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* provides an excellent model system to approach this question because of its suitability for genetic and molecular manipulation and its well-described 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 important processes. In addition, the completion of the genomic sequencing project for *Drosophila* allows the use of reverse genetic approaches such as RNA-mediated interference and targeted gene disruption as well as genome-wide expression analyses in order to address a wide variety of questions concerning the developmental biology of the fruitfly.

During the development of multicellular organisms, groups of cells assemble to form tissues that are initially homogenous. The elaboration of spatial pattern often begins by subdivision of the field of cells into smaller territories. The imaginal discs of *Drosophila* provide well-characterised experimental systems in which subdivision of the tissue depends on mechanisms that limit cell mixing to produce 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 centered on the compartment boundaries. Stable boundaries between compartments result in tightly localised sources of these signalling proteins. Intermingling of cells at the 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 that proliferate extensively during larval development to achieve a final size 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 subdivides 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 activities seek to elucidate how compartment boundaries are generated, how expression of the organising molecules Wg and Dpp is induced at the these boundaries and how growth and patterning is organised by the activity of Wg and Dpp.

Hedgehog restricts its own expression domain in the *Drosophila* wing

Stable subdivision of *Drosophila* limbs into an anterior (A) and posterior (P) compartment is a consequence of asymmetric signalling by Hedgehog (Hh) from P to A cells. The activity of the homeodomain protein Engrailed in P cells helps to generate this asymmetry by inducing expression of Hh 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 asymmetry. Gro is ubiquitously expressed but is required only in A cells that receive the Hh signal.

During the last two years, Fernando Bejarano and Lidia Pérez, in collaboration with Christos Delidakis' group in Heraklion (Crete) have analysed the role of

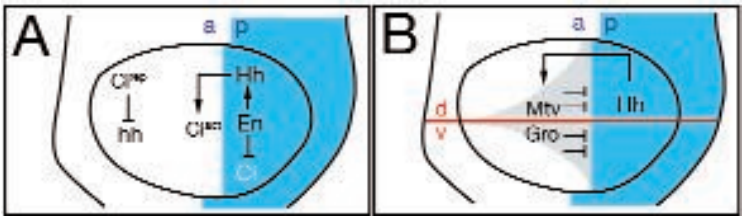

Figure 1. Two mechanisms to repress hh *expression in A cells.*

Groucho in this process. They have presented evidence that Gro exerts this action by binding to the product of *master* of *thickveins* (*mtv*), a target of Hh activity encoding a nuclear zinc finger protein. Two mechanisms are then used to repress *hh* expression in A cells (Figure 1). The first is based on Ci^{rep} acting mainly in those cells not receiving the Hh signal. The second one is based on Hh restricting its own expression domain through the activity of its target gene *mtv*. Fernando and Lidia have provided evidence that these two mechanisms are independent.

Many signalling molecules have been shown to restrict their own expression domains (*ie*, Wingless or Notch). To our knowledge, these results show for the first time that Hh is also involved in restricting its expression domain.

Mutually exclusive domains of Notch and Wingless activities confer stability to the DV boundary 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 dorsal (D) and ventral (V) compartments in the wing and adjacent rhombomeres in the hindbrain (Figure 2). The activation of the receptor Notch at the compartment boundaries, due to the activity of the Notch ligands in nearby cells, induces the expression of the signalling molecules Wingless (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 neighboring cells, thus establishing positive feedback and ensuring high activity of Notch at the compartment boundaries. Notch activity then regulates 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, during the last two years, Héctor Herranz, in collaboration with Javier Buceta's group at the Parc Científic de Barcelona, have generated a regulatory network for the establishment and maintenance of the DV boundary in the *Drosophila* wing. This regulatory network shows how short-range cell interactions, mediated 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*. Their data also present *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: refractoriness to the Wg signalling molecule. Concepts like spatially refined and polarised Notch signalling have been addressed and explained by invoking this property. A robustness analysis of the regulatory network by means of *in silico* experiments complements their results and ensures the biological plausibility of the developmental mechanism proposed.

Finally, 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 as shown by David Wilkinson's group in the UK. We therefore 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.

Self-refinement of Notch activity through the transmembrane protein Crumbs: Modulation of γ-secretase activity

Cell interactions mediated by Notch-family receptors have been implicated in the specification of tissue boundaries. Tightly localised activation of Notch is critical for the formation of sharp boundaries. In the *Drosophila* wing imaginal disc, Notch receptor is expressed in all cells. However, Notch activity is limited to a narrow stripe of cells along the DV compartment boundary, where it induces the expression of target genes. How a widely expressed protein becomes tightly regulated at the DV boundary in the *Drosophila* wing is not completely understood.

Over the last two years, Héctor Herranz, in collaboration with the groups led by Fabián Feiguin in Italy and Michalis Averof in Heraklion (Crete) have analysed the role of *crumbs* in DV boundary formation in the wing primordium. Their data indicate that the transmembrane protein Crumbs is involved in a feedback mechanism used by Notch to refine its own activation domain at the *Drosophila* wing margin (Figure 3). Crumbs is a target of Notch in the wing and is

required to reduce the activity of the γ-Secretase complex, which mediates the proteolytic intracellular processing of Notch. Crumbs exerts its function through its large extracellular part, which contains 29 EGF repeats.

Signalling centres along compartment boundaries are required to organise the growth and pattern of the surrounding tissue. However, too much of a signal has deleterious effects. The Notch signalling centre organises growth and pattern of the developing wing primordium partially through the secreted protein Wingless. Wingless activity contributes to limit Notch activity to cells immediately adjacent to the DV boundary. Our data indicate that Notch also contributes to the refinement of its activation domain through its target gene *crumbs*. Crumbs attenuates Notch signalling by repressing the activity of the γ-Secretase complex. Many loss-of-function mutations in the human homologue of Crumbs, CRB1, cause recessive retinal dystrophies, including *retinitis pigmentosa*. Given that the γ-Secretase complex also mediates the intracellular cleavage of the transmembrane protein APP, leading to accumulation of the ABeta peptide in plaques in Alzheimer disease (AD), we postulate that Crumbs is also involved in modulating AD pathogenesis. Our analysis reveals a function for the extracellular part of the Crb protein in this process. It is interesting to note that many mutations that give rise to retinal dystrophies are missense that affect EGF or LG domains of CRB1 Thus, molecular interactions mediated by the extracellular domain of Crb may be critical in both *retinitis pigmentosa* and AD.

Growth control in the proliferative region of the *Drosophila* eye-head primordium: *elbow-noc* gene complex

Notch signalling is involved in cell differentiation and patterning, as well as in the regulation of growth and cell survival. Notch activation at the DV boundary of the *Drosophila* eye-head primordium leads to the expression of the secreted protein Unpaired, a ligand of the JAK-STAT pathway that induces cell proliferation in undifferentiated tissue.

During the last three years, Carlos Luque has focused on the role of the zinc-finger proteins encoded by *elbow* and *no ocelli* in the control of growth of the eye-head undifferentiated tissue. His results show that *elbow* and *no ocelli* are expressed in the highly proliferative region of the eye-head primordium and loss of *elbow* and *no ocelli* activities induces overgrowths of the head capsule, without inducing Upd expression *de novo*. These overgrowths depend on Notch activity indicating that *elbow* and *noc* repress an Upd-independent role of Notch in driving cell proliferation. When the size of the overgrown tissue is increased, ectopic antenna and eye structures can be found.

The observation that increased size of the eye-head primordium leads to ectopic eye and antenna structures suggests that tight regulation of the size of this organ by *elbow* and *no ocelli* is crucial for proper fate specification and generation of the adult structures. We propose a model to explain how de-regulated proliferation produces tissue overgrowth that undergoes fate re-specification depending on the availability of nearby sources of organising molecules. A similar model has already been proposed by the groups of Mlodzik and Pignoni in the US to explain how field size is coupled to fate specification. (See Figure 4.)

Calderón encodes an organic cation transporter of the major facilitator fuperfamily required for cell growth and proliferation of *Drosophila* tissues

The adaptation of growth in response to dietary changes is essential for the normal development of all organisms. The Insulin Receptor (InR) signalling pathway controls growth and metabolism in response to nutrient availability. Although the elements of this pathway have been described, little is known about

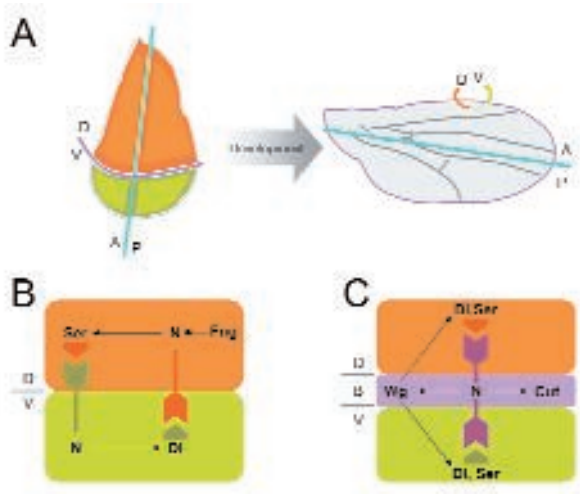

Figure 2. Short-range and long-range cell interactions in DV boundary formation.

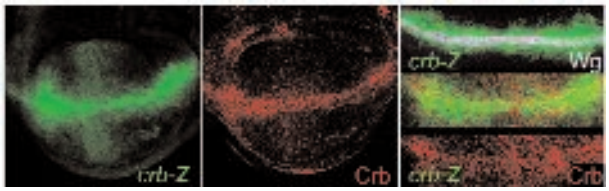

Figure 3. Expression of Crumbs at the DV boundary of the Drosophila *wing primordium.*

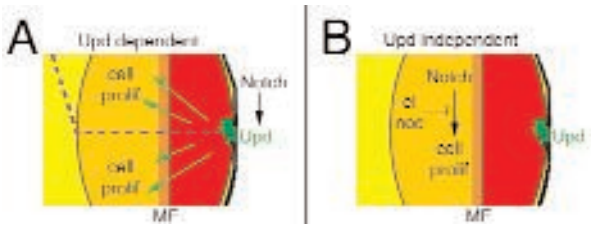

Figure 4. Upd-dependent and independent roles of Notch in inducing cell proliferation in the eye primordium.

the downstream elements regulated by this cascade. Héctor Herranz identified the *calderón* gene while he was a postdoc in Ginés Morata's lab in Madrid. He started its functional characterisation in Madrid and finished it in our lab. *calderón* encodes a protein with highest homology with Organic Cation Transporters of the Major Facilitator Superfamily. His results show that this gene is a new transcriptional target of the InR pathway. These transporters are believed to function mainly in the uptake of sugars, as well as of other organic metabolites. Genetic experiments demonstrate that *calderón* is required cell autonomously and downstream of the InR pathway for normal growth and proliferation of larval tissues. Loss of *calderón* activity mimics the phenotype of mutations in the InR pathway during embryonic and adult development. Expression of *calderón* is positively regulated by the InR downstream effectors PI3 kinase/Dp110 and TOR and its activity is required for TOR-mediated growth induction. Thus, *calderón* is a target of the PI3 kinase/TOR branch of the InR pathway required cell autonomously for insulin-mediated cell growth.

Our results indicate that the growth of imaginal cells may then be modulated by two distinct, but coordinated, nutrient-sensing mechanisms: one cell-autonomous and the other humoral.

PUBLICATIONS

Herranz H and Milán M (2006) Notch and affinity boundaries in *Drosophila*. Bioessays, 28:1-4

Herranz H, Morata G and Milán M (2006) Calderón encodes an organic cation transporter of the major facilitator superfamily required for cell growth and proliferation of *Drosophila* tissues. Development, 133:2617-2625

Herranz H, Stamataki E, Feiguin F and Milán M (2006) Self-refinement of Notch activity through the transmembrane protein Crumbs: modulation of γ-secretase activity. EMBO Rep, 7:297-302

Luque CM and Milán M (2007) Growth control in the proliferative region of the *Drosophila* eye-head primordium: the *elbow-noc* gene complex. Dev Biol, 301:327-339

Milán M (2007) Sculpting a fly leg: BMP boundaries and cell death. Nature Cell Biology, 9:17-18

RESEARCH NETWORKS AND GRANTS

Abnormal proteins in the pathogenesis of neurodegenerative disorders (APOPIS)
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Abnormal proteins in the pathogenesis of neurodegenerative disorders
Complementary grant to the European Grant BFU2004-0142-E: 2004-2006
Project Coordinator: Carlos G. Dotti

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Project Coordinator: Miguel Manzanares

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Ministerio de Educación y Ciencia: 2004-2007
Project Coordinator: Marco Milán

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Project Coordinator: Marco Milán

COLLABORATIONS

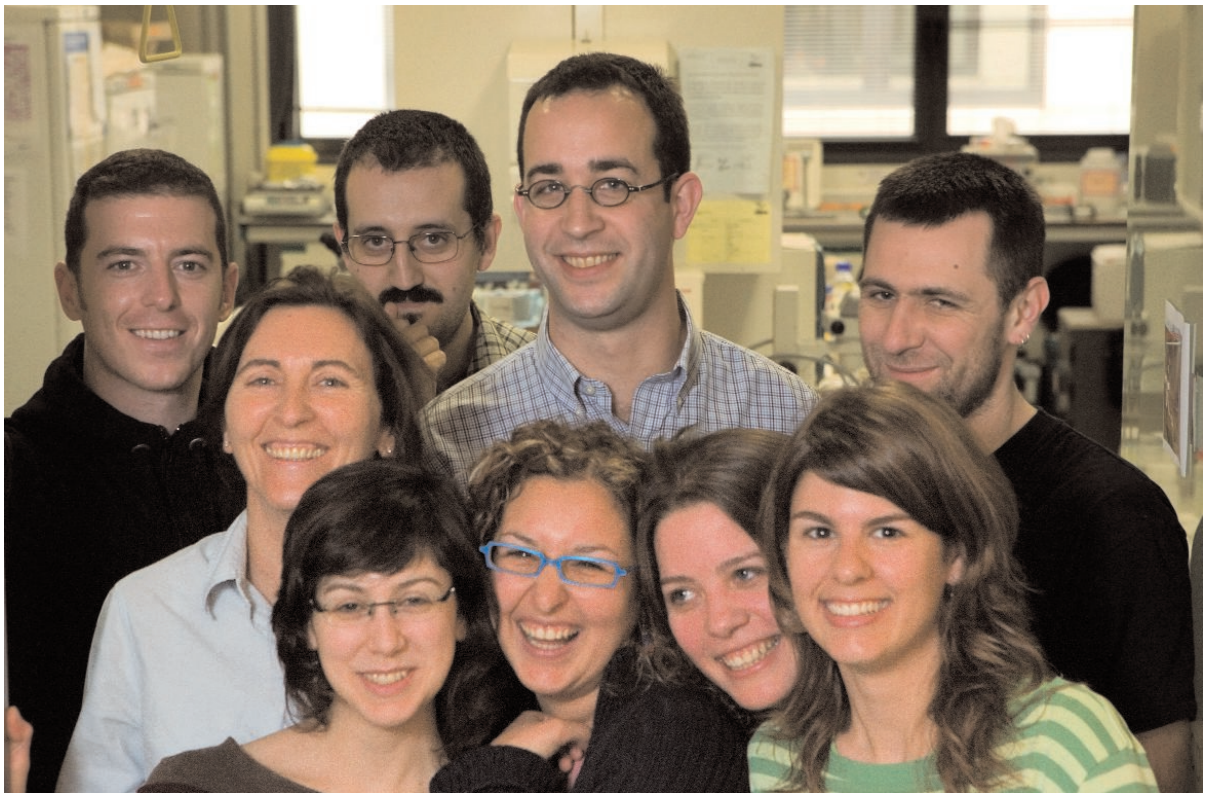
Modulation of Gamma-Secretase activity in Drosophila
Fabián Feiguin (Cavalieri Ottolenghi Scientific Institute, University of Turin, Italy)

The role of Mtv and Groucho in the repression of hh in the Drosophila *wing*
Christos Delidakis (Institute of Molecular Biology and Biotechnology, FoRTH, and Department of Biology, University of Crete, Heraklion, Greece)

The role of Crumbs in DV boundary formation
Michalis Averof (Institute of Molecular Biology and Biotechnology, FoRTH, and Department of Biology, University of Crete, Greece)

In silico *modelling of DV boundary formation in the* Drosophila *wing*
Javier Buceta, Ramon Reiguada and Frances Sahgues (Centre Especial de Recerca en Química Teòrica,

CeRQT, Parc Científic de Barcelona, Spain)
Ramon Reiguada and Frances Sagues (Departament de
Química-Física, Universitat de Barcelona, Spain)



Marco Milán's group, March 2006.