Drosophila limb development

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A central question in modern developmental biology is how the growth and patterning of tissues are controlled at a molecular and genetic level. *Drosophila melanogaster*, commonly known as the fruit fly, has become an indispensable model system to approach this question because it can be easily manipulated genetically and molecularly and there is abundant information available on its developmental biology. Systematic genetic screens for loss-of-function mutations, gain-of-function phenotypes and the detection of enhancers have revealed many of the genes involved in a number of developmentally critical processes. In addition, the completion of the genomic sequencing project for *Drosophila* makes it possible to use reverse genetic approaches, such as RNA-mediated interference and targeted gene disruption, as well as genome-wide expression analyses, to address a wide variety of issues concerning the developmental biology of the fruit fly.

During the development of multi-cellular organisms, groups of cells assemble to form tissues that are initially homogenous. The elaboration of spatial pattern often begins with the subdivision of a field of cells into smaller territories. The imaginal discs of *Drosophila* provide well-characterised experimental systems in which the subdivision of tissue depends on mechanisms that limit cell mixing, thereby producing stable boundaries. These stable subdivisions are called compartments. In the imaginal discs compartment boundaries serve as signalling centres. Short-range interactions between cells in adjacent compartments induce the expression of the signalling proteins Wingless (Wg) and Decapentaplegic (Dpp) in cells adjacent to the compartment boundaries. Wg and Dpp form long-range extracellular protein gradients centred on these boundaries. Stable boundaries between compartments result in tightly localised sources of these signalling proteins. Intermingling of cells at the compartment boundary causes disorganisation of the signalling centre with disastrous consequences for patterning and growth control.

The *Drosophila* wing imaginal disc is a monolayered epidermal sac. The wing primordium arises from the embryonic ectoderm as a group of around 30 cells, which proliferate extensively during larval development to achieve a final population of about 50,000 cells. The wing primordium is subdivided into an anterior (A) and a posterior (P) compartment by the restricted expression and activity of the homeodomain protein Engrailed in P cells. This subdivision is inherited from the embryonic ectoderm. When the wing primordium consists of around 100 cells, it becomes subdivided again into a dorsal (D) and a ventral (V) compartment by the restricted expression and activity of the LIM-homeodomain protein Apterous in D cells. Our research efforts focus on elucidating the generation of compartment boundaries, the induction of expression of the organizing molecules Wg and Dpp at the compartment boundaries, and the organisation of growth and patterning by the activity of Wg and Dpp at these boundaries. The following topics were addressed during 2007:

A novel molecular mechanism to restrict hedgehog expression in the *Drosophila* wing

Stable subdivision of *Drosophila* limbs into an A and a P compartment is the result of asymmetric signalling by Hedgehog (Hh) from P to A cells. The activity of the homeodomain protein Engrailed in P cells contributes to generating this asymmetry by inducing Hh expression in the P compartment and at the same time repressing the expression of the essential downstream component of the Hh pathway Cubitus interruptus (Ci). Ci is a transcription factor, which, in the absence of Hh signalling, is converted to a repressor form (Ci^{rep}). Ci^{rep} represses *hh* in A cells. The transcriptional co-repressor Groucho (Gro) also represses *hh* expression in A cells, thus helping to maintain the aforementioned asymmetry. Gro is ubiquitously expressed but it is required only in A cells that receive the Hh signal.

Over the last two years, Fernando Bejarano and Lidia Pérez, in collaboration with Christos Delidakis' group in Heraklion (Crete), have been analysing the role of Gro in this process (Bejarano *et al*, 2007). They have shown that it exerts its action by binding to the product of master of *thickveins (mtv)*, a target of Hh

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activity encoding a nuclear Zinc Finger protein. Two distinct mechanisms are then used to repress *hh* expression in A cells (Figure 1). The first is based on Ci^{rep} and acts mainly in those cells not receiving the Hh signal. The second is based on Hh restricting its own expression domain through the activity of its target gene *mtv*. We have shown that these two mechanisms are independent.

Many signalling molecules restrict their own expression domains (ie, Wg or Notch). To our knowledge, these results show, for the first time, that Hh does the same (Figure 1).

Robustness and stability of the gene regulatory network involved in DV boundary formation in the *Drosophila* wing

Gene regulatory networks in developing organisms have been conserved during evolution. The *Drosophila* wing and the vertebrate hindbrain share the gene network involved in the establishment of the boundary between D and V compartments in the wing and adjacent rhombomeres in the hindbrain (Figure 2). The activation of the receptor Notch at the compartment boundaries, as a result of the activity of Notch ligands in nearby cells, induces the expression of the signalling molecules Wg and Wnt-1 in boundary cells of the fly wing and the vertebrate hindbrain, respectively (Figure 2). Wg or Wnt-1 maintain the expression of Notch ligands in neighbouring cells, thus establishing positive feedback and ensuring high activity of Notch at the compartment boundaries. Notch activity then regulates the growth of the surrounding non-boundary cells and is required for maintaining the lineage restriction boundary.

By means of a Systems Biology approach that combines *in silico* and *in vivo* experiments, in collaboration with Javier Buceta's group in Barcelona, Héctor Herranz has generated a regulatory network for the establishment and maintenance of the DV boundary in the *Drosophila* wing (Buceta *et al*, 2007). This network shows how short-range cell interactions, medi-

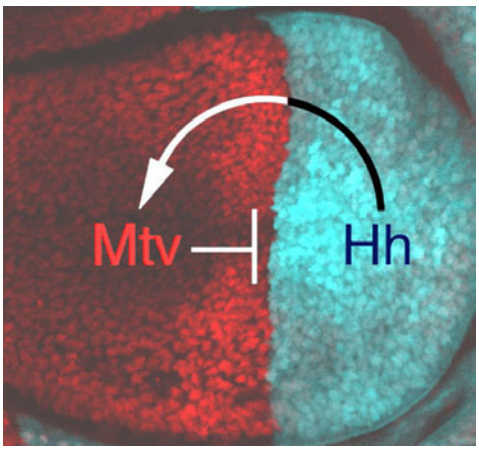

***Figure 1**. Wing imaginal disc labelled to visualize the expression of the mtv-lacZ reporter gene (antibody to β-gal, red) in a hh-GAL4;UAS-GFP background (GFP, blue).*

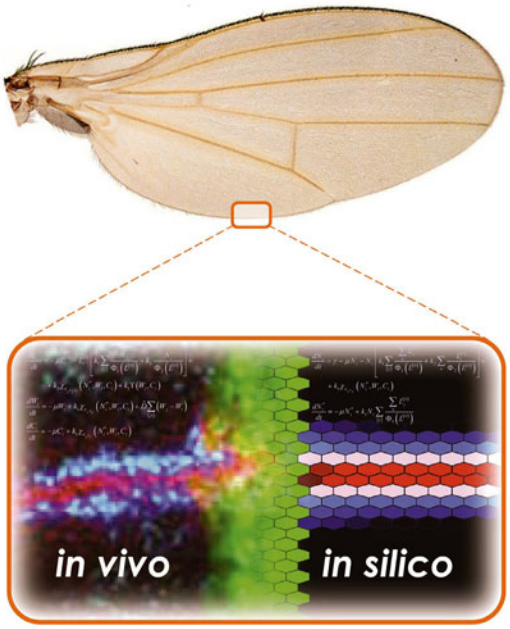

Figure 2. *DV Boundary formation in the fly wing relies on cell interactions between boundary (red) and non-boundary (white/blue) cells and is mediated by the activities of Notch and Wg.*

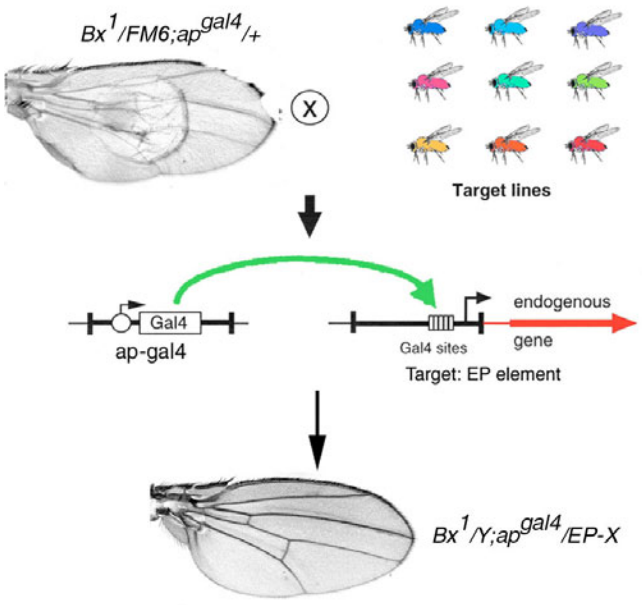

Figure 3. *Beadex^{1}/FM6; apterous^{Gal4}/CyO flies, which have a strong loss of wing margin phenotype, were crossed with a large number of independent Enhancer-Promoter (EP)-containing lines. Gal4 expressed in D cells should bind to Gal4 binding sites within the target element enhancer and activate an adjacent endogenous gene X. Those lines that rescued the wing margin phenotype were selected.*

ated by the receptor Notch and its ligands, together with long-range cell interactions, mediated by the Wg signalling molecule, shape the boundary and produce the gene expression pattern that is observed *in vivo*. The data from this research also provide *in vivo* and *in silico* evidence that a novel property, conferred by the activity of Notch in boundary cells and mediated by its target gene Cut, is required for the formation of a stable DV boundary: namely refractoriness to the Wg signalling molecule. We have addressed and explained concepts like spatially refined and polarized Notch signalling by invoking such a property. A robustness analysis of the regulatory network by means of *in silico* experiments complements these results and ensures the biological plausibility of the developmental mechanism proposed.

We wish to place our conclusions into a broader context. Boundary formation between adjacent rhombomeres in vertebrates relies on the same Wnt/Notch-dependent regulatory network. Therefore, we speculate that boundary cells also need to be refractory to the Wnt signal to generate stable boundaries. We conclude that the robustness and stability of this network, in which the interconnectivity of the elements is crucial and even more important than the value of the parameters used, might explain its use in boundary formation in other multicellular organisms (Figure 2).

A gain-of-function suppressor screen for genes involved in DV boundary formation in the *Drosophila* wing

The *Drosophila* wing primordium is subdivided into a D and a V compartment by the activity of the LIM-Homeodomain protein Apterous in D cells. Cell interactions between D and V cells induce the activation of Notch at the DV boundary. Notch is required for the maintenance of the compartment boundary and the growth of the wing primordium. *Beadex*, a gain-of-function allele of *dLMO*, results in increased levels of dLMO protein, which interferes with the activity of Apterous and results in defects in DV axis formation.

We have performed a gain-of-function screen to search for suppressors of *Beadex* when overexpressed in D cells. We have identified 53 lines corresponding to 35 genes. Loci encoding for micro-RNAs and proteins involved in chromatin organisation, transcriptional control and vesicle trafficking have been characterised in the context of *dLMO* activity and DV boundary formation.

Our results indicate that a gain-of-function screen in a *Beadex*-sensitized background to search for suppressors of the wing margin phenotype is efficient in identifying known and new genes involved in DV boundary formation as well as in the regulation of

Beadex/dLMO activity. We have shown that many of the *Beadex* suppressors involved in DV boundary formation are not essential during wing development. This observation suggests that these suppressors share redundant activities with other gene products. The gain-of-function approach has also been shown to be extremely efficient in unravelling new roles for the recently identified micro-RNAs.

Loss-of-function-based forward genetic screenings have not been as productive in this regard, probably because of the reduced size of these miRs or their redundant activities. Taken together, a suppressor gain-of-function screen in a sensitized background provides a suitable combination to identify new genes, including miRs and redundant genes, involved in a given process.

Redundancy and regulatory feedback loops contribute to the robustness of gene regulatory networks. Classical loss-of-function-based forward genetic screenings have been highly productive in identifying genes that behave as hubs in these networks. Essential genes in yeast are among those most highly connected. However, forward genetic screenings are not as effective in identifying redundant genes or regulators of these feedback loops, whose loss-of-function might not show an overt phenotype. More quantitative *in vivo* genetic screenings have been more efficient in this regard. Our results indicate that a gain-of-function *in vivo* genetic screen in a sensitized background is a strong alternative for the identification of redundant genes or regulators of feedback loops involved in developmental gene regulatory networks (Figure 3).

Publications

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Research Networks and Grants

Ayudas a grupos emergentes
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Research Director: Marco Milán

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Ministerio de Educación y Ciencia, BFU2004-00167/BMC: 2004-2007
Research Director: Marco Milán

Compartments, organizing molecules and growth control in Drosophila
EMBO Young Investigator Programme: 2007-2010
Research Director: Marco Milán

Establishment and maintenance of compartment boundaries in the Drosophila wing imaginal disc
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Research Director: Marco Milán

Collaborations

Growth control in the Drosophila wing
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In silico modeling of DV boundary formation
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Regulation of Hh expression
Christos Delidakis, Institute of Molecular Biology and Biotechnology, FoRTH, and Department of Biology, Crete University (Crete, Greece)

Awards

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