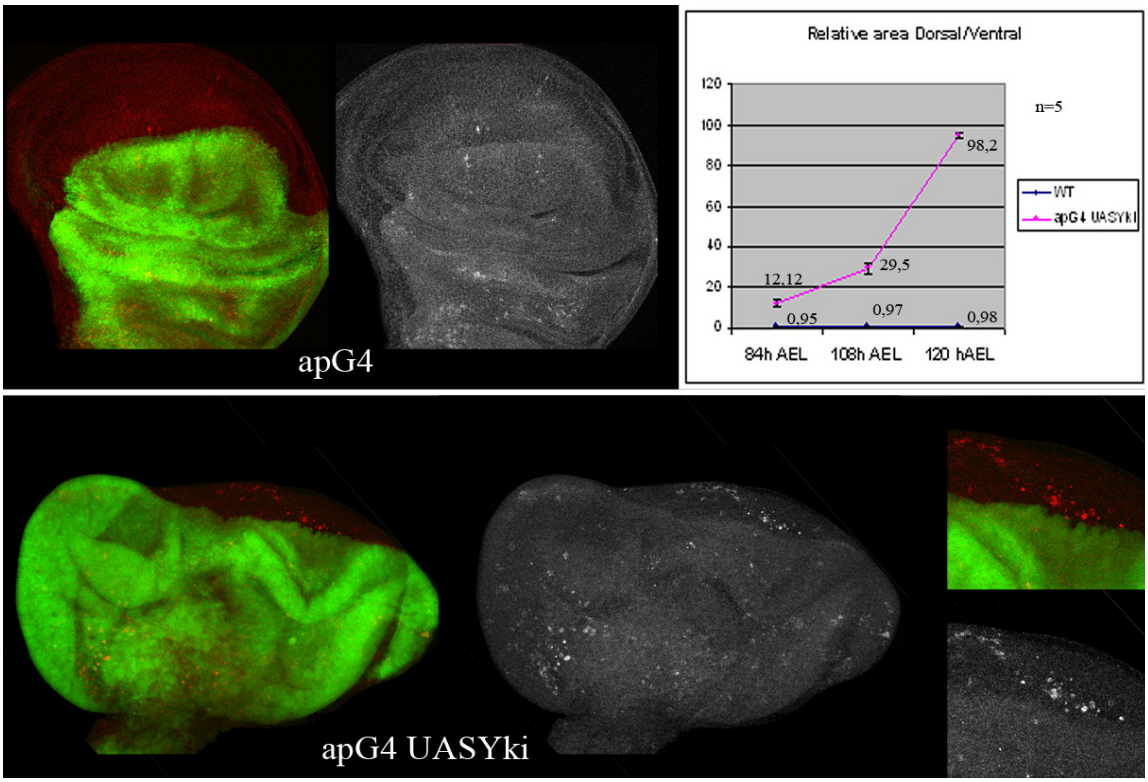


Fig.11. *yki* overexpression in dorsal compartment leads to the elimination of the ventral compartment.
In red and grey: α-activated caspase 3 immunostaining
In green: GFP signal that shows the pattern of expression of *apterous*-Gal4

A. L3 wild type imaginal disc.
B. *yki* overexpression in dorsal compartment
C. high magnification of the ventral compartment, high levels of activated caspase 3 were detected.
D. relative area between dorsal and ventral compartments at 84, 108 and 120hrs AEL.

***dmyc* competition assay**

To test if *yki* overexpressing cells still behaved as competitors when surrounded by cells expressing high levels of *dmyc,* we performed a *dmyc* competition assay inducing *yki* overexpressing clones in wing discs homogeneously expressing *dmyc* under the control of a tubulin promoter (Moreno and Basler, 2004). Under this condition, *wt* clones fail to grow and die by cell competition, while supercompetitors like *Rab5*, ras^{V12} and genes of the *dpp* pathway can confere cells survival cues and growing ability (Moreno and Basler, 2004).

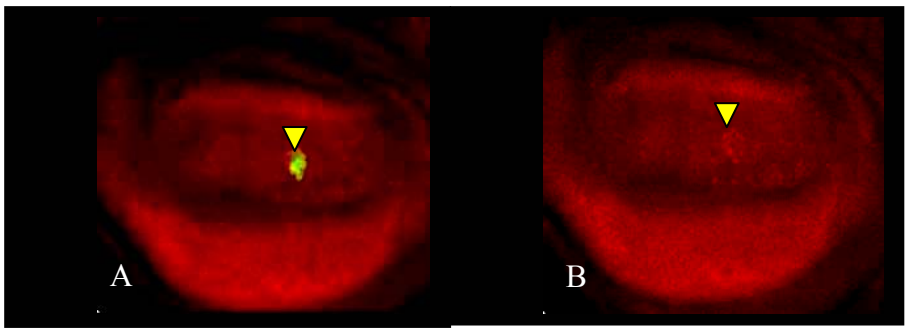


Fig.12. *yki* induced clones can compete against high *dmyc* sorrounding levels.
A. *tub-yki* clones (marked with GFP) can survive in a *tub-dmyc* background.
B. In red: immunostaining for dMyc that shows how dMyc levels inside *yki* clones (yellow arrowhead) are similar to those of the surrounding territories.

Lines crossed: *tub*>*dmyc*>GAL4; UAS-EGFP X yw; hsFLP; UAS-*yki*
Heat Shock was induced at 60hrs AEL for 45 minutes at 37°C.
yki FLP-Out induction before 45-50hrs appears to be lethal.

Here we show that *yki* cells can survive also when surrounded by homogeneous *dmyc* amounts (fig.12) and that the endogenous dMyc level inside the clones is comparable with that observed in the sorrounding cells (fig.12). So, possibly *dmyc* is the factor responsible for *yki* super-competitive ability.

To investigate this, we performed the same competition assay while scaling down *dmyc* function inside the clones. We used the pupal lethal $dmyc^{PL35}$ allele and, taking advantage of *dmyc* chromosomal position on X, we were able to analyze both female (heterozygous *dmyc* condition) and male (hemizygous *dmyc* condition) larvae where clonal *dmyc* dosage was respectively halved and

completely trimmed. In the first case, *yki* clones grew at the same rate of the previous assay (see fig.13B.), indicating that a heterozygous *dmyc* condition does not impair *yki* ability to upregulate dMyc levels, while in the second case (fig.13A.), where the absence of a *wt dmyc* copy makes it impossible for *yki* to drive its regulation, *yki* clones failed to growth and were out-competed, confirming our hypotesis that *dmyc* is the competitive factor for *yki*.

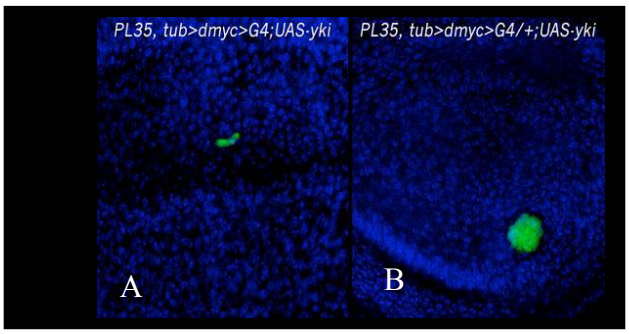


Fig.13. *dmyc* competition assay in a strong *dmyc* hypomorphic condition (PL35).

A. tub-*yki* clones hemizygous for the *PL35* allele grow poorly and die (in green) in a *PL35*, tub-*dmyc* background.
B. tub-*yki* clones heterozygous for the *PL35* allele grow poorly in a *PL35*, tub-*dmyc* background.

Nuclei were stained with DAPI (in blue)

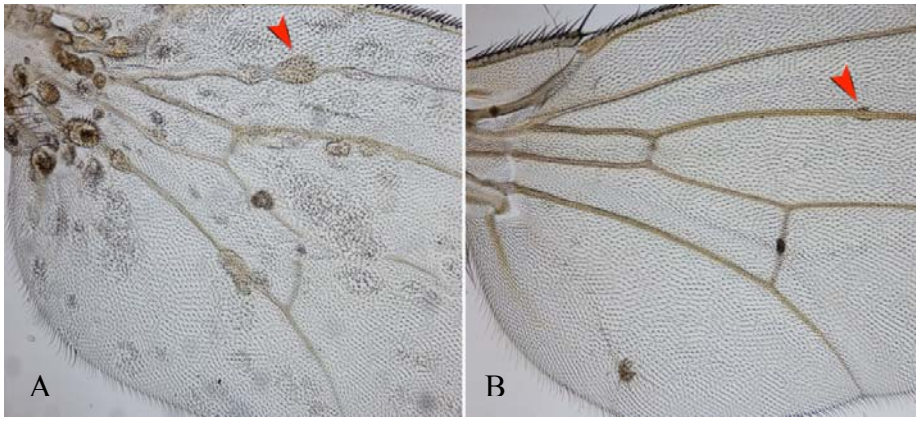


Fig.14.

A. Yki FLP-Out clones in wing of escapers;

B. Heterozygous PL35, tub-yki clones in a tub-dmyc background.

Clones were induced at 60h AEL. (in A for 5 minutes, in B for 45 minutes at 37°C).

In A, only few escapers were viable and displayed clones, in B clones were of reduced dimension and number with respect to A. Only female individuals showed clones, males (hemizygous) appeared to be normal.

***dmyc* fails to rescue *yki* LOF viability**

yki LOF clones fail to grow and die, probably for a deficiency in the proliferation machinery. Interestingly, *yki* LOF clones overexpressing dIAP1 rescue viability and growth at a rate slightly slower than a wt clones (Thompson and Cohen, 2007). This data underline that *yki* defective cells die for the activation of the apoptotic program, and the possibility that they can be eliminated by cell competition is concrete. Our first hypothesis was that yki LOF clones could fail to express *dmyc*, triggering death for competition, but we were not able to detect a decrease in dMyc level inside *yki* clones (not shown), probably due to the fact that dMyc is regulated by many different pathways.

With the aim to understand the role of *dmyc* and cell competition in the survival of *yki* deficent cells we have overexpressed UAS-*dmyc* inside *yki* LOF clones through the MARCM system (see figure 15).

Our data show that *yki* clones overexpressing *dmyc* fail to overgrow and die by apoptosis, detected by α-activated caspase 3 signal (Fig.15A) inside the clones.

This clonal death is probably due to *dmyc* overexpression, that induces an autonomous apoptotic response (Montero et al., 2008). In cells lacking the expression of the Yki targets *bantam* and *dIAP1* (see Fig.15B) dMyc pro-apoptotic response could overwhelm dMyc pro-growth effects.

This data suggest that *yki* orchestrates many dynamics that provide cells with a competitive ability; first, protection from apoptosis mediated by dIAP1 and *bantam*, that facilitate clonal onset.

In this context cell competition appears to be a support not only for clonal onset but also for clonal proliferation and expansion, but appears a relevant mechanism of apoptosis protection which allows *dmyc* upregulation that in turn confers competitivity; without this component, cells appear committed to die.

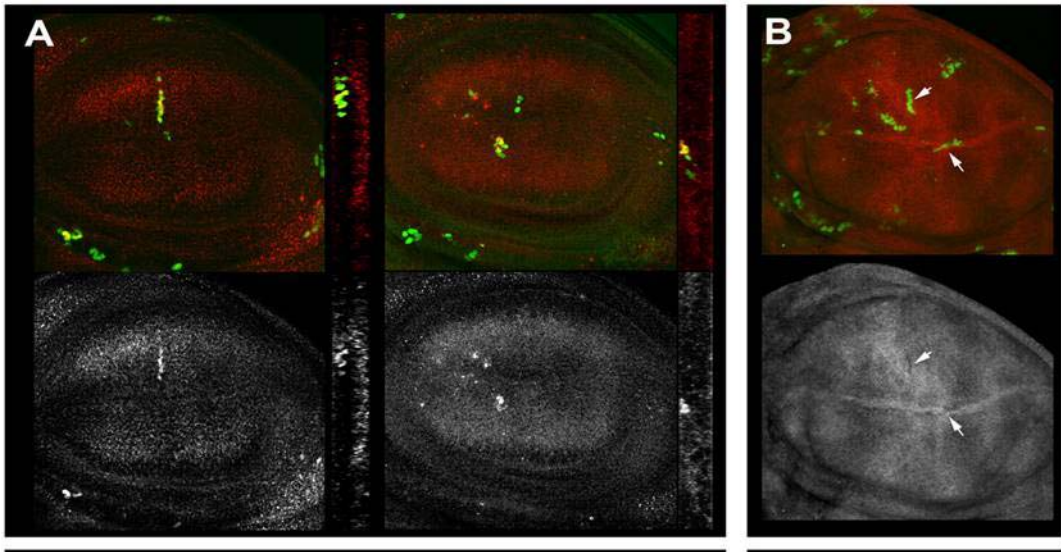


Fig.15. *dmyc* overexpression fails to rescue *yki* LOF lethality

A. yki LOF clones overexpressing *dmyc* show high levels of activated caspase 3 (merge and grey)
B. yki LOF clones overexpressing *dmyc* show low levels of dIAP1 (white arrows)

ft and *ds* clones grow preferentially in the proximal wing region

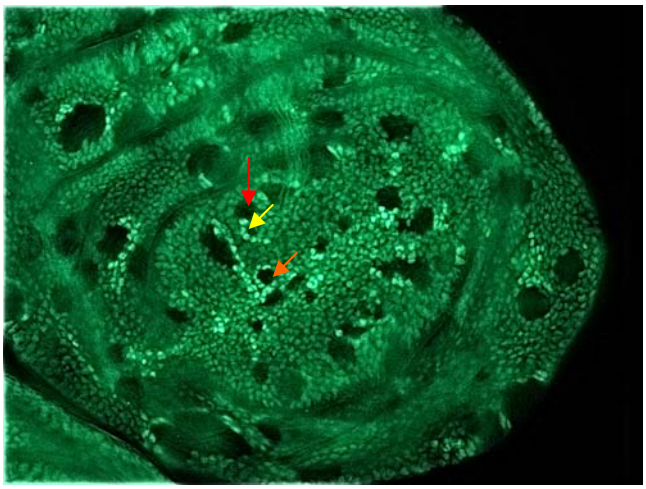


Fig. 16. *ft* clones twin analysis:

ft clones grow preferentially in the proximal region of the wing pouch (red arrow) with respect to the twin (yellow arrow, bright green).

Twins tend to grow preferentially in distal direction, and tend to surround *fat* clones, when arising proximally or flanking to it (orange arrow).

ft clones are larger when they grow in the proximal region.

By clonal analysis in adult wing Flavio Garoia and collaborators reported that *fat* clones grow preferentially in proximal direction with respect to the twin clones (Garoia et al. 2002-2004), moreover *fat* clones fail to grow in the distal region of the adult wing.

To enlight how this phenomenon can arise, we have performed a clonal analysis in imaginal discs, in which we have compared the relative distance to the PD axis (the point of intersection between AP and DV restriction boundaries) between *fat* clones and their related twins.

We have induced clones crossing hsFLP; Ubi-GFP, FRT40A female with FRT40A; $ft^{G\text{-}rv}$ males.

ft clones were marked by the loss of GFP signal and twins became homozygous for the Ubi-GFP and easily detectable within the heterozygous Ubi-GFP background.

ft clones show the tendency to be larger in the proximal regions of the wing pouch (see fig. 16.) and, in particular, twin clones tend to grow in the distal direction (fig. 16., yellow arrow).

To confirm that, we have extimated the distance from the baricenter of each *fat* clone with respect to the relative twin, calculated through ImageJ software http://rsb.info.nih.gov/ij/

The result underlines that *fat* clones tend to allocate proximally (68%; n= 34) with respect to the twin clones (20%; n=34) and in a few cases *ft* and twin clones are disposed at a comparable distance (11%; n=34).

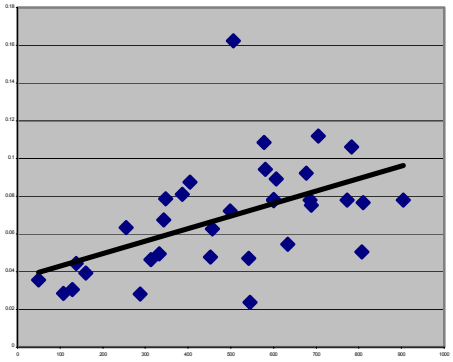
Moreover *fat* clones tend to be larger than the relative twin (1.585 +/- 0.2), and clones appear to be larger in proximal regions with respect to distal regions (P related to R^2= 0.021, regression model: y = 7E-05x + 0.0364

R2 = 0.2655, the area of each clone was standardized to the area of the respective wing pouch).

Also *ds* clones tend to be larger in proximal region (P related to R^2=0.025, regression model: y = 7E-05x + 0.0088; R^2 = 0.228) indicating a similar trend, that in the case of *dachsous* can be related to the proximal expression pattern.

This data show that *fat* activity is predominant in the proximal region of the wing, moreover we underline that *fat* can also control the allocation of the neighboring cells, in particular we underline that *fat* clones have an effect in the disposition of the twin, in fig.16 (orange arrow). We show evidence that twin

clones tend to grow preferentially in distal direction and in the cases in which the twins arise in proximal or flanking position, they tend to be outcompeted or to surround *fat* clone and run in distal direction, only in rare cases twin clones can grow in proximal direction.



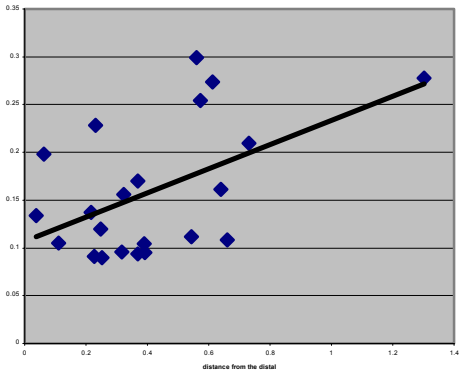


Fig.17 and Fig.18
Scatter plot representing the area of clones related to the area of the wing pouch along the PD axis.
On X where represented the distance from the distal, on the Y the area of each clone.
Regression line where represented.
In 17 ft clones, in 18 ds clones.

vg is implicated in dMyc regulation along the proximal distal axis

With the aim to understand how the expression pattern of *ds* can be modulated along the proximal distal axis, we have investigated possible genetic interactions between *ds* and *vestigial*, the wing selector gene.

The evidence that *ds* and *vg* can interact derives by the complementary pattern of expression showed by those two genes in L3 imaginal discs (see fig.19).

vestigial results expressed in almost all the wing pouch region, with a pattern that decreases from distal to proximal regions.

ds shows an opposite trend, with high levels of expression in proximal and low levels in distal regions of wing pouch.

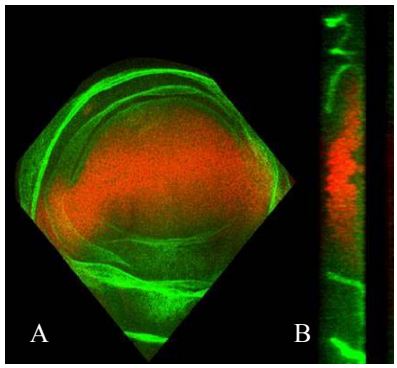


Fig.19. *dachsous* and *vestigial* show complementary expression pattern

A. double staining for Dachsous (green) and Vestigial (red)
B. z-axis projection of the same image

This evidence reinforced the hypotesis that *vg* can effectively modulate *ds* expression pattern, and to demonstrate that we have performed a series of genetic interaction experiments.

First we have attempted to exctopically express *vg* in proximal regions of the wing pouch to test if *vestigial* may affect *ds* expression pattern.

Figure 20 shows that *vestigial*, when ectopically expressed in proximal region, can strongly repress Ds expression,.

This is confirmed by a second experiment in which *vg* expression was suppressed in distal regions of the wing by the generation of vgRNAi clones through FLP-Out system. In this case a Ds overexpression was detected inside the

clone, confirming that *vg* regulates Ds expression pattern, This leads to the conclusion that Ds is gradually regulated by *vg* activity.

Understanding if also *ft* activity can be regulated by *vg*, possibly in an indirect manner by *ds* activity, could be of great relevance for comprehending how the wing is shaped in the proximal-distal direction.

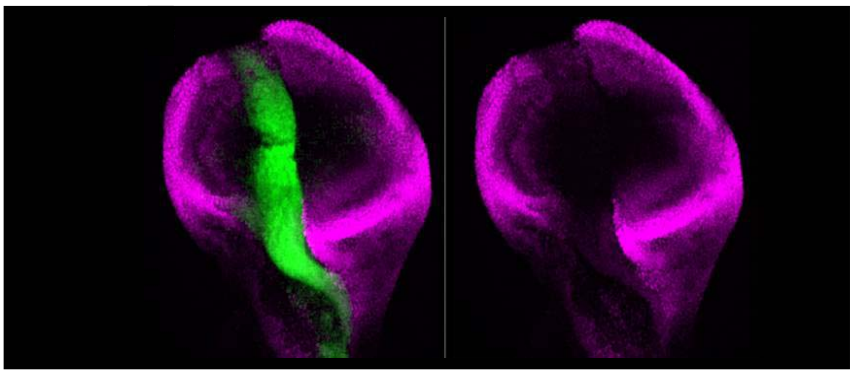


Fig.20. *vestigial* represses *dachsous*

A. UAS-vg X dpp-gal4 in ds lacz-line (Bloomingtoon stock number...)
B. in magenta, anti lacZ immunostaining

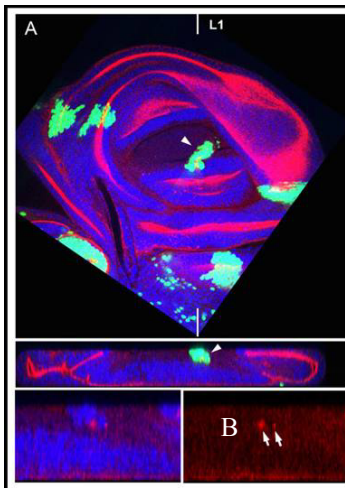


Fig.21. *dachsous* is overexpressed in vgRNAi FLP-Out clones

A. vgRNAi (in green), Ds immunostain (in red), DAPI (in blue)
B. z-axis projection that shows Dachsous overexpression in vgRNAi clones (white arrows)

Another confirmation to this analysis came by the fact that also dMyc levels are regulated by *vg* (see fig.22), both in autonomous and not autonomous manner.

This data show that *dmyc* activation by *vg* is prevalent in the proximal region of the wing, possibly provoked by the repression of *ds*.

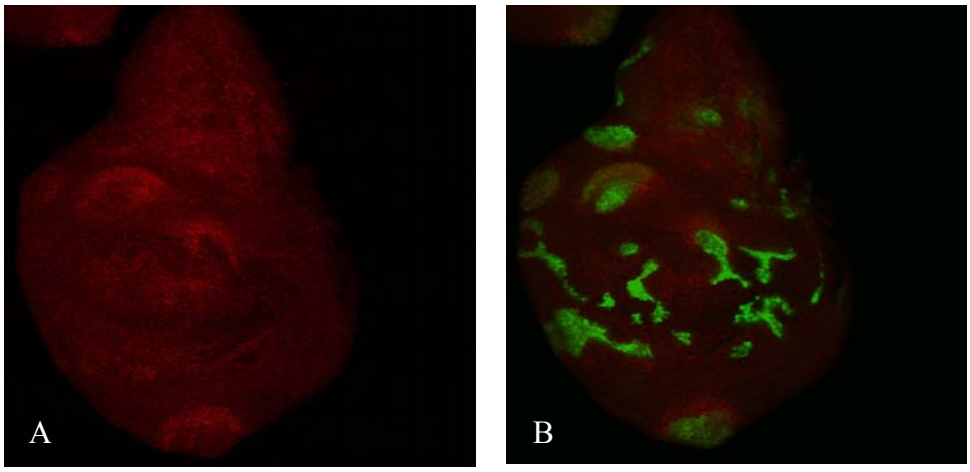


Fig.22- ectopical expression of *vestigial* increase dMyc levels autonomously and non autonomously

A. a-dMyc immunostaining
B. merged image with UAS-vg FLP-Out clones

dMyc results overexpressed non autonomously in cells surrounding clones, in particular outside the wing pouch

CHAPTER 2:

MORPHOLOGICAL ANALYSIS

Effect on the whole wing of *fat*, *ds* and *four jointed* UAS lines

With the aim to understand the cellular parameters affected in proximal-distal mutants, we have performed a phenotypical characterization inducing the overexpression of UAS-lines of genes that affect proximal distal axis through the MS1096-GAL4, expressed in all the wing pouch territory.

The UAS lines used were:

UAS-*fat*
UAS-*dachsous*
UAS-*four jointed*

With the aim to establish a relationship between the UAS lines and the mutant forms we have also examined the viable allele ft^{1}.

We have examined three parameters in three different wing regions (see fig.23A):

1.Wing Area, obtained by the breaking down of the wing in three regions, representing the Proximal, the Central and the Distal regions of the wing, and reported in mm^2.

2.Cell Area, evaluated as the inverse of cell density and reported in mm^2.

3.Cell Number, representing the number of cells for each region of the wing.

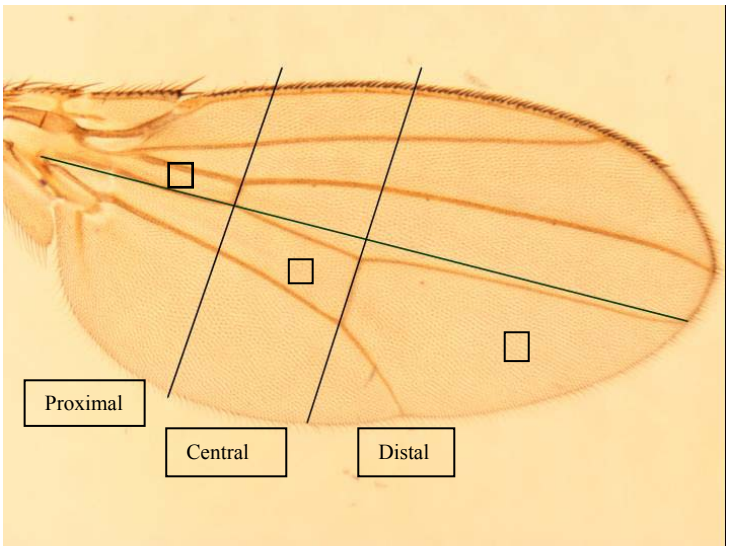


Fig23.A: In this experiment only female wings was characterized in three regions:
Proximal, from the hinge to the anterior cross vein.
Central, from the anterior cross vein to the posterior cross vein
Distal, from the posterior cross vein to the distal end of the wing.
The boundaries where traced perpendicularly to the line that crosses the proximal beginning and the distal end of the L4 vein.
In each region, **Wing Area** (in mm^2) and Cell Density (**CD**) were calculated, successively reported to mm^2. From the inverse of the CD was possible to evaluate Cell Area (**CA**) and Cell Number (**CN**) as the CD * Region Area. Wing images were collected with a Nikon 90i microscope at 40X magnification, cell count and wing measures were analyzed by ImageJ softuare (http://rsb.info.nih.gov/ij/), the statistical analysis was performed by Microsoft Excel and R.

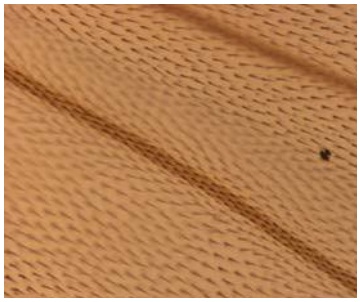


Fig23.B: 200X magnification of a wing intervein region. Each visible trichome is representative of a single cell; by counting trichomes in a standard square it is possible to go back to cell density (**CD**). Thricomes were counted only in the dorsal sheet.

Phenotipes Obtained with the MS1096-GAL4 driver (whole wing)

In false red the mutant or the UAS line overexpressed by the MS-1096 GAL4 driver is represented; in false blue, as a wild type control, we have utilized the only UAS-*ds* line not crossed with any GAL4 driver.

In each case a strong reduction along the proximal distal axis is evident, accompained by a general reduction of the whole wing dimension, in particular for UAS-*ft* and UAS-*fj*.

Overexpression was induced at the constant temperature of 25°C avoiding crowding effects.

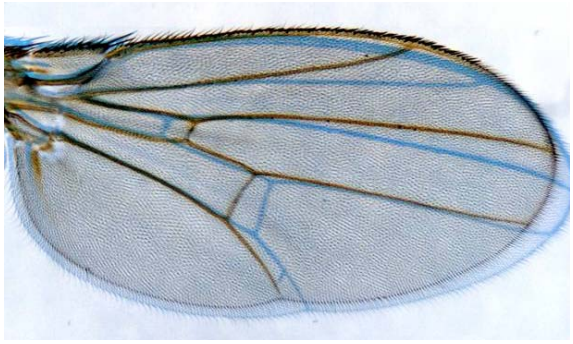


Fig.24A *fat*^{1} *MS-1096*

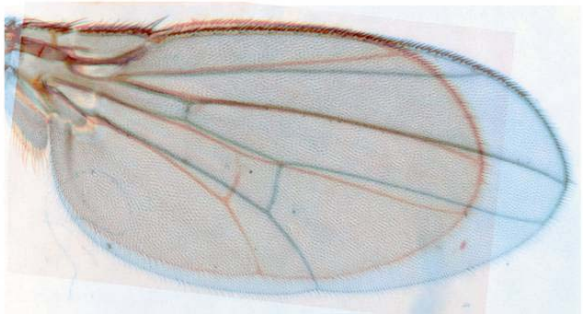


Fig.24B *UAS-fat X*

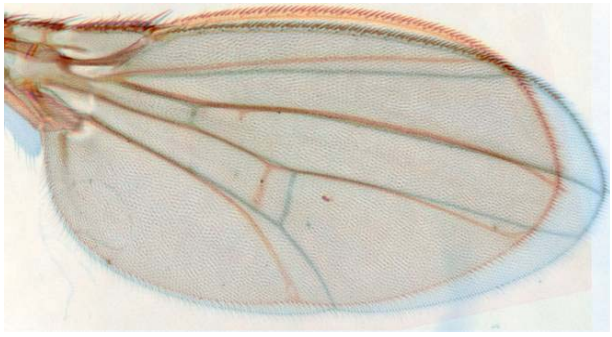


Fig.24C *UAS- Dachsous X MS-1096*

Fig.24D *UAS-four jointed X MS-1096*

Wing Area

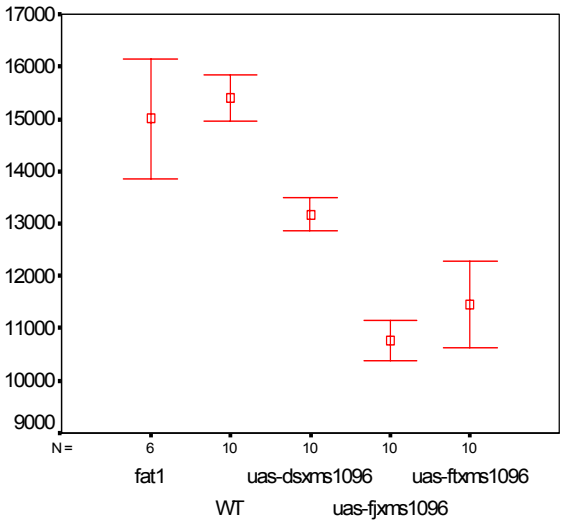


Fig.25A Total Wing Area

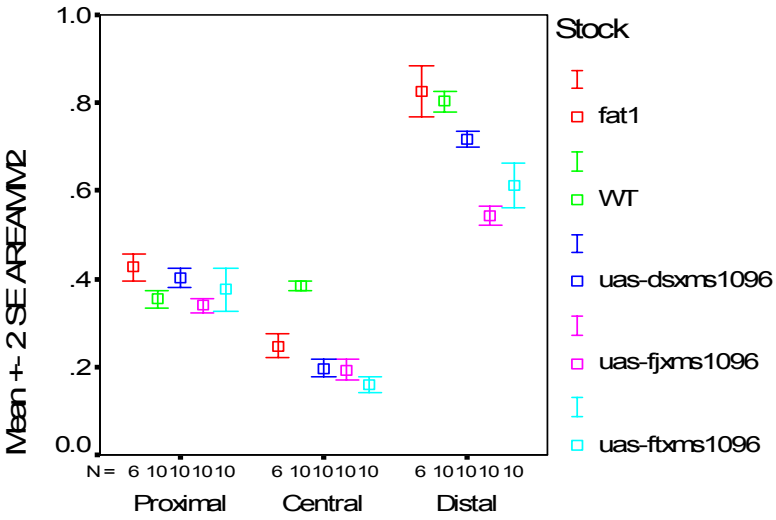


Fig.25B Region Wing Area (mm^2)

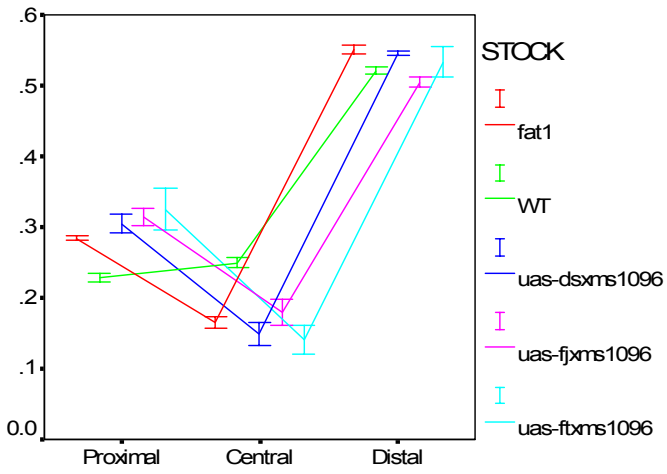


Fig.25C Wing Area
(report of each region to Total Wing Area)

In figure 25 the means regarding wing area measures are represented, the bars indicate the standard error to the means for ft^1 six wing were measured, for the other lines ten wings were measured.

The overexpression of all UAS-lines provokes a reduction in the size of the wing (Fig.25A), in all cases the mean reduction is significative (tested by ANOVA with Tukey analisys) except for ft^1 mutant that shows dimensions similar

to the control. This is a strong indication that *dachsous*, *fat* and *four jointed* can repress organ growth when overexpressed, according to their biological function (*ft* and *ds* are known as tumor suppressors). Shape variation induced by the overexpression of those genes appears surprislingly similar to the shape obtained by LOF or hypomorph alleles of those genes.

To understand how shape was perturbed along the proximal distal axis, we have decomposed wing in three regions (Fig.23A), and for each region we have estimated the mean area (Fig.25B). In the proximal region only ft^1 appears increased with respect to the control (ANOVA with Tukey), in the central region all the mutants and UAS-lines appear reduced, and in the distal region all the lines except ft^1 are reduced respect to the control.

To avoid noise due to size reduction we compared the partial areas and the total area of the wing; in this manner it is possible to evidence only allometric changes, avoiding differences due to size variation. In figure 25C the mean of each sector area in report to wing total area is shown, and major changes in shape are in the proximal region (significative in each case) and in the central region of the wing (significative in each case).

In the distal region, ft^1 and UAS-*ds* x MS1096-GAL4 show an increase while *fj* x MS1096-GAL4 tend to decrease, differences from the control in *ft* x MS1096 are not significative (ANOVA with Tukey).

This analysis shows that major changes in proximal distal axis shape affect mainly the proximal and central regions of the wing.

According to clonal analysis (Garoia 2000-2004), *ft* LOF clones overgrow mainly in the proximal region of the wing, indicating that *ft* is particularly requested in this region. *dachsous* is mainly expressed in the proximal region of the wing (Rodriguez, 2005), perhaps it is possible that major effects in wing shape changes are related to the most proximal region of the wing, where those genes are ectopically expressed. *four jointed* is mainly expressed in the distal region, and these data show that *four jointed* overexpression affects distal shape in an inverse manner with respect to *fat* and *dachsous*.

ft^1 shows an allometric change in proximal distal axis similar to its overexpression, simulating a negative dominant effect.

Despite those genes affect wing shape mainly in the proximal region, the size of the wing is strongly reduced mainly in distal region, indicating that those genes do not act only in the control of shape, but also of the whole size.

Cell Area and Cell Number

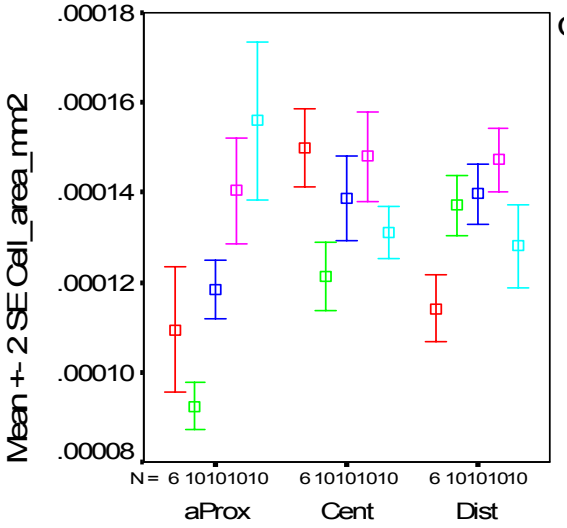


Fig.26A Cell Area (mm^2)

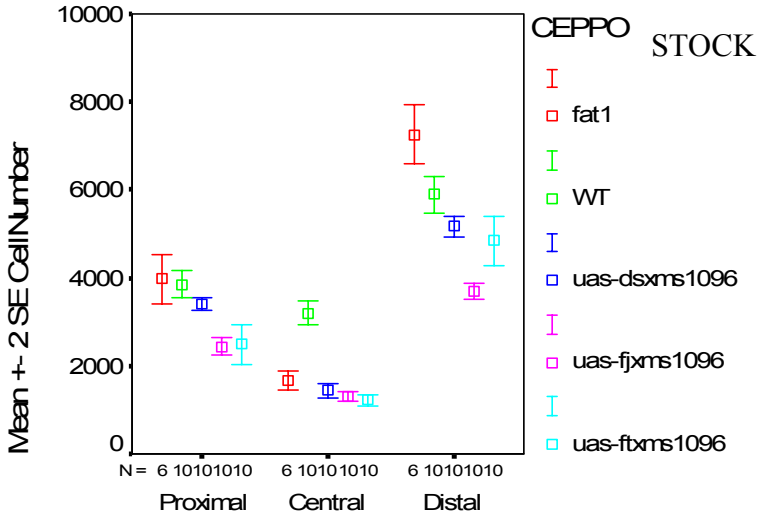


Fig.26B Cell Number

To understand how *ft*, *ds* and *fj* affect wing shape and size we investigated cell area and number.

The figure 26A reports the mean cell area variation in the three regions. These data show a significative increasing in cell area in the proximal region for each UAS line and mutant.

This trend is mantained also in the central region of the wing, when in the distal region there are no differences from the control except for ft^1, that shows a significative decrease in cell area. Figure 26B reports the mean of cell number along the proximal distal axis.

These data show that the strong reduction of wing area is mainly associated with a strong reduction in cell number that results significative in all the regions for all the UAS lines.

ft[1] shows no difference only in the proximal region; a strong reduction in cell number is instead visible in the central region, according to the reduction of wing area in this region. *ft*[1] also shows a significative increase in the distal region, contrarywise to the UAS-lines.

Tab.1 Wing Area, Cell Number and Cell Area ANOVA Referred to fig.25B,26A,26B

			Proximal		Central		Distal	
		df	Mean Square	F	Mean Square	F	Mean Square	F
W.A.	Stocks	4	1.01E-02	*4.8	7.8E-02	*89.93	1.30E-01	*47.4
	Residuals	41	2.12E-03		8.69E-04		1.30E-01	
C.N.	Stocks	4	4767892.63	*18.4	6728033	*85.05	1.352E+07	*33.86
	Residuals	41	258617.23		79103.59		3.99E+05	
C.A.	Stocks	4	6.1E-09	*19.04	1.26E-09	*7.62	1.21E-09	*8.9
	Residuals	41	3.2E-10		1.65E-10		1.357E-10	

The mean difference is significant at the .05 level.

Model: $X_{ir} = \mu + S_i + Er_{(i)}$

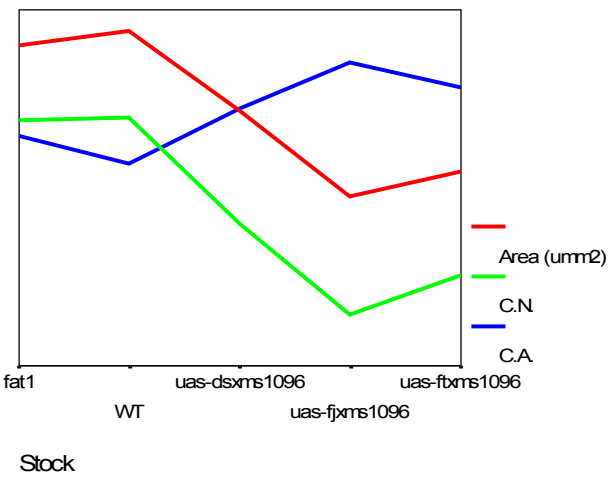


Fig.27A

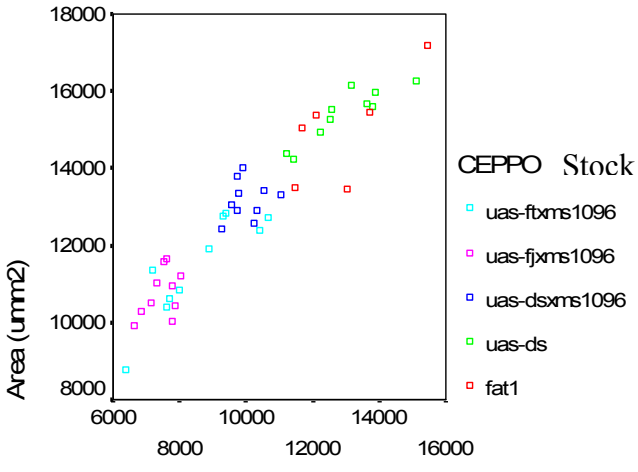


Fig.27B

In Fig. 27A the mean lines of Total Wing Area (in red), Cell Number (in green) and Cell Area (in blue) are evidenced. The graph shows that cell number is directly related to wing dimension; in particular, UAS-*four jointed* and UAS-*fat* show the greatest reduction in size.

On the contrary, the mean cell area shows an inverse relationship respect to the wing area, possibly imputable to mechanisms of compensation due to the strong reduction of cell number. However, the clear increase in cell area only in the proximal and central regions of the wing (Fig.4A) is not explainable with the reduction in cell number, that is more evident in the central and distal regions.

These data show that *ft*, *ds* and *fj* regulates cell size in an opposite manner respect to cell proliferation.

The scatter plot in Fig. 27B underlinse the correlation between cell proliferation and cell area of the wing in all the lines by now examined. The correlation is significative ($R^2 = 0.88$; p value associated = $1.6x10^{-63}$) indicating that wing size is mainly reduced through cell number.

Continuos analysis along the proximal distal axis

Following to the preliminary characterization, with the subdivision of the whole wing area in three regions along the PD axis, it emerged that wing area is variable along this axis. The wild type strain shows more compacted cells in the proximal region with a gradual increment in their area in more distal regions of the wing (fig.4A in green).

ft, *ds* and *four jointed*, when ubiquitously expressed, affect cell area only in the proximal and central regions of the wing. In distal regions, cell size appears not to be affected, opening the question of how cell size and cell proliferation act to control wing shape.

The former analysis does not allow to highlight how cells are actually redistributed in wings in which shape is perturbed by the ectopic expression of *ft*, *ds* and *fj*. This is due to the fact that anterior and posterior cross veins are arbitrary landmarks, that do not allow a continuous analysis necessary to understand those dynamics, and more mappings of cell number are necessary to understand if this parameter changes along the entire axis.

To answer those questions we performed a whole wing analysis of the PD axis, in which it was possible to evaluate differences in wing area (WA), cell area (CA) and cell number (CN) in a continuous manner.

To do that we took a series of high magnification (200X) photographs along the proximal distal axis of the wing in the B compartment that can be considered representative of the PD axis of the whole wing, in particular we have selected this compartment for the absence of cross veins and for its position in the mid region of the wing.

Images was partially overlapping and by an image assembler software (PanVue Image Assembler 3) it was possible to perform a reconstruction of the whole B compartment.

Successively a line was traced intersecting the point in which L2 and L3 veins fuse together in the proximal region and the mid point between the end of L2 and L3 veins at the distal region (see fig.28).

Along this line we built a series of squares of the same area, that allowed to evaluate the trend of cell density (by the count of the trichomes contained in the squares) along the entire PD axis.

Each square was successively associated to the sector area of intervein comprised between L2 and L3 veins.

It was so possible to evaluate in a continuous way the trends of variation in wing area, cell area and cell number between the control and the UAS lines overexpressed in all the wing.

At least five wings were analized for the control and for each line.

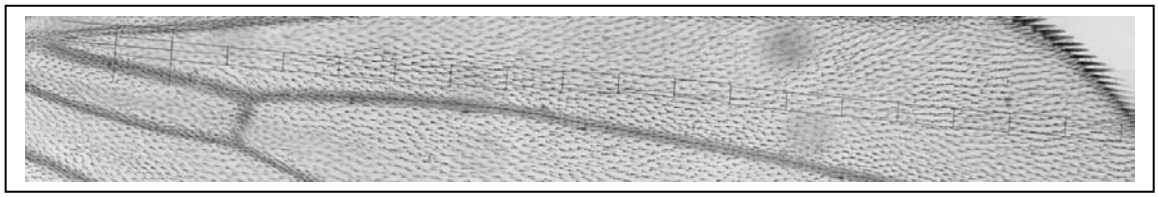


Fig.28 – Recostruction of the whole B compartment by a series of overlapping 200x images. Cell density was taken inside each square.

Trend in Cell Area Variation along the proximal distal axis

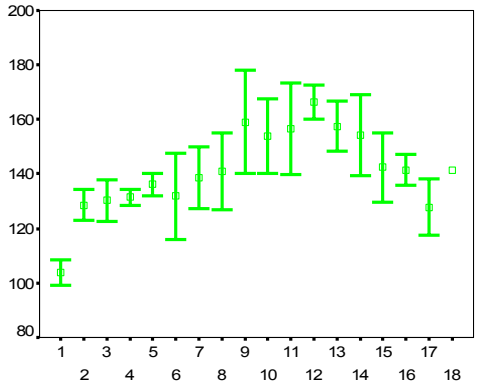


Fig.29 - Trend in Cell Area Variation along the proximal distal axis of the wild type wing. A total of six wings was analyzed.

Figure 29 shows how cell area changes along the proximal distal axis, confirming the previous analysis. Cells tend to be smaller in the proximal region, and gradually incrase their area in the central and central-distal regions. In far distal region cells tend to clench again.

ANOVA
Trend in Cell Area Variation Referred to fig.29

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19242.179	17	1131.893	7.087	1.824E-09
Within Groups	10860.792	68	159.718		
Total	30102.971	85			

Trends of Wing Area, Cell Number and Cell Area of the UAS lines overexpressed with MS1096-GAL4

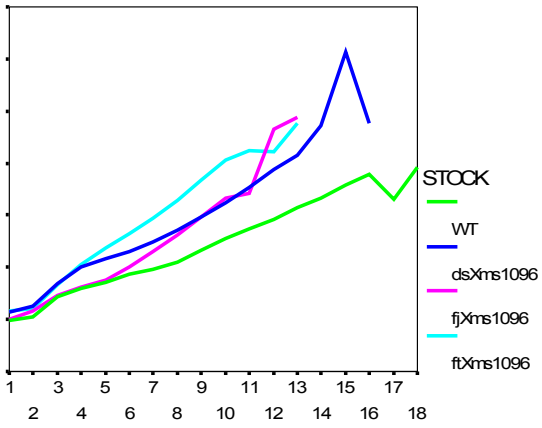


Fig.30A –Wing Area Variation along the P-D axis

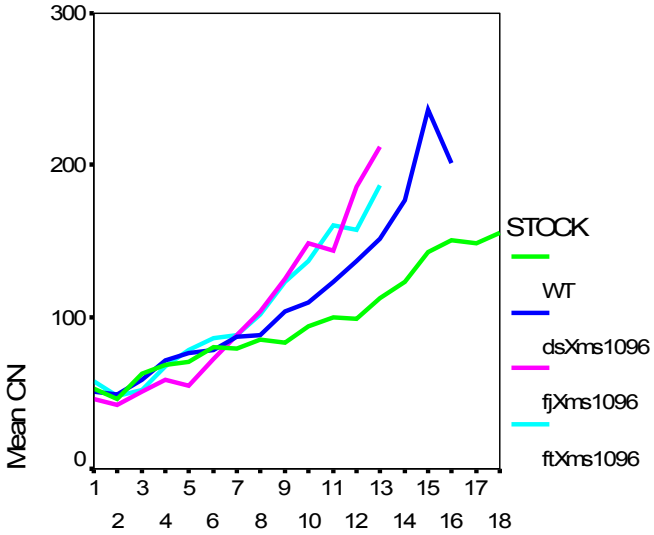


Fig.30B –Cell Number Variation along the P-D axis

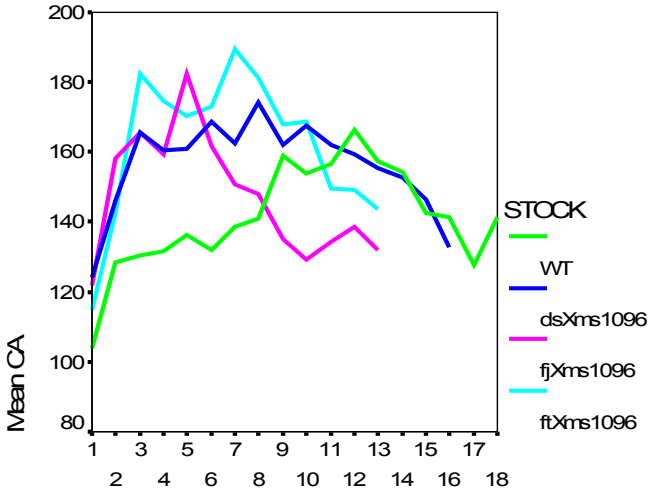


Fig.30C –Cell Area Variation along the P-D axis

Figure 30A shows the trend of mean Wing Area (WA) along the proximal distal axis of B compartment.

Wing area increases from the proximal to the distal direction, probably due to the joining of L2 and L3 veins. For the effect of the overexpression of UAS-*ft*, *ds* and *fj* this angle increases. Increasing the area of the sectors, but the reduction in wing dimension is reflected in a significative reduction of wing length, relevable by a significative reduction in sector number. The decrease in wing area shown in the WT and UAS-*ds* in far distal region is a consequence of the curvature of the wing at the distal margin of B compartment.

Figure 30B represents the trend of mean Cell Number (CN) obtained reporting cell density of each square to the WA of each sector.

These data show small differences in CN in proximal regions of the wing between all the UAS lines and the control, according to the previous analysis (Fig.4B).

Significative differences in cell number are evident in central and distal regions, where UAS lines shows an increase in CN related to the increase in WA.

As a confirmation of the previous analysis, UAS lines induce e significant effect on Cell Area (figure 30C) in proximal regions.

Ectopic Patterned Expression along the Proximal Distal Axis

Data shown here underline that all the UAS-lines show a similar proximal distal reduction in wing shape when overexpressed ubiquitously, moreover all the lines reduce body size through an effect on cell number and strong effects are visible in the control of wing area in the proximal region of the wing, that appears the major parameter affecting wing shape in this region.

These results suggest that those genes act in a similar manner to modulate the shape, but data in literature evidence that *ft*, *ds* and *fj* are characterized by different patterns of expression (see intro).

In particular *ds* shows high levels of expression in the proximal region, *ft* is ubiquitously expressed and *fj* localizes only in the distal region of the wing, moreover *ft* represses *four jointed* expression through the Hippo pathway and the regulation of *ft* activity by *dachsous* is strongly suspected.

This means that a finely tuned mechanism is involved in the definition of shape, in which the expression pattern may play a central role, therefore ubiquitary expression of those genes may produce similar phenotypes through a perturbation of feedback mechanisms among *ft*, *ds* and *four jointed*.

To avoid this noise and to better characterize the function of those genes, we performed a pattern-specific overexpression along the PD axis of UAS-*ft*, UAS-*ds* and UAS-*fj*.

To do that we firstly characterized a series of GAL4 drivers with a pattern of expression distal or proximal.

We have obtained eleven GAL4 ehnancer trap lines with a putative proximal or distal patterns, kindly provided by the Kyoto NP-Consortium (Kyoto Institute of Technology) and reported in the GETDB data base.

Successively we have performed a X-GAL staining in L3 imaginal discs and in pupal wings to choose a GAL4 line for the proximal and one for the distal regions of the wing.

The X-GAL staining highlights the pattern of expression of a GAL4 line crossed with the UAS-lacZ line (n° of Bloomington stock center 3955).

Figures 31A and 31C show the expression pattern of the NP4657-GAL4 line in L3 instar imaginal disc and pupal wing. The pattern of expression of this line is more intense in the hinge and proximal wing presumptive regions, and absent in the distal region. In pupal wings the expression is very low and is mainly localized in the hinge region (black arrowhead) and along the L1 vein, the white arrow indicates the two cross veins.

Figures 31B and 31D show the expression pattern of the NP4284-GAL4 line in L3 instar imaginal disc and pupal wing. The expression pattern is distal, except for the DV boundary, and the pupal wing demonstrates that this gene is active until the last stages of development only in the distal region of the wing.

UAS-lacZ X NP4657-GAL4
(proximal)

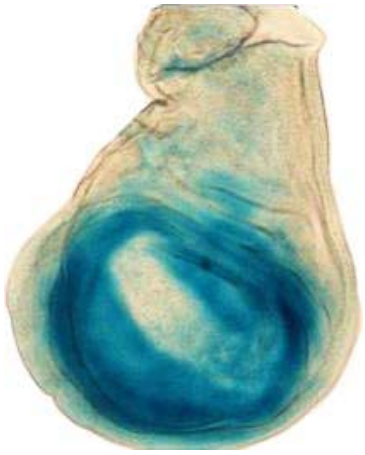


Fig.31A

UAS-lacZ X NP4284-GAL4
(distal)

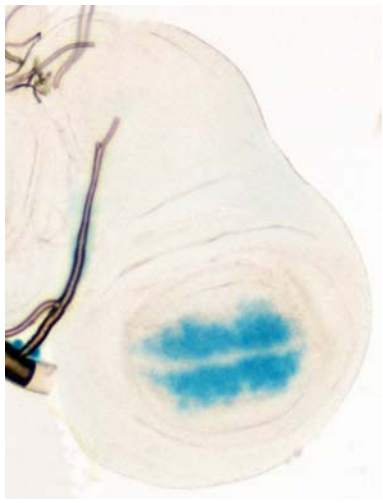


Fig.31B

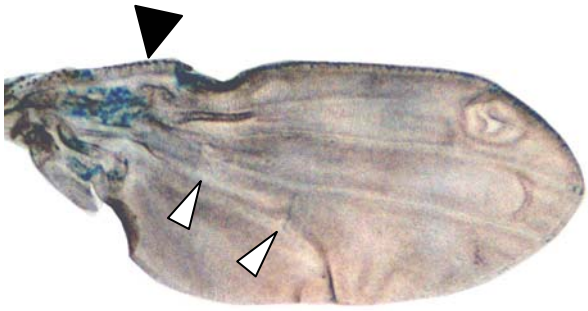


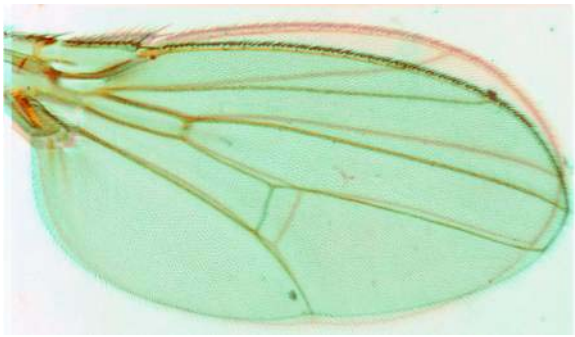
Fig.31C

Fig.31D

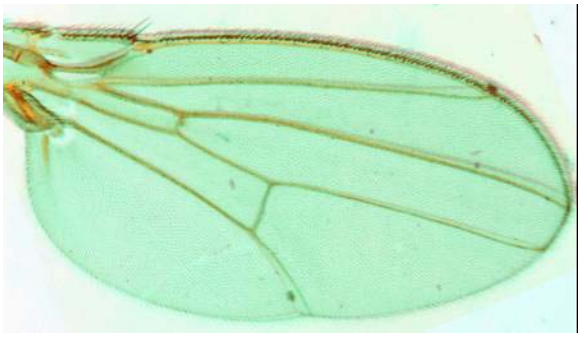
Fig. 32 - Effect of Ectopical Expression in Proximal and Distal regions

Proximal Expression **Distal Expression**

A - UAS-*ft* X NP4657-GAL4



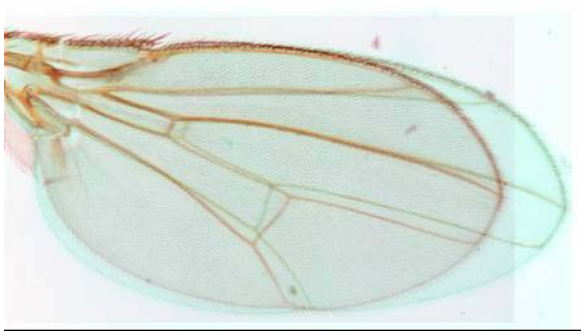
B - UAS-*ft* X NP4284-GAL4



C - UAS-*ds* X NP4657-GAL4

D - UAS-*ds* X NP4284-GAL4

D - UAS-*fj* X NP4657-GAL4



E - UAS-*fj* X NP4284-GAL4

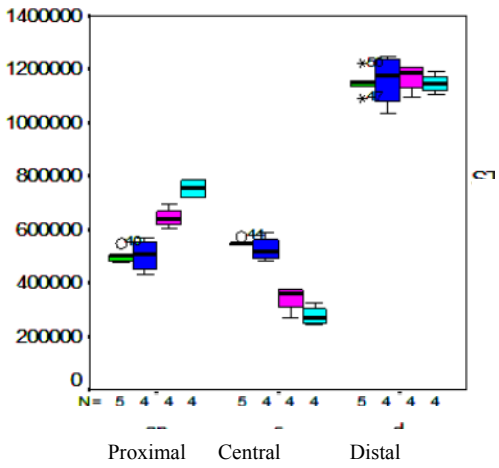


Fig.33A – Wing area variation in proximal ectopically expressed lines

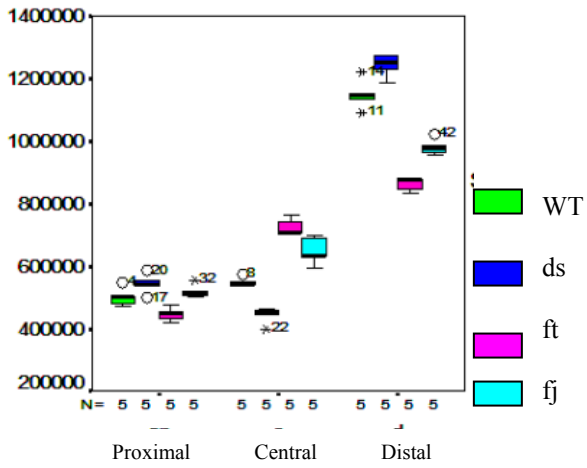


Fig.33B – Wing area variation in distal ectopically expressed lines

The boxplot in figure 33A shows the mean wing area variation of the three wing regions obtained by the overexpression of the UAS lines by the NP4657-GAL4, with proximal expression pattern.

UAS-ft and UAS-fj when overexpressed in the proximal region show significative differences with respect to the wild type where a proximalization is clearly present that is provoked both by an enlargement of the proximal area and by a reduction of the central area.

The lines do not show any difference in the distal region, underlining the autonomous effect of those genes.

dachsous ectopical expression in the proximal region does not show differences in shape variation with respect to the wild type (visible also in the figure 32C).

This is possibly due to the normal *ds* expression pattern that is typically proximal and the NP4657-GAL4 partially resembles this pattern.

The boxplot in figure 33B shows the mean wing area variation of the three wing regions obtained by the overexpression of the UAS lines by the NP4658-GAL4, with distal expression pattern.

Also in this condition *ft* and *fj* ectopic expression induce a variation from the control that can be interpreted like an effect of proximalization; the central area indeed results increased at the expenses of the proximal area.

On the contrary, *ds* expression in the distal wing, where *ds* is ususlly expressed at a low level, induces a phenotypical distalization of the wing respect to the wild type, in which the central region results reduced, with an increase of the distal wing region.

The results provide some interesting suggestions: first, *ds* acts in an opposite manner with respect to *ft* ans *fj* in the organization of the proximal distal axis; second, *ds* overexpression where it is usually expressed does not apparently affect the shape of the wing.

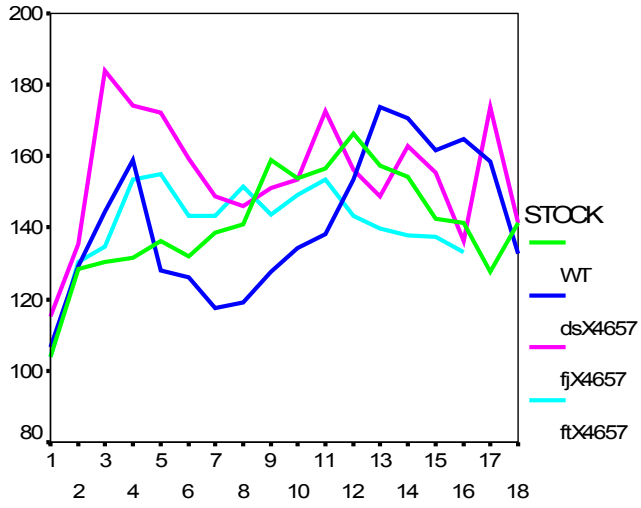


Fig.34A – trend of Cell Area in 4657-GAL4 lines

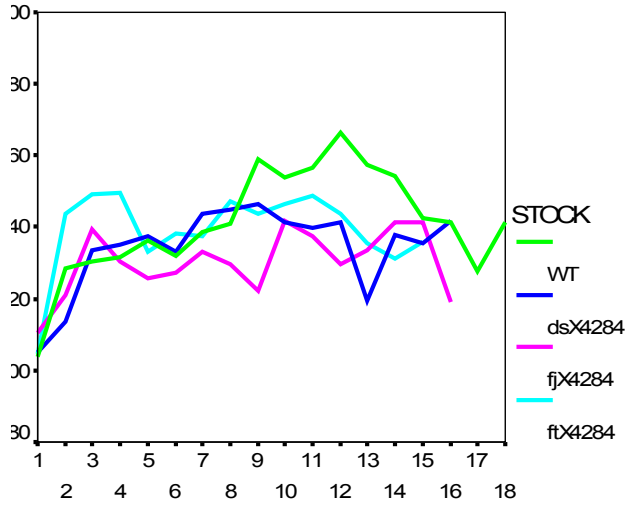


Fig.34B – Trend of Cell Area in 4284-GAL4 lines

Figure 34A shows the trend of mean Cell Area variation of *ds*, *four jointed* and *fat* when overexpressed in the proximal region by the 4657-Gal4 line.

In all cases an increase in Cell Area is evident in the proximal region. *ds*, besides an increase of the proximal region, shows a strong reduction in cell area in the region crossing the border of expression of the GAL4 line.

Figure 34B shows the trend of mean Cell Area variation of the UAS lines distally overexpressed by the NP4284-GAL4. In this case, lines do not show any

difference from the control in the most proximal regions and in a similar manner show reduced cell area in the distal region of the wing.

Ds expression pattern constrain the shape of the wing

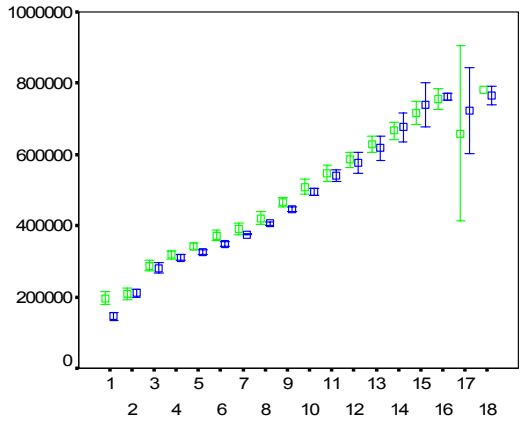


Fig.35A – Sector Area

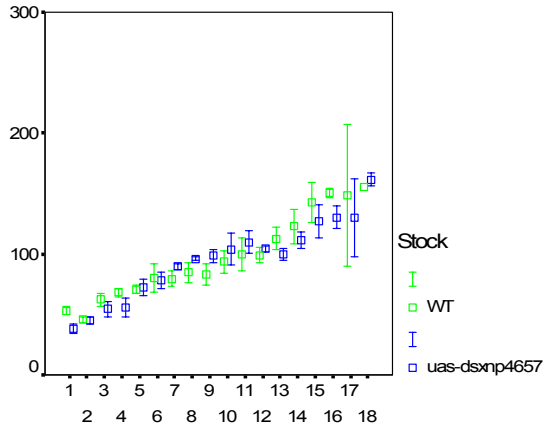


Fig.35B – Cell Number

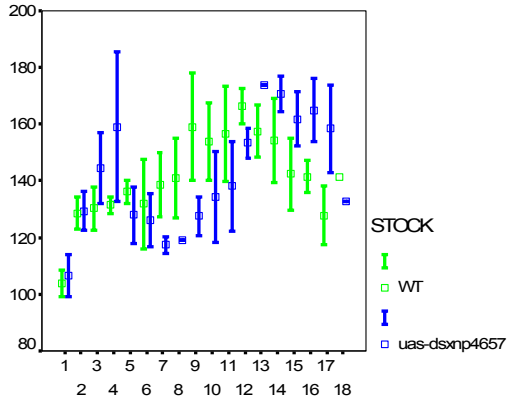


Fig.35C - Cell Area

The absence of change in wing dimensions and allometric components shown in Figure 32C and 33A underline that when *ds* is overexpressed in its wild type pattern it does not affect the shape of the wing. Possibly *ds* is limited by another interacting factor, so explaining why its overexpression (that does not induce pattern variations) does not change wing shape.

Figures 35A and B show that sector area and cell number display the same trend, with a small decrease in the sector area of *ds,* in particular in the proximal-central area of the wing.

Figure 35C shows the trend of mean cell area; in this case we report significative differences between *ds* overexpressed line and the control, particularly relevant in sectors 7, 8 and 9, where *ds* shows a strong reduction in cell area ($P=7.16E^{-5}$; F=21.65). In this region also the sector area is smaller than the control ($P=6.95E^{-30}$; F=2930.19), but not the cell number ($P=2.99E^{-5}$; F=24.72), indicating a reduction for an effect in cell area.

This may appear a compensatory effect for the increased cell area in proximal sectors (in particular the 4, one tailed t-test, $P=5.91E^{-9}$), accompained by an increase in sector area (one tailed t-test, $P=2.43E^{-2}$) and by a decrease in cell number (one tailed t-test, $P=1.62E^{-9}$) , probably by a *ds* effect on proliferation.

This analysis show that small effect in the shapening of the wing are in action, but is evident how the increase of a region is compensated by a decrease of the flanking region, this compensatory effect leads to a wing shape constrain.

TABLES

Tab.1 Wing Area ANOVA

ANOVA
AREAMM2

Source of Variation	df	Proximal Mean Square	Proximal F	Proximal Sig.	Central Mean Square	Central F	Central Sig.	Distal Mean Square	Distal F	Distal Sig.
Stocks	4	.010	4.808	*.003	.078	89.933	*.000	.131	47.413	*.000
Residuals	41	.002			.001			.003		

* Sig. is the F associated significativity; The mean difference is significant at the .05 level.

Model: Xir = u+Si+Er(i)

Tab.2 Total Wing Area ANOVA

	df	Mean Square	F
Stocks	4	.391	*45.171
Residuals	41	.009	

The mean difference is significant at the .05 level.

Model: $X_{ir} = \mu + S_i + Er_{(i)}$

Tab.3 Mean Cell Area ANOVA

	df	Mean Square	F
Stocks	4	1.192E-09	*14.965
Residuals	41	7.963E-11	

The mean difference is significant at the .05 level.

Model: $X_{ir} = \mu + S_i + Er_{(i)}$

DISCUSSION AND PERSPECTIVES

In this thesis, genetic and cellular mechanisms have been considered representing the basis for the development of the proximal-distal axis of *Drosophila* wing.

In particular, we examined cellular mechanisms yet uncharacterized that can be of considerable relevance in the correct development of organ shape and size. We showed that a stricy correlation exists between shape and size, since genes analyzed act modulating both traits.

fat, genes of the Hpo pathway and their effector *yki,* evolutionarily conserved in metazoans, act as molecular brakes in limiting proliferation and driving programmed cell death.

Our data and recent papers (Tyler et al., 2007; Li and Backer, 2007) highlight the evidence that genes of the Hpo pathway also act in controlling cell competition, a phenomenon affecting cells with a reduced proliferative potential. In particular, in mutant or defective conditions, we showed that *dmyc* is responsible for Yki competitive ability, which regulates it transcriptionally. The role of cell competition seems thus to be that to favor cells within an organ showing a comparable proliferation rate.

About Cell Competition

Cell competition appears to be a very sophisticated mechanism and the evidence that it is associated to genes involved in the control of an axis form suggests the possibility of being itself involved in the definition of this trait.

How this thesis shows, dMyc expression pattern is not homogeneous within wing imaginal disc, neither within compartments. Such a pattern would not be possible if some mechanisms safeguarding competition were not at work; in wild type imaginal wing discs apoptosis is indeed in average very low. Cell competition and its consequences, such as apoptosis, result from success of a particular cell lineage over another during an organ development. Dale Purves (1984) and Martin Raff (1984), on the basis of Rita Levi Montalcini findings, proposed that cell death is the default fate of all metazoan cells, and that a series

of survival signals are required to permit cells to survive (cytokines, hormones, physical heterotipyc interactions with neighboring cells or extracellular matrix).

With the same outcome Moreno, Basler and Morata (Moreno et al., 2002) described the cell competition phenomenon like a Darwinian selection between the fittest cells within a developing tissue, and Santiago Ramon y Cajal, Edelman and D'Amasio have also proposed a series of models of neural Darwinism (Edelman, 1987) to explain apoptosis in neural cells due to an insufficent synaptical connectivity.

Competition between cells can be translated into competition between modules (Klingemberg 1998) or organs (Nijhout and Elmen, 1998), but this mechanism can be instrumental in the onset of local autonomy inside a developmental structure with a low degree of compartmentalization (Minelli, 2003), such the wing blade proximal distal axis.

For competition to have a role in shape determination, a strict regulation is necessary. In this thesis we enlight that genes involved in cell competition control are regulated in a pattern-related manner in determining proximal-distal axis formation. When ectopically expressed, *yki* appears to be active and behave as a super-competitor in an ubiquitous manner. In a wild-type condition, Yki is inactivated by gene owing to the Hpo pathway, whose activity is modulated upstream by Fat and Ds which in turn act determining proximal-distal axis form in a pattern-dependent manner. In particular, a null condition of Ds, normally expressed in the proximal regions of the wing, induces in this territory a dramatic effect in cell growth accompanied by a strong increase in dMyc abundance, giving cells a competivite advantage and favoring cell proliferation.

Here we propose a model in which the correct *ds* expression is controlled by *vg*, the wing selector gene, which further to provide cells with a wing identity, plays a key role in the determination of the organ shape. This, in turn, modulates *ft* activity that is higher in the proximal regions and this implies that Hpo pathway and Yki are regulated in a gradual manner along this axis. How *ft* activity is regulated is still an open question, but it is highly presumable that *ds* exerts a positive role in this, possibly acting as an insoluble ligand, so *vg* could act as a repressor in *ft* signaling.

On Genes and Form

This thesis illustrates the fact that cell area is not constant within *Drosophila* wing blade, but it rather changes along the proximal-distal axis. It further demonstrates that cell area is heavily affected from shape variations due to *ft*, *ds* or *fj* misexpression.

Cell area variation seems to be the parameter related the most to shape allometric variations. Indeed, as it can be observed in Fig. 25C, in the proximal region of the wing, where changes in shape reach a peak, misexpression effects are mainly visible in changes in cell area. This observation apparently countertrends LOF phenotypes of the same genes, which mainly act in proliferation control. A possible cellular mechanism operating in the control of cell size without altering cell number is cell competition, that can thus explain how these changes in shape may occurr, integrating those produced by cell allocation which better explain what happens in the distal region of the wing where changes in shape seems not to be attributable to cell area variations. Do genes dedicated to cell shape actually exist? This is a crucial question for developmental biologists (for details, see Minelli, 2003), and the answer is not that easy.

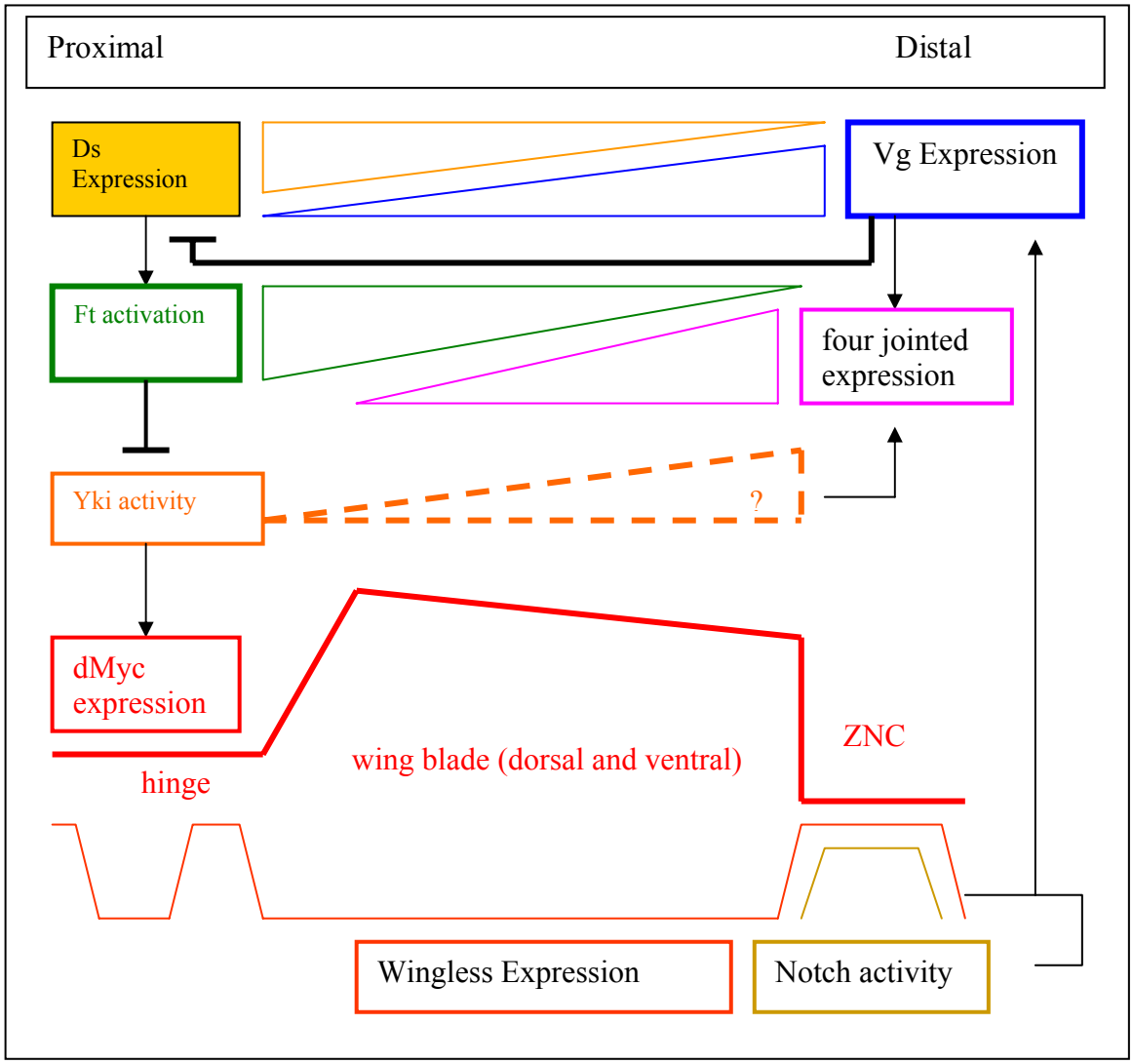
The form of an organ is the sum of a wide series of cellular mechanisms, such proliferation, control of cell growth, apoptosis or cell competition, oriented cell divisions or migration leading to cell allocation.

A wide series of genes are involved in the control of those dynamics (probably the majority of the genes), but not all the genes are responsible for organ shapening.

To shape an organ it is necessary to canalize those dynamics, and the genes analyzed in this tesis, *ft*, *ds* and *fj*, converging on Hpo pathway, play a pivotal role in all the described dynamics.

Are those genes masters in shape control?

The answer is yes if we are searching for genes involved in orchestrating mechanisms establishing a correct allometric relationship among the different components of an organ. At the same time, we have to consider the importance of the expression pattern and the modulation of their activity.



On the Evolution of Animal Form

Drosophila wing is an excellent model for studying shape evolution. Especially in the study of the minimal shape variations occurring in natural population, very sensitive morphometric methods have been developed to distinguish small allometric variations from those related to dimensions. Among these, we mention methods for analyzing relationships among different wing regions and compartments (Cavicchi et al., 1985), the angular off-sets analysis that summarizes allometrical changes in shape through the analysis of the angles found among a series of landmarks (Weber et al., 1990) or the statistical Fourier

analysis (Cavicchi et al. 1991), geometric morphometric methods based on procustes analysis (Hurley and Cattell, 1962; Klingemberg et al., 2000).

These methods allowed to discover that, though strongly constrained, wing shape is subjected to variations during natural population evolution; furtherly, in laboratory populations under artificial selection, shape changes the first, also before dimensions. (Santos et al., 2006).

Artificial selection experiments had shown that Very small regions can respond to selection almost independently (Weber, 1992), suggesting that the control of wing pattern formation must involve many genes and that many units of selection, other than major wing compartments, can exist.

Finding genes involved in shape variation in populations under selection is very hard and the main analyses in this field are QTL studies and linkage disequilibrium mapping (Palsson and Gibson 2004) or mycroarrays analysis (Weber et al., 2007).

Concerning wing shape in *D. melanogaster*, a number of studies have demonstrated moderate to high heritability for the phenotype (Weber 1990; Birdsall et al. 2000; Zimmerman et al. 2000; Palsson and Gibson 2004; Mezey et al. 2005), and there is little evidence for constraints on the evolution of shape (Mezey and Houle 2005).

Complementation deficiency mapping and polymorphism studies identified members of the EGFR pathway in the explaination of natural variation in wing shape (Palsson and Gibson 2004; Dworkin et al.2005; Palsson et al. 2005).

Another significative result was reported by random P-element insertions that produce heritable wing shape variations in isogenic backgrounds (Weber et al., 2005). Rescue of the region flanking the new insertion allows to identify candidate genes.

The EGFR *locus* or genes associated to this pathway appear involved in some cases in natural variation of wing shape (Palson and Gibson, 2004; Palson et, al.,2005).

Ian Dworkin and Greg Gibson have reported that EGFR mutants can affect wing shape in a size independent manner and in the whole wing, finding also a role for *scalloped* gene (the binding partner of Yki and Vestigial) (Dworkin and Gibson, 2006), considering also of fundamental importance the indirect effect of

competitive growth between cell populations (Klingemberg and Nijhout 1998; Nijhout and Elmen, 1998). In this scenario they propose that changes in growth patterns in one region of the wing imaginal disc are compensated for in other regions, resulting in global shape changes in the wing; moreover they suggest to use clonal analysis in *Drosophila* to distinguish it from possible direct effects.

This thesis is perfectly in agreement with these data, for genes involved, for compensatorial effects reported between different regions of imaginal disc, for the competitive activity and for the genetic approaches proposed.

ft is directly implicated in the EGFR pathway modulation (Garoia et al., 2004) acting as a repressor; we have also demonstrated that *vestigial* can modulate *ft* activity.

EGFR pathway is implicated in dMyc protein stabilization, while *fat* acts in the opposite manner in this function and *dmyc* can be responsible for the competition between different regions of the disc, generating compensatorial effects.

A recent publication (Weber et al. 2007) focused on a bi-directional selection of wing shape through the angolar off-sets method, and gene expression was evaluated by microarray analysis.

These data reported genes that affect shape through changes in proliferation such as *vestigial*, *Delta* and *Notch*. In agreement with our data, modulating *vestigial* expression it is possible to modulate wing shape. Weber and collaborators have found also *reaper* (White et al.,1996), a gene involved in apoptosis through *hid* pathway, the same used by *dmyc* to induce cell competition.

Our data show that a massive expression is not able to induce *per se* changes in shape; as an example, *ds* overexpression in the proximal region of the disc is incapable to affect shape consistently; ectopical expression is rather more effective and this is not detectable through a microarray analysis from samples derived by the whole disc.

Nevertheless, the use of developmental genetic methods is not sufficiently sensitive in evaluating subtle changes that can affect expression pattern of genes involved in natural population shape variation, but it is possible that these differences are relevable in macroevolutive events.

In prospect, we propose to investigate if differences in expression pattern are present among related species in which those gene functions are conserved and may be responsible for shape variation.

Within *Drosophila genus*, hawaiian species offer some of the most dramatic examples in morphological and behavioral evolution (Edwards et al., 2007). Nearly 1000 species are present in this archipelago, following a single event of colonization hypotisized 26 milion of years ago (Carson and Kaneshiro, 1976; Carson and Clague, 1995 In: Wagner WL, Funk VA, eds. Hawaiian Biogeography: Evolution on a hot spot archipelago. Washington D.C.: Smithsonian Institution Press. pp 14–29; Carson, 1997; Remsen and DeSalle, 1998).

This impressive founder gene effect has generated a wide range of speciation in this group, and mainly the wing of different species presents different morphologies in size, shape and pigmentation pattern.

In the collection presented by Edwards and collaborators (Edwards et al., 2007) samples are exposed of wings that in some cases present marked differences.

This can allow, through a series of immunostainings of wing imaginal discs and through a morphometrical analysis, to enlight if genetic patterning can affect shape evolution.

We speculate that possible differences can be detected in Ds and Vg expression along the proximal-distal axis of these different species.

Dachsous

Proximalized wing

Vestigial

Distalized Wing

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