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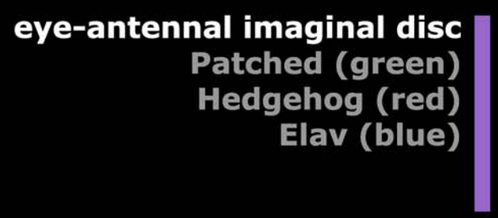

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Biologia Experimental e Biomedicina

THE ROLE OF *ODD-SKIPPED* FAMILY GENES IN THE *DROSOPHILA* HEAD DEVELOPMENT

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Catarina Brás Pereira has participated in the elaboration of the work presented in the following publications under the name of **Bras-Pereira C.**:

Publications:

Bras-Pereira C. and Casares F. (2008)
An antennal-specific role for *bowl* in repressing supernumerary appendage development in *Drosophila*. (*submitted for publication*)

Bras-Pereira C., Bessa J., Casares F. (2006)
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Publications in collaboration:

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Odd-skipped genes encode repressors that control kidney development. ***Dev Biol***. 301(2):518-31

Ao avô Braz

e à avó Lurdes,

com saudade!

O desenvolvimento de organismos multicelulares requer uma coordenação correcta entre a proliferação e a especificação das células. As mesmas vias de sinalização extracelular que controlam a especificação da identidade das células também regulam a proliferação das mesmas. O principal objectivo desta tese foi compreender o papel da família de genes *odd* durante a especificação celular e a organogênese do olho e da antena de *Drosophila*.

A família de genes *odd* é composta por quatro genes: *odd-skipped (odd)*, *brother of odd with entrails limited (bowl)*, *drumstick (drm)* and *sister of odd and bowl (sob)*, os quais apresentam extensas semelhanças nos domínios de zinco de ligação ao DNA. Diferentes estudos mostraram que estes genes estão implicados na formação do padrão de distintos tecidos, como é o caso do intestino, patas e epiderme embrionária.

Nesta tese, demonstrámos que a família de genes *odd* é expressa ao longo da margem posterior do disco de olho, um centro especializado de sinalização. Este domínio é requerido para o início do desenvolvimento da retina através da produção da molécula de sinalização *hedgehog (hh)*. Nas células da margem, *bowl* é necessário para a activação de *hh* e, consequentemente, para o desenvolvimento do olho. Portanto, a família de genes *odd* é essencial para o desenvolvimento da retina. Além disso, a expressão ectópica de *odd* e *drm* nas células indiferenciadas do domínio anterior do olho é suficiente para induzir a expressão de *hh* com a concomitante formação de olhos ectópicos. Assim sendo, os genes *odd* são essenciais para definirem o domínio a partir do qual a retina se começa a diferenciar (Chapter I).

No seguimento do trabalho, comprovámos que *bowl* também é necessário durante o desenvolvimento da antena para a repressão da expressão de *wg (wingless)*, na região onde normalmente se expressa a molécula BMP2/4, Dpp (Decapentaplegic). Esta activação de *wg* no domínio de expressão de *dpp* origina um novo eixo proximodistal (PD) que, por sua vez, gera o desenvolvimento de antenas extra. Estes resultados podem ser explicados não apenas com base na simples acção repressora da transcrição de *wg*, mas também se considerarmos que *bowl* é responsável pela supressão do desenvolvimento de um primórdio cefálico, normalmente 'silenciado'. Em contraste com o que foi mostrado no desenvolvimento da pata, a família de genes *odd* parece não ter nenhuma função na segmentação da antena (Chapter II).

Mostrámos que a cassete Drm/Lin/Bowl, descrita como funcional durante o desenvolvimento do intestino e da epiderme embrionária, está também em funcionamento durante o desenvolvimento do disco imaginal de olho e antenna. Em ambas as situações, na especificação da margem e do eixo da antenna, Drm, muito

provavelmente em associação com Odd, é necessário para aliviar o efeito repressor de Lin sobre a função de Bowl (Chapter I and II).

Em colaboração com o Dr. José Luis Gomez-Skarmeta, demonstrámos que a família de genes *odd* pode estar implicada no normal desenvolvimento dos tubos renais (tubos de Malphigian) de *Drosophila*, da mesma forma que no desenvolvimento renal de *Xenopus* e zebrafish, onde ambos os genes *odd* de vertebrados, Osr1 e Osr2, são suficientes e necessários para o correcto desenvolvimento dos pronefros (Appendix).

The development of multicellular organisms requires the correct coordination between proliferation and specification of cells. The same extracellular signaling pathways that control cell fate specifications also regulate proliferation. The main objective of this thesis was to understand the role of odd family genes during specification and organogenesis of the *Drosophila* eye and antenna.

The *odd* family genes is composed of four genes *odd-skipped (odd)*, *brother of odd with entrails limited (bowl)*, *drumstick (drm)* and *sister of odd and bowl (sob)* that display extensive homology in the zinc DNA-binding domains. Different studies have shown that these genes are involved in the patterning of distinct tissues, such as gut, legs and embryonic epidermis.

In this thesis, we have shown that *odd* family genes are expressed along the posterior margin of the eye disc, a specialized signalling center required for the initiation of retinal development by producing the *hedgehog (hh)* signaling molecule. In the margin cells, *bowl* is necessary for the activation of *hh* and therefore for eye development. Thus, *odd* family genes are essential for retinal development. In addition, misexpression of *odd* and *drm* in anterior, undifferentiated eye cells is sufficient to induce *hh* expression with concomitant formation of ectopic eyes. Therefore, odd genes are essential for defining the retina initiation center (Chapter I).

Further investigation revealed that b*owl* is also required during antennal development for the repression of *wg (wingless)* expression in territories that normally express the BMP2/4 molecule Dpp (Decapentaplegic). This de-repression of *wg* in the *dpp*-expressing domain generates a novel proximo-distal (PD) axis that results in the development of an extranumerary antenna. These results can be explained if rather than simply acting as a block of *wg* transcription, *bowl* were suppressing the development of a cephalic primordium that remains normally "silent". In contrast to what has been shown in leg development, *odd* family genes do not seem to have any role in antennal segmentation (Chapter II).

In addition, we have demonstrated that the Drm/Lin/Bowl cassette, described to be functioning during gut and embryonic epidermis development, is also at work during the development of the eye-antennal imaginal disc. In both situations, margin specification and antennal axis specification, Drm, most likely in association with Odd, is required to relief the repressor effect of Lin on Bowl function (Chapter I and II).

In collaboration with Dr. José Luis Gomez-Skarmeta, we have shown that *odd* family genes may be involved in proper renal (Malphigian) tubules development in *Drosophila*, like it occurs in *Xenopus* and zebrafish kidney development, where both

vertebrate *odd* genes, Osr1 and Osr2, are sufficient and required for proper pronephros development (Appendix).

General Introduction

1. *Drosophila* eye as a model to study cell specification and pattern formation during organogenesis

A key process in development is the control of growth of tissues by cell proliferation through the action of signaling molecules. Cells unable to respond to these external regulatory signals may either under-proliferate, undergo programmed cell death, or proliferate in a random and uncontrolled manner, typical of tumor overgrowths. Indeed, growth control is critical for an organism proper development and improper growth control is a hallmark of many diseases. Inductive signals are also required to convey positional information to cells and specify their developmental fates. Thus, the development of multicellular organisms requires coordination of proliferation of cells with their specification, patterning and differentiation. These processes are controlled by signaling molecules which are required to convey positional information to cells, specify their developmental fate and control the growth of tissues by cell proliferation. These signals and their pathways are conserved during evolution and belong to a limited number of gene families: Wnt, Hedgehog (Hh), Transforming Growth factor-ß/Bone Morphogenetic Protein (TGFβ/BMP), Epithelial Growth Factor (EGF), Fibroblast Growth Factor (FGF) (Freeman and Gurdon, 2002).

The eye of the fruit fly *Drosophila melanogaster* is an excellent model to study in genetic and molecular terms how the control of growth and differentiation are coordinated in the development of an organ. The adult eye of *Drosophila* contains between 750 and 800 ommatidia, or eye units, each of which harbors eight photoreceptor (R1-R8) cells and 12 accessory cells: four cone cells, six pigment cells and one mechanosensory bristle, organized in a regular hexagonal array. Thus, an extra cell, a missing cell or a wrongly specified cell will disrupt this precise structure.

In addition, *Drosophila*, apart from having a short life cycle and offering an easy husbandry, is an excellent genetic model organism, with a 'tool kit' of genetic techniques that allows the induction of genetic changes in groups of genetically marked cells or tissues at particular developmental time points (Blair, 2003).

In this thesis, we took advantage of these genetic tools to study mechanisms operating during the specification and organogenesis of the *Drosophila* eye and antenna. These two sensory organs develop from a compound primordium, the so-called eye-antennal imaginal disc. Through the study of the Odd-skipped (odd) gene family, we have tried to clarify the mechanisms involved in the triggering of retinal differentiation. In addition, we have carried out the study of the molecular mechanisms that permit the correct development of the proximo-distal axis of the antenna.

2. Eye Morphology

The *Drosophila* compound eye comprises approximately 800 unit eyes, called ommatidia (Figure 1). Each ommatidium is a precise assembly of photoreceptor and accessory cells in which each cell can be identified by its specific morphology, gene expression and position. An ommatidium contains 20 cells: 8 photoreceptors (R) neurons – 6 outer cells, R1-R6 and 2 inner cells, R7-8, plus 12 accessory cells: cone, pigment and bristle cells (Harris et al., 1976; Ready et al., 1976; Tomlinson, 1988). The core of the ommatidium contains the 8 photoreceptors, and is characterized by microvillar extensions of their apical membranes, called rhabdomeres that carry the photosensitive opsins (Cook and Desplan, 2001; Montell, 1999). Photoreceptor cells can be classified into three functionally distinct types: R1-R6, R7 and R8. These three classes of photoreceptors have different spectral sensitivities and express different photosensitive pigments (Fryxell and Meyerowitz, 1987; Harris et al., 1976; Ligoxygakis et al., 1998; O'Tousa et al., 1985; Zuker et al., 1987). The six outer photoreceptors, retinula cell 1 to 6 (R1-6), carry the blue-sensitive rhodopsin (Rh1) and are arranged in a trapezoidal conformation. The R7 expresses one of the two UV-sensitive rhodopsins (Rh3 or Rh4 type) (Fryxell and Meyerowitz, 1987; Zuker et al., 1987) and the R8 one of the blue-green-sensitive opsin (Rh5 or Rh6 type) (Chou et al., 1996; Papatsenko et al., 1997; Salcedo et al., 1999). R7 is located on top of R8, so that this R pair acts as a detector of light quality. Each of the three types synapses in the optic lobes in different positions: R1-R6 extend short axons which synapse in the lamina ganglion, while the R7 and R8 project long axons which synapse at different levels in the medulla ganglion, deeper in the lobes. Above the photoreceptors lies the lens system consisting of a fluid-filled pseudocone, bordered on top by the corneal lens, laterally by the two primary pigment cells and basally by the four cone cells. The cone cells are 4 flattened cells and are responsible of the secretion of the dioptic elements, which are the lens and the crystalline cone (Perry, 1968). Surrounding this central group of photoreceptors, cone cells and primary pigment cells there is a ring of secondary and tertiary pigment cells, which are shared with neighbouring ommatidia. These pigment cells surrounding each ommatidium achieve the optical isolation of each of them. The ommatidial array is hexagonal and, at each alternate vertex of the pattern, a mechanosensory bristle projects from the eye.

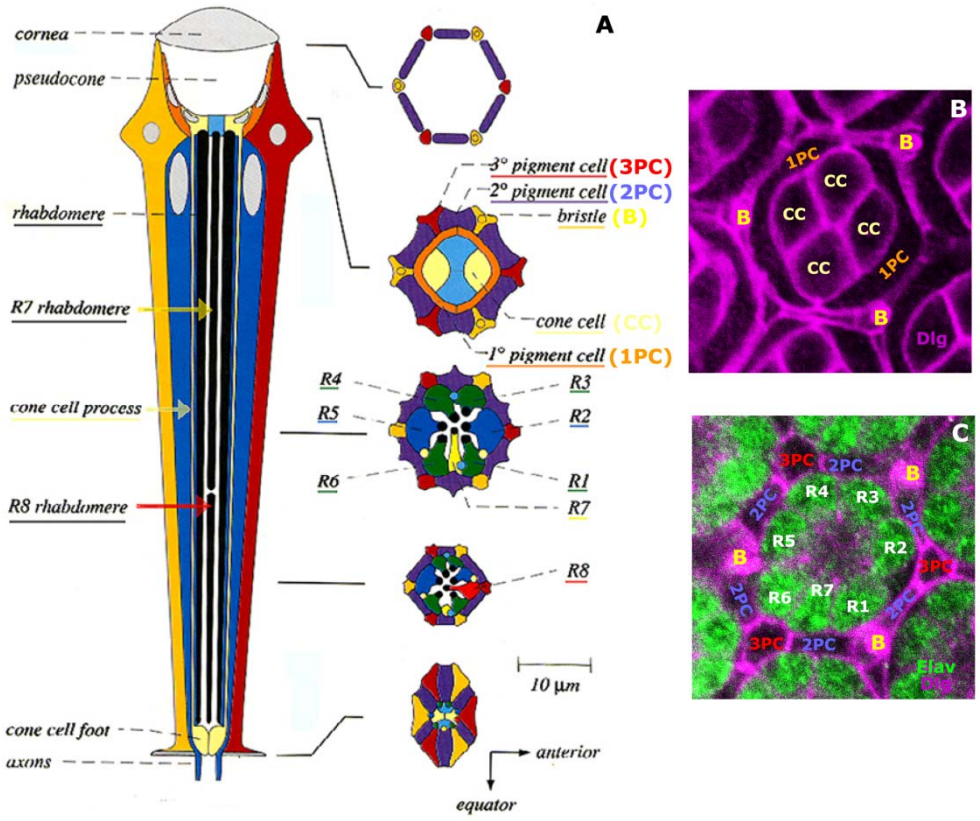

Figure 1. The *Drosophila* adult ommatidium. (A) Schematic representation of a transversal view of an ommatidium (adapted from Wolff and Ready, 1993). (B, C) Confocal images of a pupal retina. Apical (B) and basal (C) view of an ommatidium: membranes are stained with Disc-Large (Dlg, purple) and photoreceptors are labelled with the neural marker Elav (green). The adult eye is composed by approximately 800 hexagonal ommatidia arranged in regular quasi-crystaline array. Each ommatidium contains eight photoreceptor (R) cells, each one associated with a rhabdomere. The outer photoreceptors cells (R1 to R6) surround the inner photoreceptors (R7 and R8), forming a trapezoid. Above the photoreceptors cells, four lens-secreting cone cells (CC) are laterally surrounded by two primary pigment cells (1PC). Each ommatidium is optically isolated by six secondary pigment cells (2PC) and three tertiary cells (3PC) that are shared with adjacent ommatidia. A mechanosensory bristle is present at each alternate vertex of the ommatidium.

The precise shape, position and orientation of the cells within each ommatidium and between ommatidia is crucial for image formation (correct optical alignment and neural connection) and environmental perception, once each unit is focused on a point in space 2º away from its neighbours (Franceschini, 1975). Thus, the function of a compound eye depends on a very precise arrangement of fixed numbers of different cell types. Its quasi-crystalline structure can be disrupted if any cell in the ommatidium is missing, in excess or show a deformed shape. Therefore, the formation of a mature, functional eye requires the tight control of developmental processes, such as proliferation, differentiation and patterning.

All external adult head structures (eye, antenna, ocelli, palpus and surrounding head capsule), with the exception of the proboscis, develop from a pair of

symmetrical epithelial sacs, called eye-antennal imaginal discs (Cohen et al., 1993; Haynie and Bryant, 1986) (Figure 2C, D). Eye development begins in the early embryo. The eye-antennal imaginal disc primordium results from the fusion of at least three groups of cells located in different embryonic cephalic parasegments (Cohen et al., 1993; Younossi-Hartenstein, 1993), i. e., approximately twenty cells are set aside during embryonic stage and invaginate from the ectoderm, giving rise to the whole disc (Garcia-Bellido and Merriam, 1969). Each eye antennal imaginal disc is a flattened epithelial sac, and each one of its opposing layers has a distinct morphology and developmental fate. The main epithelium (ME) is composed by columnar cells, and it will give rise to most adult head structures, including the eye, the antenna and part of the head capsule; and the peripodial epithelium (PE) characterized by the squamous morphology of its cells, and which will form the surrounding head capsule structures (Haynie and Bryant, 1986) (Figure 3A, A').

Both epithelial layers interact during development. Signals from the PE control retina development in the ME (Cho et al., 2000; Gibson and Schubiger, 2000). On the other hand, signals from the ME control gene expression in the PE: thus, overexpression of the EGF receptor, its ligand *spitz (spi)* or the activation of the EGFR pathway in the ME affects gene expression in the PE (Firth and Baker, 2007). This communication between both epithelia occurs probably through apical membrane extensions detected in the lumen (space between them) (Cho et al., 2000; Gibson and Schubiger, 2000).

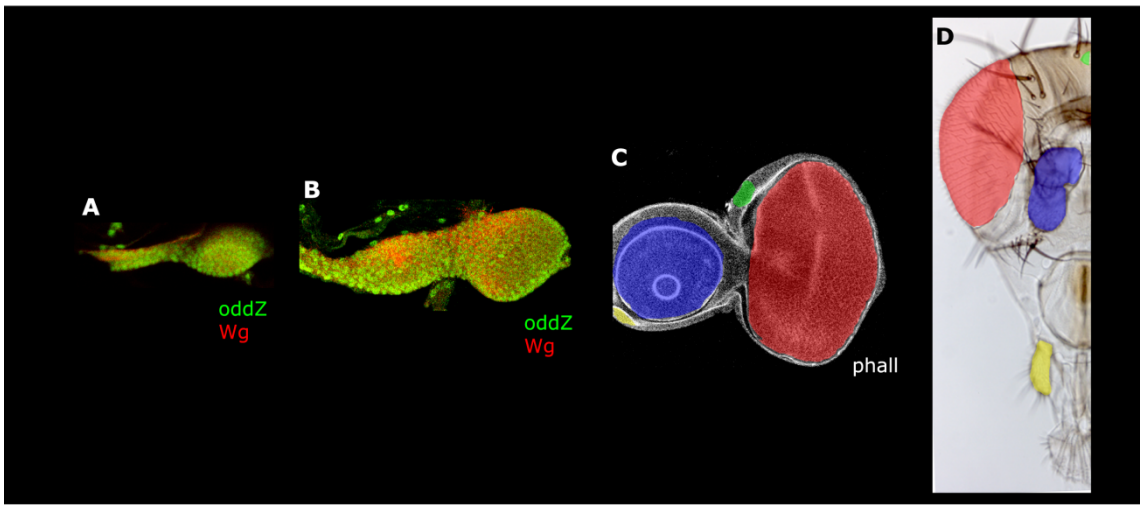

Figure 2. *Drosophila* head development during larval stages. The adult *Drosophila* head (D, shows a hemi-head) derives from a pair of eye-antennal imaginal discs (A-C). The development of these structures occurs during the three larval stages (or instars) of the fruitfly. The eye-antennal disc primordium is a group of cells that are set aside during embryogenesis and that grows by proliferation during first (A) and second (B) instar (L1 and L2). (B) During early second instar, the specification of the eye domain in opposition to the antennal domain occurs. These two domains are visible at this stage as

two lobes within the disc and are generally called antennal and eye discs. In the anterior, antennal domain, Wg protein (red) is detected in the dorsal part, whereas *odd* shows ventral expression. *odd* expression is detected using a reporter line (*oddZ*, green). In the posterior, eye domain, *wg* is expressed in the anterior region, while *odd* stains the surrounding margin. At early third instar (C), different domains that will give rise the distinct adult structures (D) are detected within the eye imaginal disc. Retinal differentiation (red domain) and antennal segmentation (blue domain) processes occur. During this phase, ocelli (green domain) and maxillary palps (yellow domain) start to differentiate. Phalloidin staining (grey) allows the visualization of cellular morphology and the global structure of the imaginal disc.

During the first (L1) and second (L2) larval stages (also known as instars), the eye-antennal imaginal disc grows by asynchronous proliferation (Figure 2A, B). It is during L2 that the antennal and eye fields are specified in the ME. These fields are seen as two lobes in the disc (Figure 2B and Figure 11A, B). The anterior lobe becomes specified as antenna (antennal disc) and expressed the antennal-specific gene, *cut* (*ct*). The posterior lobe becomes specified as eye (eye disc) and retains the expression of the eye selector gene, *eyeless* (*ey*) (Kenyon et al., 2003) (Figure 11B). Once the cells are committed to become the eye primordium, signals from the most posterior cells in the disc are responsible for the induction of retina development in this primordium. Then, the differentiation of the retina progresses in a posterior-to-anterior direction in a wave-like fashion, so undifferentiated cells begin to assemble into ommatidial preclusters and to differentiate retinal cells (Figure 3B). This wave of differentiation that sweeps across the eye disc is marked by an apical constriction of the ME and is called morphogenetic furrow (MF) (Ready et al., 1976; Tomlinson and Ready, 1987) (Figure 3C). Indeed, August Weisman (1864) was the first to describe the MF, but he did not notice that it moved as development proceeds. The MF functions as a boundary that separates the pluripotent progenitor cells, anterior to it, from differentiating cells, behind it (Heberlein and Moses, 1995). Thus, eye development is a progressive process that includes, first the specification of the eye field, and then retina differentiation. This latter proceeds through two steps: retinal induction (or retinal triggering) and progression of the retinal differentiation.

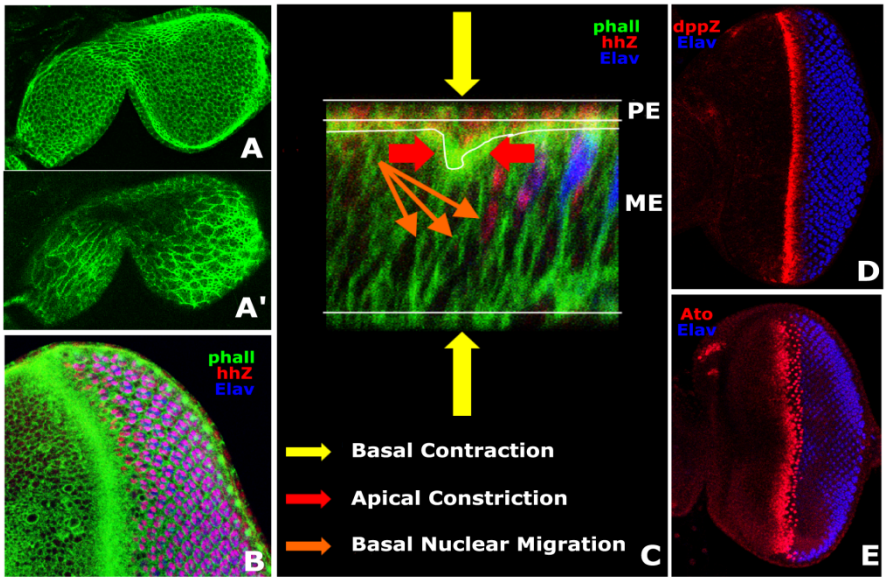

Figure 3. The wave of differentiation and the formation of the morphogenetic furrow. (A, A') Early third instar (L3) eye-antennal imaginal disc where actin is labelled with phalloidin (phall). Each imaginal disc is a flattened epithelial sac with two opposing epithelial layers show here in two different confocal plans: the main epithelium (ME, A) and the squamous, peripodial epithelium (PE, A'). Retinal differentiation occurs in the ME and starts in more posterior cells of eye domain. (B) An apical optical section of the most posterior region of a mid L3 where the differentiating ommatidia are detected: *hh*-expressing (red), differentiating photoreceptors express the neuronal marker Elav (blue). Cellular constriction (high levels of polymerized actin, phall in green) detected ahead of the differentiated region determines the position of the morphogenetic furrow (MF) and separates this region from the undifferentiated domain, which lies anterior to it. (C) In the cross section of this portion of the disc (B) it is possible to detect the distinct morphologies of both epithelia that form the imaginal disc: the columnar main epithelium versus the squamous peripodial epithelium. The formation of the MF depends on the coordination of three different cell processes: apical constriction, basal contraction and nuclear migration. These processes are accomplished along with gene expression changes. Differentiating photoreceptors express *hh* (B, C), which diffuses anteriorly and activates *dpp* within the MF (D). Hh is also responsible of the induction of the expression of the proneural gene, *atonal (ato*) at the MF and just anterior to it (E).

3. Eye Specification

3.1. The Retinal Determination Gene Network– Establishment of retinal fate

In *Drosophila*, the eye primordium is specified as a sub-domain of the Pax6-expressing cells in the center of the eye disc, by the co-expression of a set of retinal determination genes (Dominguez and Casares, 2005; Pappu and Mardon, 2004). These genes form a network called the retinal determination gene network (RDGN) due to their role in *Drosophila* eye specification, although they work in several other different developmental processes as well. In addition, this network has been shown to be evolutionary conserved (Donner and Maas, 2004; Silver and Rebay, 2005). In *Drosophila*, the core of this network is composed of a group of nuclear factors: two Pax6-like paired-type homeodomain proteins (*eyeless*, *ey* and

toy, *twin of eyeless*), *eyes absent* (*eya*), the Six family transcription factor, *sine oculis* (*so*) and a novel nuclear protein with a putative DNA-binding ability, *dachshund* (*dac*). However, other genes important for eye specification are members of this RDGN: the Pax genes, *eyegone* (*eyg*) and *twin of eyegone* (*toe*); the Six family gene, *optix* (*opt*); *homothorax* (*hth*) and *teashirt* (*tsh*) (Dominguez and Casares, 2005; Pappu and Mardon, 2004).

A key characteristic of RDGN genes is that they are fundamental for eye development: loss of their function results in reduced or completely absent eyes [*ey* (Quiring et al., 1994), *eya* (Bonini et al., 1993), *dac* (Mardon et al., 1994) and *so* (Cheyette et al., 1994; Serikaku and O'Tousa, 1994)]. Also, the ectopic expression of some of these genes, alone or in combination, results in the formation of ectopic eye structures [*ey* (Halder et al., 1995), *eya* (Bonini et al., 1997), *dac* (Shen and Mardon, 1997)].

Guided by the studies in *Drosophila*, gene families of the vertebrate homologues of the RDGN were discovered, and shown to be expressed during vertebrate eye development: Pax6, Eya1-3, Six3 and Dach1 and 2 (Davis et al., 2001; Hammond et al., 1998; Oliver et al., 1995; Walther and Gruss, 1991; Walther et al., 1991; Xu et al., 1997).

Evolution has maintained the use in eye development of the entire genetic cassette, rather than simply conserving the use of individual RDGN elements (Wawersik and Maas, 2000). This evolutionary conserved eye patterning cassette, where genes of the RDGN gene families participate, was incorporated into the development of others sensory organs, such as lens and nasal placodes (Purcell et al., 2005) and muscle (Heanue et al., 1999).

3.1.1. Retinal Determination Genes: Pax6, Eya, Six and Dach

In *Drosophila*, two homologues of vertebrate Pax6 were identified: *ey* and twin-of-eyeless (*toy)* that function in eye development (Czerny et al., 1999; Quiring et al., 1994). These are highly related transcription factors that harbour a paired-type homeodomain (HD) as their DNA binding domains. However, only the HD of *ey*, but not that of *toy*, is able to downregulate *Distal-less* (*Dll*), which is a key process in the eye specification cascade. This indicates that the DNA-binding domains of Toy and Ey have different functions (Punzo et al., 2004). Different results indicate that *toy* positively regulates *ey* expression. *toy* is expressed earlier than *ey* in the eye anlagen and ectopic expression of *toy* activates *ey* expression, inducing ectopic eye formation (Czerny et al., 1999). This, together with the fact that an *ey* enhancer has several Toy binding sites (Czerny et al., 1999; Hauck et al., 1999), indicates that *toy* acts upstream of *ey*. Both *toy* (Czerny et al., 1999) and *ey* (Quiring et al.,

1994) are expressed in the eye anlagen as early as these structures can be detected, with their expression during subsequent larval stages becoming restricted to cells anterior to the MF. Different data suggest that *toy* and e*y* behave like eye selector genes. Indeed, based on their characteristics, they were named as 'master control gene for eye development' (Gehring, 2002).

Epistasis analysis indicate that *toy* and *ey* act upstream of *so*, *eya* and *dac* (Bonini et al., 1997; Chen et al., 1999; Halder et al., 1998; Pignoni et al., 1997).

The Six family genes are transcription factors that contain a highly conserved homeodomain (HD) and a second motif, the Six domain, a 110 aa region immediately 5' of the HD, required for protein-protein interaction with Eya and other proteins (Pignoni et al., 1997). Drosophila Six genes, *so* and *optix,* are both expressed in the developing eye but have different expression patterns, showing coexpression in the furrow and in adjacent cells; whereas the expression of *dsix4*, another family member, has not yet been reported in the eye (Cheyette et al., 1994; Seimiya and Gehring, 2000). Toy and Ey proteins act in a concerned manner to directly regulate *so* transcription through an eye-specific enhancer (Niimi et al., 1999; Punzo et al., 2002). On the other hand, *optix* is directly regulated by Ey (Seimiya and Gehring, 2000).

Eya belongs to a novel family of proteins identified in many animals: flies, worms and vertebrates. Four Eya homologs, Eya1-4, were identified in the mouse, while in *Drosophila* only one single gene exists, although with two isoforms originated by alternative splicing, which differ by 23 aa at the N-terminus (Bonini et al., 1993; Leiserson et al., 1998). Analysis of the vertebrate Eya gene product shows that Eya domain (ED) (Xu et al., 1997) is a highly conserved 271 aa C-terminal motif. In *Drosophila* Eya binds both So and Dac through this domain (Chen et al., 1997; Pignoni et al., 1997). At the N-terminus of Eya lies the Eya domain 2 (ED2), a moderately conserved domain, which contains a non-conserved proline-, serine-, threonine-rich (PST) region (Zimmerman et al., 1997) required for Eya's transcriptional activator function (Silver et al., 2003; Xu et al., 1997). The expression of *Drosophila eya* is first detected during second instar larval (L2) in the eye domain restricted to its posterior region (Bonini et al., 1993).

The Dach family proteins, encoded by vertebrate Dach1 and Dach2 and *Drosophila dac* genes share 2 highly-conserved domains with *ski* proto-oncogene and *sno*, a ski-like gene: Dach Domain 1 (DD1): N-terminal domain known as ski domain, and Dach Domain 2 (DD2): C-terminal domain which contains an unusual extended helical coiled-coil motif (Davis et al., 2006; Davis et al., 1999; Hammond et al., 1998; Mardon et al., 1994). *Drosophila* Dac binds directly to Eya protein through the C-terminal fragment (DD2), which contains DachC box and the coiled-coil motif

(Chen et al., 1997). DD1 contains the DachN box, which may be involved in transcriptional activation (Chen et al., 1997). Genetic epistasis analysis has placed *dac* as the most downstream element among the known components of RDGN. *dac* expression depends primary on Ey, Eya and So (Anderson et al., 2006; Chen et al., 1997; Kenyon et al., 2005; Pignoni et al., 1997).

Ectopic expression of *eya* and *dac* induce ectopic eyes at lower frequency than *ey*. When coexpressed, they act synergistically, increasing dramatically the penetrance of ectopic eyes (Chen et al., 1997) and inducing *ey* expression. The same occurs when *eya* is expressed together with *so* (Bonini et al., 1997). Thus, the induction of ectopic eyes by *eya*, *so* and *dac* induces *ey* expression and requires *ey* function, but does not induce *toy* expression (Bonini et al., 1997; Chen et al., 1997; Halder et al., 1998; Pignoni et al., 1997; Shen and Mardon, 1997). Eya and either So or Dac form protein complexes (Chen et al., 1997; Pignoni et al., 1997), and these protein-protein interactions might explain the synergistic effect of their coexpression on the induction of ectopic eyes. *ey* induction is not necessary inconsistent with the idea that *ey* is upstream of a regulatory hierarchy of genes that control eye development (Halder et al., 1995). Indeed, these genes form a network with complex series of positive feedback loops (Chen et al., 1997). The fact that *ey* is unable to induce ectopic eyes in the absence of either *dac* or *eya* or *so* and that misexpression of *ey* strongly induces *dac* expression (Halder et al., 1998; Shen and Mardon, 1997) indicates that *ey* acts upstream of *dac* and *eya*, suggesting that *ey* is upstream of this genetic hierarchy involved in eye formation, and that later on in development the expression of *eya*, *so* and *dac* locks in the eye fate through positive feedback loops that mutually reinforce their expression (Desplan, 1997).

3.2. Interactions between RDGN and signaling pathways

The RGDN is not only composed by nuclear factors that regulate eye development. Different signaling pathways are known to interact with the members of this network. Additionally, these interactions occur bidirectionally: signaling pathways influence the expression of RDGN genes and, on other hand, the activity of RDGN products are required for the activity of those signaling pathways (Donner and Maas, 2004; Silver and Rebay, 2005).

The proper expression of RDGN genes is established by the positive input of Hh and Dpp signaling pathway and the repressor effect of wg signaling pathway. The regulation of RDGN genes by these pathways is at the transcription level, whereas EGFR signaling pathway contributes positively at the protein level.

ey, *so*, *eya* and *dac* are expressed in domains that overlap with *dpp* and *hh* prior to the start of differentiation (Desplan, 1997). Eye-specific mutations that disturb Hh and Dpp signaling pathways (Figure 5) present a reduced eye phenotype similar to the one of RDG mutants (Heberlein et al., 1993; Ma et al., 1993; Masucci et al., 1990) and these pathways are required for the initiation of R differentiation (Borod and Heberlein, 1998; Burke and Basler, 1996; Wiersdorff et al., 1996). *hh* and *dpp* play no role in regulating *ey*, but are required for *eya*, *so* and *dac* expression, which is important for MF initiation. Even so, the expression of these genes is independent of Dpp once the MF is in motion (Curtiss and Mlodzik, 2000).

Furthermore, functional Eya protein seems to be required to maintain expression of *dpp* and can induce *hh* expression in the eye imaginal disc (Hazelett et al., 1998; Pignoni et al., 1997).

Hh signaling positively regulates *eya* expression. Removal of the repressor form of *ci* leads to the de-repression of *eya* (Pappu et al., 2003). Moreover, Dpp together with Eya, but not Dpp alone, can rescue the eye phenotype of the removal of *hh* signal. On the other hand, mutant clones of Hh receptor, *smoothened (smo)*, downregulate *eya* and *dac* expression at MF initiation (Curtiss and Mlodzik, 2000; Pappu et al., 2003). In addition, the efficient induction of ectopic eyes upon misexpression of RDGN members requires active Dpp and Hh signaling pathways (Chen et al., 1999; Pappu et al., 2003).

Wg regulates *eya* expression and appears to be regulated by Eya itself, since Wg levels are upregulated in *eya* mutant clones (Hazelett et al., 1998). Indeed, Wg seems to maintain separated the retina and the adjacent head structures by inhibiting the expression of *eya*, *so* and *dac* (Baonza and Freeman, 2002).

Moreover, *eya* seems to act downstream of EGFR pathway (Rebay et al., 2000). In addition, Eya protein displays two MAPK phosphorylation sites and Eya phosphorylation, in response to EGFR/Ras/MAPK signal, positively regulates Eya activity *in vivo* (Hsiao et al., 2001).

4. Retinal induction

4.1. Eye margin as the 'signaling center' for furrow induction

Eye specification takes place during second larval instar (L2) (Kenyon et al., 2003; Kumar and Moses, 2001a). These 'eye primordium' cells remain undifferentiated and proliferating randomly until the beginning of third instar (L3). During L2, two signaling molecules are key in promoting the proliferation of the primordium and in the establishment of the different axes with the eye disc. The expression of *wg* from the dorsal anterior eye disc organizes the dorso-ventral (DV) axis by indirectly setting a line of Notch activation at the DV midline of the disc (Blair, 1999; Irvine,

1999). Notch, in turn, induces proliferation by establishing an organizing center required for eye growth (Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). The Notch target gene, *eyegone* (*eyg*), is activated along the DV axis (Chao et al., 2004; Dominguez et al., 2004) and is required downstream of N for eye growth. *wg* expression is somehow required for proliferation although this function, although its potential interaction with Notch has not been fully explored (Dominguez and Casares, 2005). It has been proposed that only after enough growth has happened during L2 a *wg*-free domain is generated (Figure 2B). It could be in this domain where retinal differentiation might start (Kenyon et al., 2003).

The point of intersection between the DV axis (Notch signaling domain) and the most posterior cells of the eye disc determines the point where the MF initiates, called 'firing point' (Silver and Rebay, 2005). This point coincides with the position of the optic stalk, which serves as scaffold for the formation of the optic nerve.

The 'firing point' is formed by the most posterior cells of the margin. The margin is a row of cells that surround the eye domain (Figure 4A). These cells are not eye-committed cells but play an essential role for retinogenesis, since they are responsible for sending the necessary signals to induce furrow initiation. In addition, these margin cells give rise to the adult head cuticle that surrounds the eye, the posterior head capsule (phc) (Haynie and Bryant, 1986) (Figure 4B). Margin cells are specialized PE cells, of cuboidal morphology. Squamous to cuboidal morphological transition occurs seamlessly. On the contrary, margin cells and ME are separated by a fold (Bessa, 2007). Lineage experiments using an *odd*-Gal4 (*odd-skipped* Gal4) line have shown that a restriction border between PE and ME exists (see Figure 3 in Chapter I (Bessa, 2007)). This means that cells originated in the PE rarely appear in the ME. Thus, this compartment border physically lies in the fold separating ME and the margin. Indeed, the posterior margin plays a key role as the site of production of retinal inducing signals (Treisman and Heberlein, 1998). More recently, a study has also demonstrated that the margin functions as a non-autonomous inducer of planar cell polarity within the eye primordium (Lim and Choi, 2004). However, genes required for the functionally specification of the margin as signaling center were not known.

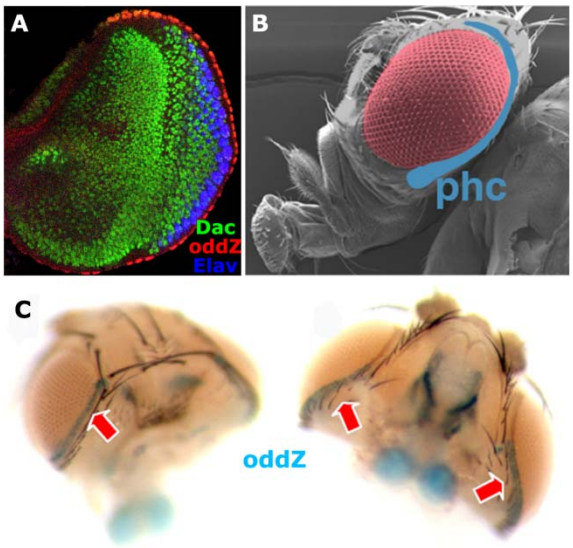

Figure 4. Characterization of the eye-surrounding margin. (A) The margin, a row of cells that surround the eye field, is characterized by the expression of *odd* (*odd* reporter, *oddZ*, red). This margin is a specialized signaling center that promotes retinal induction. The retinal determination gene, *dac* (green), is expressed in the eye field, where photoreceptors cells differentiate (neuronal marker, Elav, blue). (B) In the adult head, these margin cells give rise to the posterior-ventral rim of head cuticle abutting the adult eye (red), the posterior head capsule (phc, blue) and maintains the expression of *odd* (arrows mark the X-gal histochemical stain in heads of *odd-Z* pharate adults, C).

4.2. Furrow initiation

Ommatidial assembly is initiated in the MF, a dorsal-ventral indentation in the ME epithelium which moves anteriorly during L3. The MF coincides with four classes of cellular events: coordinated changes in cell shape (apical constriction- greatly reduced apical surfaces; apical-basal contraction; basal nuclei migration, Figure 3C) (Tomlinson, 1985; Wolff and Ready, 1991); changes in gene expression; synchronization of the cell cycle; and specification of a regular array of ommatidial founder cells. Along the length of the furrow, a column of ommatidia begins to assemble and the furrow subsequently moves anteriorly, leading to one new ommatidial column every 2 hours (Basler and Hafen, 1989a).

Once the furrow is triggered, it sweeps across the disc being continuously reinitiated along the lateral margins (Ma et al., 1993). The reinitiation of the MF from the lateral margin is known as MF 'reincarnation'. This process depends on positive signals from the Hh, Dpp, EGFR, Notch and JAK/STAT pathways and negative signals from the Wg pathway (Baonza and Freeman, 2001; Borod and Heberlein, 1998; Chanut and Heberlein, 1997; Curtiss and Mlodzik, 2000; Dominguez and Hafen, 1997; Kumar and Moses, 2001b; Pignoni and Zipursky, 1997; Wiersdorff et al., 1996). A description of the Hh and Dpp signaling pathways can be found in Figure 5.

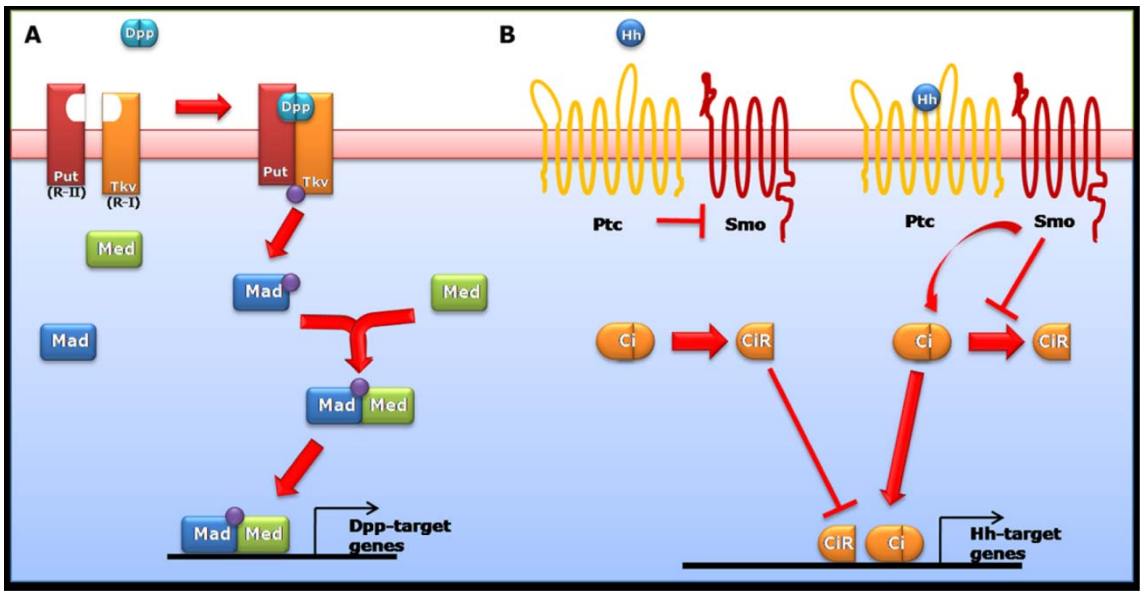

Figure 5. The Hh and Dpp signaling pathways. (A) The binding of the Drosophila BMP-like ligand, Decapentaplegic (Dpp) brings together both type I (Tkickveins, Tkv) and type II (Punt, Pnt) coreceptosr serine&threonine kinases. This complex is activated by the phosphorylation of Tkv by its partner Pnt. As a consequence, Mad (Mother against dpp) is directly phosphorylated and activated by the activated Tkv, type I receptor. Phosphorylated Mad forms complex with Medea (Med) that is translocated to the nucleus, where it regulates target gene transcription. (B) In the absence of Hedgehog, the 12 transmembrane spanning protein, Patched (Ptc), prevents Smoothened (Smo), a 7 transmembrane spanning protein, from activating downstream components. Upon Hh reception, inhibition of Smo by Ptc is released, leading to activation of the Hh pathway, that blocks the proteolysis of the transcription factor Cubitus interruptus (Ci), which normally leads to a short repressor Ci (CiR). The block of the proteolytic event yields a long Ci form that behaves as a transcriptional activator in cells receiving Hh.

Domínguez and Hafen described for the first time the initial expression pattern of Hh, before retinal induction, along the posterior eye disc margin (Dominguez and Hafen, 1997). Before that study, it was believed that *hh* was not involved in the triggering of the furrow, since *hh* expression was only detected in developing photoreceptors, i.e., after the differentiation had started. Hence, *hh* has a critical role in the initiation of retinal differentiation (Borod and Heberlein, 1998; Dominguez and Hafen, 1997). Accordingly, the onset of *hh* expression precedes the beginning of retinogenesis (Cavodeassi et al., 1999; Cho et al., 2000). The activity of the Notch pathway along the prospective DV border is required to increase the levels of *hh* transcription at the firing point (Cavodeassi et al., 1999). Once retinal differentiation starts, *hh* is expressed in R cells. Hh produced at these R cells diffuses at short-range anteriorly, activating the BMP2/4 gene *decapentaplegic* (*dpp*) anterior to them, within the furrow (Blackman et al., 1991). Dpp in turn acts as a long-range signaling molecule. Moreover, dpp positively regulates its own expression in the eye disc: *dpp* is not expressed in *mad* mutant clones (Chanut and Heberlein, 1997; Wiersdorff et al., 1996). In addition, a positive regulatory loop

between Dpp and Hh was described as being essential for the MF initiation. In fact, *dpp* expression pattern can be divided into domains: *hh*-dependent *dpp* expression along the posterior margin and at the advancing MF (Borod and Heberlein, 1998; Dominguez and Hafen, 1997), and *hh*-independent *dpp* expression along the lateral margin (Chanut and Heberlein, 1997; Wiersdorff et al., 1996), which is repressed by *wg* in the anterior disc margin (Baker, 1988; Ma and Moses, 1995; Treisman and Rubin, 1995). *dpp* is genetically downstream of *hh* (Chanut and Heberlein, 1997; Heberlein et al., 1993; Ma et al., 1993) and it is partially redundant with it during MF progression (Greenwood and Struhl, 1999).

The initiation of retinal differentiation fails in either Hh or Dpp signaling mutants, while its progression is only slowed down (Curtiss and Mlodzik, 2000). Dpp seems to be required for furrow initiation: blocking Dpp signaling pathway, differentiation fails to initiate and *wg* is expressed ectopically (Chanut and Heberlein, 1997; Wiersdorff et al., 1996) and, in addition, *dpp* misexpression along the margin blocks *wg* expression and induce the triggering of ectopic furrow (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997) and missexpression of *dpp* in the anterior margin is sufficient to activate *hh*, even in the absence of ectopic photoreceptor differentiation (Borod and Heberlein, 1998; Dominguez and Hafen, 1997).

Epistatic analysis suggests that EGFR acts upstream of Notch and upstream of Hh signaling during MF initiation and that EGFR and Notch act upstream of *dpp* function during MF reincarnation (Kumar and Moses, 2001b).

4.3. Hh and Dpp signaling pathway: requirement for furrow propagation

After many years of contradictory data about the signaling pathways necessary and sufficient for furrow progression, work by Fu and Baker (Fu and Baker, 2003) clarified the role of Hh, Dpp and Notch by clonal analysis where they removed the receptor and the nuclear effector of one, two or all three pathways. They concluded that either Dpp or Hh signals are sufficient for eye differentiation, but neither is absolutely required, due to their partial redundancy. In addition, they showed that Notch potentiates Dpp signaling. It was already known that cells must be able to respond to either Hh or Dpp in order to differentiate (Curtiss and Mlodzik, 2000; Fu and Baker, 2003; Greenwood and Struhl, 1999; Heberlein et al., 1993) and that Notch also contributes to this process (Baker and Yu, 1997; Li and Baker, 2001). Other authors argued that Hh is required for furrow progression (Heberlein et al., 1993; Ma et al., 1993).

This model of Hh having a primary role for MF progression was further supported by experiments of activation of Hh signaling pathway by ectopic Hh expression: Hh is sufficient to induce ectopic furrows in the anterior undifferentiated region (Heberlein et al., 1995; Strutt et al., 1995) as well as loss of *pka-C1* (Pan and Rubin, 1995; Strutt et al., 1995) or of *ptc* (Chanut and Heberlein, 1995; Ma and Moses, 1995; Strutt and Mlodzik, 1995; Wehrli and Tomlinson, 1995), which causes a cell autonomous, ligand-independent signaling of the *hh* pathway.

Initially, it had been proposed that the major role for Hh signaling is the stabilization of full length Ci (Ci^{act}, the activator form of Ci), preventing the production of Ci^{rep} (its repressor form) (Pappu et al., 2003). This model is based on the observation that *smo* mutant clones lack retinal differentiation in contrast with *ci* mutant clones where retinogenesis occurs as normal (Fu and Baker, 2003). However, it seems that this analysis was not correct. Indeed, *smo* mutant clones can differentiate in response to Dpp, although they show a delay in PR differentiation (Curtiss and Mlodzik, 2000; Dominguez, 1999; Fu and Baker, 2003; Greenwood and Struhl, 1999; Strutt and Mlodzik, 1997). Dpp and Notch signaling are dispensable for differentiation (*mad* and Su(H) double mutant clones - (Fu and Baker, 2003) and *Dl* and *medea (med)* double mutant clones - (Baonza and Freeman, 2001)) if *ci* gene was not removed. This means that hh signaling does not function only to prevent Ci cleavage, but requires the active form to drive differentiation (Fu and Baker, 2003).

dpp signaling is sufficient to induce differentiation in the absence of Hh and N pathways (removal of *ci* and *Su*(H)), nevertheless this differentiation is delayed. This defect is overcome if N pathway is restituted (*ci* mutant clones). In the presence of others signaling pathways, *dpp* function is not required for furrow progression (Wiersdorff et al., 1996). Different data suggest that *dpp* signaling alone is not sufficient to induce ectopic differentiation everywhere in the eye disc (Baonza and Freeman, 2001; Greenwood and Struhl, 1999; Pignoni and Zipursky, 1997). This could be due to the repressor activity of Su(H) (Hsieh and Hayward, 1995; Morel and Schweisguth, 2000), that in the absence of N signaling slows down the wave of differentiation (Li and Baker, 2001). Thus, *dpp* signaling is sufficient to promote eye differentiation or furrow progression (Chanut and Heberlein, 1997) but does not seem to be required for neither of the two processes (Greenwood and Struhl, 1999; Strutt and Mlodzik, 1997). Blocking the reception of Dpp by removing the type I or II receptor (*punt* or *tkv*, respectivelly), the furrow progresses and ommatidial development occurs normally (Burke and Basler, 1996; Greenwood and Struhl, 1999). Based on experiments of ectopic expression and hypomorphic mutant analysis, different data attribute to Dpp a crucial role for normal MF

initiation, taking in account its capability of activating its own expression and repressing *wg* transcription (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997).

Notch on its own (removal of *smo* or *ci* and *tkv*) cannot drive differentiation (Fu and Baker, 2003; Greenwood and Struhl, 1999). Notch potentiates differentiation in response to Dpp signaling but is not required for differentiation in response to Hh (Baonza and Freeman, 2001; Fu and Baker, 2003). Thus, Notch signaling is neither required nor sufficient for differentiation.

4.4. *wg*: the opposite force of furrow progression

The Wnt-1 homologue, *wingless (wg)*, is expressed at the anterior edges of eye disc where it functions as an antagonist of furrow initiation and progression (Baker, 1988; Ma and Moses, 1995; Treisman and Rubin, 1995) by inhibiting *dpp* activity. It has been shown that Wg blocks *dpp* function in the eye development not only by repressing *dpp* transcription, but also by blocking retinogenesis downstream of *dpp* receptor within the presumptive eye domain (Hazelett et al., 1998).

Anterior-lateral margin, especially the one at the anterior-dorsal side, fires the furrow precociously when *wg* function is removed during larval stage using a *wg* temperature-sensitive allele (Baker, 1988; Ma and Moses, 1995; Treisman and Rubin, 1995). The induction of differentiation by the removal of *wg* requires *hh* function, i. e., the absence of *wg* is not sufficient for retinogenesis (Borod and Heberlein, 1998).

On the other hand, the elimination of Dpp signaling pathway (*mad* mutant clones) in regions near the eye margin autonomously allows *wg* expression (Wiersdorff et al., 1996). Thus, it seems that the primary function of Dpp is the repression of *wg* and thereby it is required for MF initiation (Chanut and Heberlein, 1997; Dominguez and Hafen, 1997; Pignoni and Zipursky, 1997; Wiersdorff et al., 1996).

5. Retinal differentiation

Based on observations in the ant and the precise lattice arrangement of the insect retina, Bernard (1937) proposed that it develops by precise cell linage, i. e., it was thought that each ommatidium was clonally derived from a single precursor cell (Bernard, 1937 cited in (Lawrence and Green, 1979)). Later on, different studies demonstrated a clear absence of repeatable lineage relationship between cells of an ommatidium, and that photoreceptors and accessory cells differentiate from a pool of equivalent cells (Hotta and Benzer, 1970; Lawrence and Green, 1979; Ready et al., 1976). From the nonclonal origin for the ommatidium, it was inferred that cells choose their differentiation pathways depending on the environmental cues.

The morphogenetic furrow (MF) sweeps across the eye domain during two days, leaving behind a new column of precisely spaced ommatidial founder cells (photoreceptor cell 8, R8) approximately every two hours (Basler and Hafen, 1989a; Basler and Hafen, 1989b; Ready et al., 1976; Tomlinson, 1988; Wolff, 1993). Photoreceptors differentiate in a fixed and sequential order: R8 is the founder photoreceptor, followed by the pair of photoreceptors R2/R5, R3/R4, R1/R6 and finally R7 (Tomlinson and Ready, 1987). Then, the accessory cells differentiate: cone cells, pigment cells and bristle. Cone cells star to differentiate during larval stages but the correct ommatidial maturation occurs during pupal stages. Ommatidia exist in two chiral forms disposed along a line of mirror-image symmetry, the equator, that divides the eye domain in the dorsal and ventral domain (Ready et al., 1976).

Thus, during L3, different domains can be distinguished within the eye domain: proliferation, determination and differentiation domain (Figure 6A). In the most anterior domain, cells proliferate asynchronously. When they receive the signals coming from the furrow, these cells undergo a synchronous mitosis (First Mitotic Wave, FMW) to then arrest temporarily their cell cycle in G1 phase. These G1-arrested cells become eye-determined by acquiring a so-called preproneural (PPN) state (Greenwood and Struhl, 1999). Posterior in this determination domain, cells close to the furrow acquire a proneural fate. Behind the furrow, cells start to differentiate and cluster into forming ommatidia. However, in this differentiation domain, an extra round of mitosis is required to ensure the correct number of progenitor cells. Thus, cells that do not belong to the differentiating ommatidial precluster enter synchronously in S phase (Second Mitotic Wave, SMW). (For a detailed characterization of the different domains please go to Figure 6B, C.)

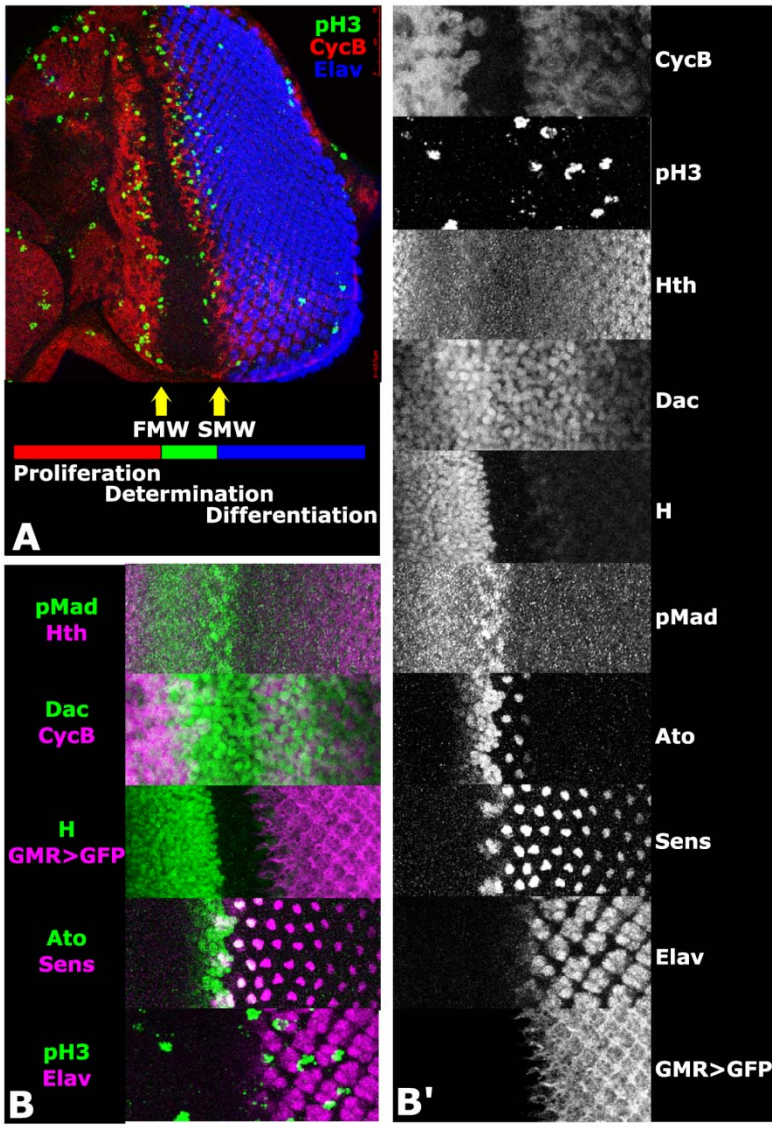

Figure 6. Characterization of the distinct domains within the eye field. Once the retina starts to differentiate, three different domains can be distinguished within the eye domain. In the anterior region cells proliferate asynchronously, and accumulates the G2-cyclin, CyclinB (CycB). This proliferating domain is characterized by the expression of homothorax (hth). Signals coming from the furrow induce these cells to synchronously perform a round of mitosis (marked by phosphohistone3, pH3), the so-called First Mitotic Wave (FMW, arrow) to then arrest in G1 phase. Hth is transcriptionally downregulated by the Dpp signal coming from the furrow. The activation of the *dpp* pathway is visualized by the detection of the phosphorylated form of Mad (pMad). Another effect elicited by the *dpp* signal is the activation of *h* (*hairy*). Together, these gene regulation events induce cells to acquire a preproneural (PPN) state. The retinal determination gene (RDG), *dachshund* (*dac*) presents a complementary expression pattern to that of *hth*. In the posterior region, where retinogenesis is taking place, the downregulation of *dac* coincides with the expression of *hth* in non-neuronal cells. Just ahead of the furrow, highest levels of pMad, together with the contribution of the Notch pathway downregulate, *h* and activate *atonal* (*ato*), inducing the acquisition of a proneural state. Immediately behind the furrow, within the differentiating domain, Notch pathway acts to restrict the expression of *ato* until it is expressed in just one cell, R8, which is the founder cell of each ommatidium. During this process, *senseless* (*sens*) expression is activated in R8 and maintained throughout retinal development. After the formation of the precluster, cells that do not form part of it, enter synchronously in S phase and undergo one more round of mitosis, the Second Mitotic Wave (SMW, arrow). Differentiating photoreceptors express the neuronal marker Elav. The Glass Multimer Reporter (GMR) Gal4 driver is specifically active in all cells within the retinal differentiating domain.

5.1. Cell cycle regulation ahead of the MF

During development, patterning and growth are tightly associated. Cell proliferation and differentiation are regulated spatially and temporally. During retinal

development, highly ordered cell fate specification and differentiation events are associated with patterned cell proliferation (Wolff, 1993). The way to ensure coordinated regulation of pattern formation and cell cycle synchronization is to regulate them using the same regulatory signaling pathways (Hh, Dpp, N, EGFR).

The induction of eye differentiation results in the formation of the MF that is a basal contraction of the epithelium, which is a physical consequence of the constriction of the apical actin cytoskeleton rings (Corrigall et al., 2007; Escudero et al., 2007; Ready et al., 1976; Wolff and Ready, 1991). At the same time, all cells that read signals from the furrow withdraw from the cell cycle (first mitotic wave – FMW) and remain in G1 phase. This means that furrow formation is coincident with a band of G1 cell cycle arrest (Baker and Yu, 2001; de Nooij and Hariharan, 1995; Thomas et al., 1994). This process seems to be, at least partially, under the control of Dpp (de Nooij et al., 2000; Dong et al., 1997; Horsfield et al., 1998; Penton et al., 1997). Gain- and loss-of-function experiments have demonstrated that Dpp signaling pathway is sufficient and required for *hth* repression (Bessa et al., 2002), which together with Tsh and Ey, is required for the proliferating of the undifferentiated cells anterior to the furrow (Bessa et al., 2002). Therefore, in the absence of *dpp*, *hth* plus *ey* and *tsh* could maintain cells proliferating. However, Firth and Baker proved, by clonal analysis, that cell-cycle arrest in G1 ahead of the MF is not only dependent on Dpp signal: if cells cannot respond to Dpp they arrest later in response to Hh. Although this process depends primarily on Dpp, Dpp and Hh are partially redundant in inducing a cell cycle arrest (Firth and Baker, 2005). These data are coherent with the study of the role of Hh, Dpp and Raf pathways in furrow progression performed by Greenwood and Struhl (Greenwood and Struhl, 1999). They conclude that Hh controls the rate of furrow progression by inducing *dpp* expression that acts long-range and induces, in turn, cells to shift from an undifferentiated state to a 'pre-proneural' (PPN) state. In the absence of dpp signal, the rhythm of this shift is decreased, which leads to a delay in the advance of the furrow. Thus, Dpp signal is sufficient but not absolutely required to establish the PPN state (Greenwood and Struhl, 1999).

G1 is a critical phase of the cell cycle. Cells developmentally maintained in this phase can respond to extracellular signals to initiate another round of cell division, to withdraw temporarily from the cell cycle or to differentiate.

Different targets of Hh and Dpp are necessary for cell-cycle synchronization. *stg*, the mitotic inducer cdc25 homolog (Edgar and O'Farrell, 1990), and *rux* (Thomas et al., 1994) are thought to contribute to cell cycle synchronization, acting on different points of the cycle: *stg* force cells in G2 to enter mitosis, ensuring that they reach the G1 phase (Heberlein et al., 1995; Mozer and Easwarachandran, 1999); on the

other hand, *rux* prevents cells in G1 phase from re-entering in S phase (Avedisov et al., 2000; Dong et al., 1997; Escudero and Freeman, 2007; Foley et al., 1999; Foley and Sprenger, 2001; Sprenger et al., 1997; Thomas et al., 1994; Thomas et al., 1997).

So, Hh and Dpp signaling pathways are both involved in cell-cycle control, forcing cells to arrest in G1 phase and in the process of differentiation, contributing for R8-founder cell specification and for ommatidial differentiation through the activation of *ato* expression (Heberlein and Moses, 1995; Heberlein et al., 1995; Heberlein et al., 1993; Ma et al., 1993). The combination of these pathways with other regionalized inputs separates these processes in time and space: G1 arrest always occurs before differentiation.

5.2. Proneural Genes and the R8-founder cell (Figure 7)

G1-arrested cells express the proneural gene, *atonal* (*ato*), a basic helix-loop-helix (bHLH) transcription factor (Jarman et al., 1994; Jarman et al., 1995). *ato* expression is activated by Hh signal coming from the developing photoreceptors. Therefore, *hh* signaling not only activates *dpp* in the furrow but also is responsible for the neuronal commitment of the cells in front of the furrow, by inducing *ato* expression (Heberlein and Moses, 1995; Heberlein et al., 1995; Heberlein et al., 1993; Ma et al., 1993). *ato* functions together with Daughterless (Da) (Brown et al., 1996; Brown et al., 1995; Jarman et al., 1994; Jarman et al., 1995; White and Jarman, 2000), a dimerization partner that is uniformly expressed. On the other hand, Atonal is functionally inhibited by Hairy (H) and Extramacrochaetae (Emc) (Brown et al., 1991; Brown et al., 1995; Ohsako et al., 1994). H and Emc, both HLH proteins are expressed anterior to the furrow to avoid precocious neuronal differentiation ahead of the furrow. Binding of H or Emc to Ato blocks its transcriptional function. Removal of both H and Emc results in ectopic *ato* expression anterior to the furrow (Brown et al., 1995). On the other hand, even when Hairy is maintained in cells lacking both N and Hh pathway, differentiation could occur (Fu and Baker, 2003). Although Dpp does not appear to be essential for the activation of *h*, it seems to contribute to elevate its expression (Fu and Baker, 2003; Greenwood and Struhl, 1999). Notch and Hh activation are independently sufficient to downregulate *h* expression (Baonza and Freeman, 2001; Fu and Baker, 2003). Thus, ahead of the furrow, the PPN domain is characterized by the transduction of Dpp signaling that contributes to the high levels of expression of both transcriptional repressors, *h* and *emc* (Greenwood and Struhl, 1999), and plays a role in cell cycle synchronization (Penton et al., 1997). This region is also defined by the upregulation of *da* (Brown et al., 1996; Brown et al., 1995).

ato has three phases of expression, with two steps of spatial restriction in the developing eye (Dokucu et al., 1996; Greenwood and Struhl, 1999; Sun et al., 1998) (Figure 7). The first phase occurs just anterior to the furrow and is characterized by uniform *ato*-expression. Gradually, this uniform expression is reduced to small 'intermediate group' (IG) and then to single cells. The IGs probably coincident with the 'rosettes' described by Wolff and Ready (Wolff and Ready, 1991) are detected just posterior to the furrow, in the first column, and are the first distinguishable clustering of cells, formed by four or five cells in the core plus approximately fifteen surrounding-cells (Baker and Zitron, 1995; Wolff and Ready, 1991). Only IG maintains the expression of *ato*. (Baker et al., 1996; Baker and Yu, 1997; Dokucu et al., 1996; Jarman et al., 1995). At this step, all cells of the IG are functionally equivalent (also called 'equivalent group'). The refinement of *ato* expression is achieved in the third column, where *ato* remains expressed in just one cell, the R8 founder cell. Inside the five-cell cluster, called precluster (PC), the most posterior cell is the founder R8. *ato* expression in the R8 cell is maintained until the sixth or seventh column (Baker et al., 1996; Baker and Yu, 1997; Dokucu et al., 1996; Jarman et al., 1995). Since *ato* expression is transient, the differentiation of R8 cells can be followed by *senseless* (*sens*) expression, which is maintained throughout the eye disc thereafter (Nolo et al., 2000) (Figure 6B, B').

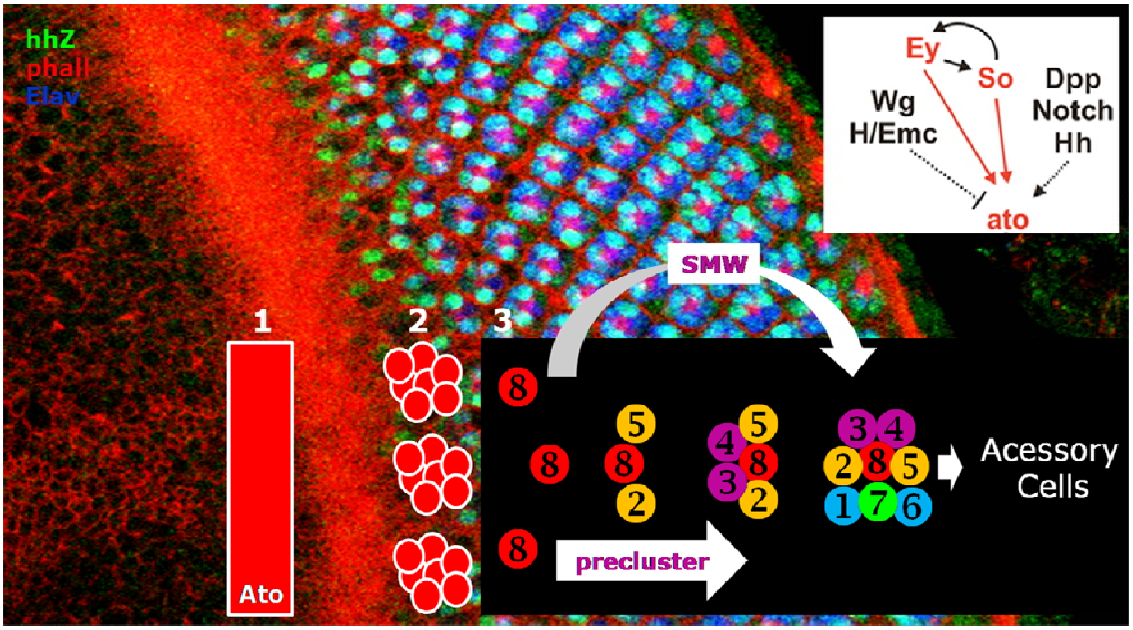

Figure 7. Ommatidial differentiation. The bHLH transcription factor *atonal* (*ato*) is the proneural gene required for the specification of the first photoreceptor of each ommatidium, R8, which is the ommatidium's founder cell, since it is required for the recruitment of the other ommatidial cells. *ato* expression, as indicated in the diagram (adapted from Zhang et al., 2006), is under the positive control of Dpp, Notch and Hh signaling and the determination genes *eyeless* (*ey*) and *sine oculis* (so). In contrast, Wg signaling and the HLH transcription factors Hairy (H) and Extramacrochaetae (Emc) are

responsible for the repression of *ato* expression. *ato* has three distinct phases of expression with two steps of restriction. Ahead of the furrow, all cells express *ato*. Once the furrow passes, *ato* expression is downregulated in some group of cells, being maintained in cells of an 'equivalent group'. In a second step of restriction, *ato* expression is restricted to R8s. These R8 cells are precisely spaced and function as the organizing center for ommatidial development. The R8 founder cells express the EGFR ligand, Spitz (Spi) that activates in the neighbouring cells the EGFR pathway. Cells that receive this signal are recruited to the ommatidium: first the pair of photoreceptors R5 and R2, followed by the pair of photoreceptors R4 and R3. In contrast, cells that do not receive the EGF signal activate the Notch pathway through receiving its ligand Delta (Dl), which induces a synchronously entry in S phase. Further reiterative cycles of EGFR and N signaling pathway allows the recruitment of the other photoreceptors cells: the R1 and R6 pair, and the last photoreceptor, R7; and, in addition, the accessory cells (cone cells, pigment cells and bristle cells) later in pupal stage

The first phase of *ato* expression is dramatically affected when Hh signal pathway is removed. In fact, Hh signaling pathway seems to be a direct activator of *ato* transcription (Dominguez, 1999) and *hh* loss-of-function results in the failure of IG specification (Heberlein and Moses, 1995; Ma et al., 1993). Hh is secreted by the developing ommatidia and acts anterior to the furrow (Dominguez, 1999; Greenwood and Struhl, 1999; Heberlein and Moses, 1995; Heberlein et al., 1995; Heberlein et al., 1993; Ma et al., 1993; Strutt and Mlodzik, 1997).

Cagan and Ready (1989) proposed a simple model for early eye patterning where the precise and dispersed pattern of founder *ato*-expressing cells is obtained by the balance between the Hh inductive signal and the Notch inhibitory signal acting through lateral inhibition (Cagan and Ready, 1989). Indeed, Notch, together with the Hh signaling pathway, modulates the IG formation and consequently the spacing between R8-founder cells that ultimately is reflected on the ommatidial array. In addition, the removal of N or its ligand *Delta* (*Dl*) suggests that Notch signaling is required for *ato* restriction and IG spacing (Baker and Zitron, 1995; Cagan and Ready, 1989). However, more exhaustive studies have revealed that Notch enhances proneuronal competence of *ato*-expressing cells, induced by Hh, before inhibiting *ato* expression through lateral inhibition (Baker et al., 1996; Baker and Yu, 1997; Ligoxygakis et al., 1998). However, Notch only induces *ato* expression in the PPN region signated by Dpp, through the downregulation of the *ato* repressors *h* and *emc*. The upregulation of *ato* and downregulation of *h* and *emc* induce cells to acquire the proneuronal state (Baonza and Freeman, 2001).

The initial uniform expression of *ato* in the MF is controlled by cis-regulatory sequences that lie 3' to the *ato* coding sequence, whereas the following expression, first in IGs and then in the R8 founder cells, depends on regulatory elements that lie 5' to it and requires *ato* function. 3' *ato* enhancer analysis has revealed binding sites for the RDG, Ey and So, and for the effectors of Dpp and Notch signaling

pathway, Mad and Suppressor of Hairless (Su(H)), respectively (Sun et al., 1998; Zhang et al., 2006). The diagram in Figure7 shows the genes and the pathways involved in the regulation of *ato* expression.

5.3. EGF receptor and Notch signaling pathway: the yin&yang of retinal differentiation

The signal transduction pathway downstream of tyrosine kinase receptors (RTK) involving Ras and Raf has been shown to have many roles in cell fate specification during the development of the *Drosophila* eye, most notably in mediating signals by the Sevenless and EGF receptor (EGFR). EGFR and Notch pathways interact in different ways, such that cells integrate and interpret these signals in time and space (see schematic representation of both pathways in Figure 8). Thus, these pathways are often involved in the same processes, where they may cooperate or antagonize each other (Doroquez and Rebay, 2006; Sundaram, 2005).

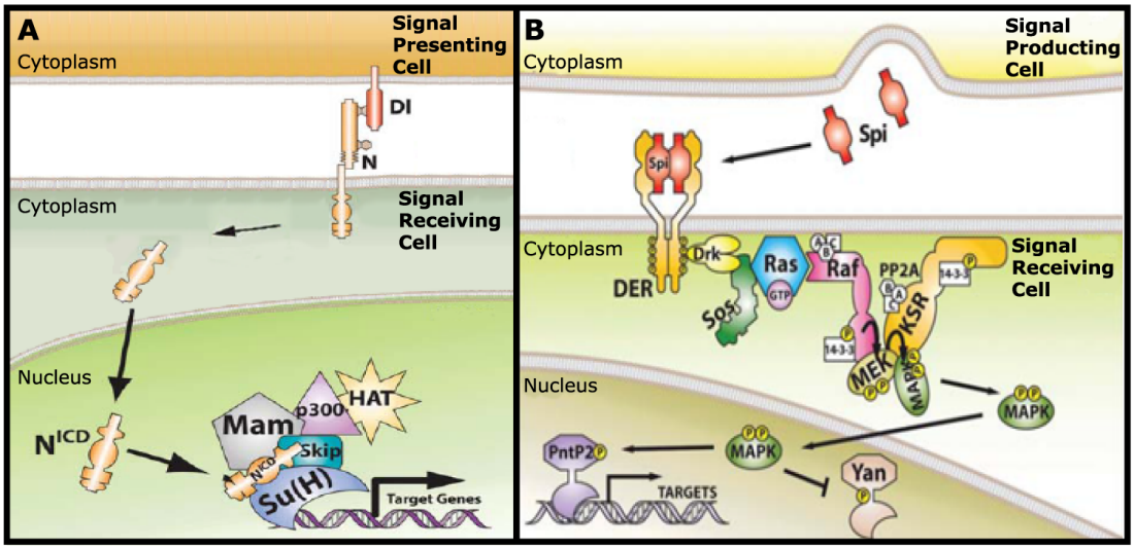

Figure 8. Notch and EGFR signaling pathways. (A) Notch pathway activation. Following binding of a ligand, Notch is proteolytically cleaved on its extracellular side. Further cleavages permit the release of N^{ICD} (Notch intracellular domain) into the cytoplasm. In unstimulated cells, Su(H) (Supressor of Hairless) mediates transcriptional repression in association with a Smrter (Smr) or a Hairless (H)/Groucho (Gro) repressor complex. Stimulation of the pathway promotes conversion of Su(H) into an activator by N^{ICD} and Mastermind (Mam), recruiting an activator complex. (Skip, Ski-interacting protein; HAT; histone acetyl transferase). (B) EGFR pathway activation. In non-stimulated cells, Ras exists in a GDP-bound, inactive state. The phospho-binding-protein 14-3-3 binds to phosphorylated Raf and Ksr (Kinase suppressor of Ras), retaining them in the cytoplasm. In this situation, Yan, a Notch-target gene and Ets transcriptional regulator, represses target gene transcription. EGFR activation by Spitz (Spi) leads to its activated, GTP-bound state. PP2A (Protein Phosphatase 2A) dephosphorylates Raf and Ksr and displaces 14-3-3. This permits Raf and Ksr to re-localize with Ras near the membrane to activate the kinase cascade that results in the double phosphorylation of MAPK (Mitogen Activated Protein Kinase). The dpMAPK (di-phosphorylated MAPK) translocates into the nucleus promoting, by phosphorylation, the Ets

transcriptional regulator PntP2 (Pointed-P2) to activate target gene transcription and the concomitant exporting of Yan from the nucleus. (DrK, Downstream of Receptor Kinase; Sos, Son of sevenless; MEK, Map/Erk Kinase). (Adapted from Doroquez and Rebay, 2006)

R8 photoreceptor cell is responsible for the recruitment of the other photoreceptors into the growing cluster (Tomlinson and Ready, 1987). Episodic activity of EGFR and Notch signaling pathways allows the staggered recruitment of all retinal cells to one of the precursor cell fate (Freeman, 1997; Voas and Rebay, 2004).

The *ato*-expressing R8 is responsible of the recruitment of two pairs of photoreceptors: R2/R5 and R3/R4 that form the precluster (Figure 7). This recruitment is achieved by the activation of the EGFR pathway in the presumptive R2/R5 and R3/R4 by the ligand Spitz (Spi), which is secreted by R8 cell. Thus, R8 cells are required for the EGFR-dependent recruitment of other cell types of each ommatidium (Dominguez et al., 1998; Jarman et al., 1994; Kumar et al., 1998; Lesokhin et al., 1999; Tio and Moses, 1997). Accordingly, the removal of *spi* does not affect R8 specification, but does prevent specification of all other photoreceptors (Tio and Moses, 1997). The activation of EGFR in the precluster ensures that these cells do not re-enter the cell-cycle by inducing the transcriptional upregulation of the cycE/cdk2 antagonist Dacapo (Dap) (Firth and Baker, 2005). The effector of the EGFR pathway, Pointed (Pnt), which is required for the differentiation and cell cycle arrest (Yang and Baker, 2003), directly regulates *dap* expression (Sukhanova et al., 2007).

Cells that are neither R8 cells nor recruited by them, which comprise the remaining 65% of the cells, synchronously re-enter the cell cycle, in the second mitotic wave (SMW) (Figure 7). Through this process more cells are produced to add up to fifteen more cells per ommatidium. Accordingly, most ommatidia are incomplete when the SMW is blocked by the expression of the $p21^{CIP1/WAF1}$ homologue, *dap*. However, even under this condition all fates still occur, indicating that the SMW is not required for any particular fate specification (de Nooij and Hariharan, 1995).

The remaining cells, that do not have EGFR activity, are activated by Dl which leads to trigger G1/S transition. Indeed, *Dl* expression is activated by EGFR pathway in the precluster. Thus, Notch activated by its ligand Dl and concomitant EGFR inactivation is required for cells to progress in the cycle through the G1 to S transition. Cells cannot enter into S phase in the absence of Notch signal (Baonza and Freeman, 2005; Firth and Baker, 2005). Once in S-phase, cells progress through the cycle until G2-phase. Entry into mitosis depends on an EGFR-dependent signal coming from differentiating Rs. In this way, cells born in the SMW are also in G1 when they differentiate.

6. *Drosophila* appendage development

As it is the case for the antenna in the head, all of the *Drosophila* adult appendages develop from primordia called imaginal discs (Cohen, 1993).

Besides their very different structure and function, Snodgrass (1935) proposed that arthropod segmented appendages are homologous on the basis of their development and anatomy, and that the evolutionary ground state of arthropod limbs was composed of two segments: a basal segment, the coxopodite and a distal segment, the telopodite (Snodgrass, 1935).

Drosophila antennae, legs, genitalia and analia are serially homologous appendages, despite their morphological differences, are thought to share a number of basic developmental mechanisms. These ventral appendages depend on different selector genes for their unique identity, that is, their specific structure and function. The activity of different selector genes acting upon a common ground state gives rise to different appendage morphologies. The loss of selector gene activity results in a leg-like appendage that would represent the developmental ground state. For example, the removal of both *Antennapedia (Antp)* and homothorax (*hth)* from T2 leg and only *hth* function from the antenna originates an appendage with the same structure that would represent this ground state (Casares and Mann, 2001; Struhl, 1981). However, this structure is only formed by two segments along the PD axis: proximal segment and distal tarsus (Casares and Mann, 2001), while WT legs are formed by five distinct segments. Surprisingly, the loss of selector gene function does not affect the underlying positional information along the proximo-distal (PD) axis (Casares and Mann, 2001).

Although ventral homologous appendages, leg and antenna have very different structure and function. Legs are composed by 10 segments and are required for locomotion, while the antenna is composed by only three segments and an arista and performs a variety of sensory function, including olfaction, audition, hygrosensation and thermosensation (Carlson, 1996; Eberl, 1999; Gopfert and Robert, 2001; Sayeed and Benzer, 1996; Snodgrass, 1935) (Figure 9A).

Based on the homology between leg and antenna segments (Postlethwait and Schneiderman, 1971), it has been shown that genes involved in generating the PD axis of these appendages have different expression patterns in each one, indicating that the PD axis of legs and antenna are differentially subdivided (Dong et al., 2001) (Figure 9B, C). However, it seems unlikely that these relative differences in their expression pattern are due to variations in *dpp* and *wg* expression. Indeed, comparative analysis of leg and antenna development suggests that only some of the pattern-forming genes have leg and antenna expression patterns that coincide with the homology regions described by Postlethwait and Schneiderman (1971).

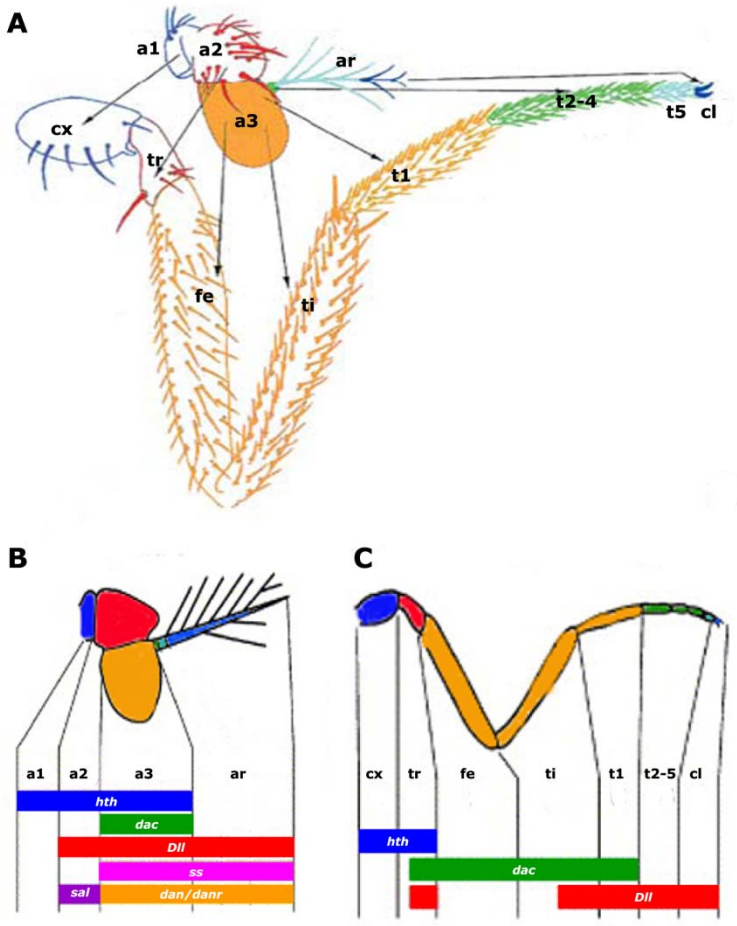

Figure 9. Comparison between the two ventral homologues appendages, leg and antenna. (A) Correspondence map of antenna and leg structures based on position-specific transformations in homeotic antennae of Antennapedia mutants (adapted from Cummins et al., 2003). (B, C) Expression of proximo-distal (PD) domain genes in (B) antenna and (C) leg of *Drosophila* (*hth*, *homothorax*; *dac*, *dachshund*; *Dll*, *Distal-less*; *sal*, *spalt*; *ss*, *spineless*; *dan*, *distal antenna*; *danr*, *distal antenna related*) (adapted from Angelini and Kaufman, 2005). Abreviations: a1, a2 and a3: first, second and third antenna segments; ar, arista; cl, claw organ; cx, coxa; fe, femur; ti, tibia; t, 1-5 first to fifth tarsal segments; tr, trochanter.

The developmental choice between leg and antennal development lies in a single selector gene, the Hox gene *Antennapedia* (*Antp*), function (Postlethwait and Schneiderman, 1971): *Antp* seems to limit the overlap between the domains of *hth* and *dll* expression in the leg disc, avoiding *hth* expression in distal and medial leg cells (Casares and Mann, 1998) and in that way promotes leg development instead of antennal development.

hth encodes a TALE (three amino acid loop extension) class homeodomain transcription factor (Burglin, 1997) that is required for antennal development and sufficient to induce antennae when ectopically expressed in domains expressing *Dll*. Hence, *hth* is consider to be an antenna selector gene (Casares and Mann, 1998; Dong et al., 2000). Indeed, the removal of *hth* function is sufficient to cause the transformation of antennal into leg-like appendages, without de-repression of *Antp* or any other HOX gene. The same occurs with the loss of *extradenticle (exd)* function (Casares and Mann, 1998; Gonzalez-Crespo et al., 1998; Gonzalez-Crespo and Morata, 1996). The PBC class homeodomain protein, Exd, is broadly transcribed and translated (Rauskolb et al., 1993) and forms complexes of relatively high specificity with Hth and Hox proteins by direct binding (Mann and

Chan, 1996). The Hth-Exd interactions promote the Exd nuclear localization that otherwise remains in the cytoplasm (Aspland and White, 1997; Kurant et al., 1998; Mann and Abu-Shaar, 1996; Pai et al., 1998; Rieckhof et al., 1997). On the other hand, Exd is necessary to prevent the degradation of the Hth protein (Abu-Shaar and Mann, 1998). Exd is an obligatory partner of Hth, and therefore the loss of either *hth* or *exd* results in similar developmental defects.

Dll encodes a HD-containing transcription factor that function as a selector gene for all ventral appendages (Gorfinkiel et al., 1997), which includes all limbs (Cohen et al., 1989). Lineage studies demonstrate that all segments, except the most proximal one, the coxa, derive from *Dll*-expressing cells, even if later during development *Dll* expression becomes restricted to distal segments. This reflects different temporal requirements for *Dll* along the PD axis of the leg: medial cells lose their requirement for *Dll* earlier than distal cells do (Campbell and Tomlinson, 1998). *Dll* expression is lost from presumptive proximal cells during either embryogenesis or L1, while its loss from medial leg cells happens either before or during the L2. *Dll* is continually expressed in presumptive distal cells throughout leg development (Weigmann and Cohen, 1999). In the antenna, by analogy to the leg, it is thought that proximal-most *Dll* expression is lost during either embryogenesis or the L1. *Dll* is not only required to specify distal cell fates, but also to specify antennal versus leg fates together with *hth* (Panganiban, 2000).

Antp is expressed in the cells that give rise to all three leg disc during embryogenesis and its expression persists during early leg disc development. However, later on, *Antp* expression is restricted to more proximal regions because it represses its own transcriptional activator, *hth* (Casares and Mann, 1998). In contrast, the antenna develops in the absence of *Antp* function (Struhl, 1981). Indeed, *Antp* seems to block the acquisition of the antennal fate by repressing genes essential for its identity like *hth* (Abu-Shaar and Mann, 1998; Casares and Mann, 1998). Nevertheless, *Antp* and *hth* are coexpressed in presumptive proximal leg, indicating that Antp may need a cofactor to repress *hth*. Dll is likely to be this cofactor, since it is expressed in distal leg, where Antp represses *hth*, and Dll is known to repress *hth* in legs (Abu-Shaar and Mann, 1998; Casares and Mann, 1998; Gonzalez-Crespo et al., 1998). Accordingly, the absence of *Antp* expression in the presumptive antenna enables the coexistence of Hth and Dll. *hth* and *Dll* are independently regulated during antenna development (Dong et al., 2000).

6.1. PD axis formation

Signaling mechanisms responsible for anterio-posterior (AP), dorso-ventral (DV) and proximo-distal (PD) patterning appear to be identical in the developing leg and antenna primordia (Brook et al., 1996; Campbell, 1995; Lecuit and Cohen, 1997).

The first and fundamental subdivision occurs between A and P compartments and is established. In legs, this happens during embryogenesis, before the discs are formed (Garcia-Bellido et al., 1976; Lawrence and Morata, 1977). During early embryogenesis, P cells from each segment express Hh that diffuse and reach A cells that along AP boundary compartment activate *wg* expression. Later on, *wg* expression becomes restricted to a dorsal patch and a ventral stripe in each segment. The limb primordia are allocated as clusters of cells that include *wg*-expressing cells at the dorsal edge of the ventral stripe and *dpp*-expressing cells positioned further dorsally (Campbell et al., 1993; Cohen et al., 1993). Nevertheless, in the antenna, the establishment of an effective AP restriction occurs much later during development (in L2)– and, actually, it happens in the eye-antennal imaginal disc (Morata and Lawrence, 1979).

In all *Drosophila* appendages, during larval stages, the P compartment is characterized by the activity of the selector gene *engrailed* (*en*) that programs these posterior cells to express and secrete Hh while, simultaneously, blocks Hh signaling in these *en*-expressing cells. Thus, only anterior cells are capable to read and transduce the Hh signal (Dahmann and Basler, 2000; Lawrence and Struhl, 1996). This AP subdivision is kept by 'clonal boundary': cells born in the anterior compartment never gives rise to posterior cells and vice-versa.

The DV and PD subdivisions appear during postembryonic development of the disc (Abu-Shaar and Mann, 1998; Lecuit and Cohen, 1997; Morata, 2001), however, unlike the AP subdivision, they are not maintained by a cell lineage mechanism in ventral appendages (Mann and Morata, 2000).

In the wing disc, Hh is known to activate *dpp* in anterior cells along the AP boundary, which can diffuse to both sides, acting at long range. Hence, Hh, indirectly through Dpp, controls growth and patterning in both compartments (Dahmann and Basler, 2000; Lawrence and Struhl, 1996). In both antenna and leg, this mechanism is more complex. *dpp* and *wg* are activated similarly by Hh, showing similar relative expression patterns and exhibiting similar mutual antagonism in both appendage primordia (Figure 10). Thus, DV subdivision results from the localization of Dpp and Wg signals: *dpp* and *wg* are expressed in opposite wedges along the AP compartment boundary in response to Hh signal (Basler and Struhl, 1994; Diaz-Benjumea et al., 1994). Complementary patterns of *dpp* and *wg* expression are maintained by mutually repressive interactions (Brook and Cohen,

1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996). The antagonism between the Dpp and Wg pathways ensures that the two domains (D and V) are kept developmentally segregated, i. e., the dorsalizing and the ventralizing activities of Dpp and Wg, respectively, are restricted to opposite sides of the leg primordium. However, *dpp* and *wg* expression domains are not strictly exclusive and overlap at the center of the primordium, where they jointly specify the distal tip of the future appendage. Further, the combination of Dpp and Wg signals induces growth and activates distinct genes along the PD axis (Basler and Struhl, 1994; Campbell et al., 1993; Campbell, 1995; Diaz-Benjumea et al., 1994).

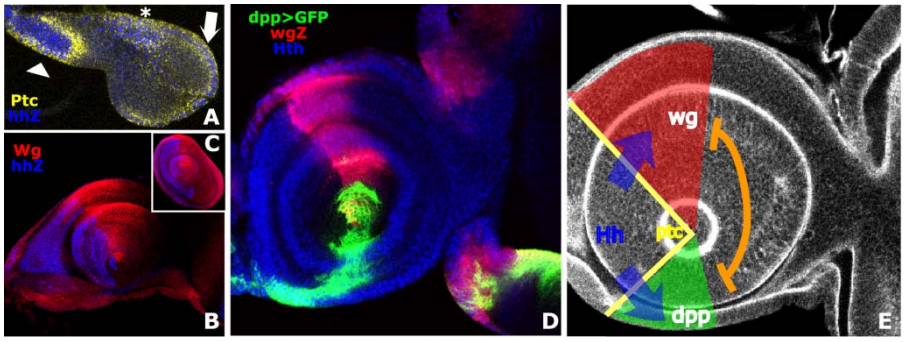

Figure 10. Establishment of the proximo-distal (PD) axis of the antenna. (A) A second instar (L2) eye-antennal disc shows the expression of *hedgehog* (*hhZ*, blue) in the anterior domain of the antenna (arrowhead), along the margin (arrow) and in the ocellar domain (asterisk). The expression of *patched* (*ptc*, yellow), the Hh receptor, reflects the activation of hh signaling. (B, C) As a short-range morphogen, Hh (hhZ, blue) diffuses and activates *wg* (Wg, red) in dorsal domain of the antenna (B) and in ventral domain of leg (C) discs. Note this apparent inversion of dorsal (D)/ ventral (V) domains in the antenna. It is just the result of assigning D or V position relative to the eye disc. Nevertheless, the *wg*- and *dpp*-expressing domains of antennae and legs are homologous. (D) Hh activates *dpp* (*dpp*>GFP, GFP expression driven by dppGal4 driver reflects *dpp* expression, green) in the ventral domain of the antenna, which results in opposing wedges of *dpp* and *wg* activation (*wgZ*, red). The mutual repression between Wg and Dpp signaling maintains separated the dorsal and ventral domains, except in the center of the disc, where *wg* and *dpp* are coexpressed. This domain of *dpp* and *wg* coexpression establishes the distal tip of the antenna and triggers the concomitant formation of the P/D axis. The TALE-homeodomain gene, *homothorax* (*hth*, blue), required for antennal specification, is initially expressed throughout the antennal disc, but becomes absent from the its most distal portion during L3. (E) Schematic representation of the establishment of the PD axis in the antennal disc.

Studies on late larval leg development have shown that PD patterning becomes Wg- and Dpp-independent after 84h AEL. Downregulation of Wg or Dpp pathways after this time point results in a normal PD organization, despite ventral or dorsal patterning defects, respectively (Galindo et al., 2002).

6.2. Leg Vs Antenna

An essential difference between leg and antenna development is their developmental origin. Leg derives from independent imaginal disc, whereas the

antenna derives from the eye-antennal imaginal disc complex (Figure 11A-C). In addition, while leg imaginal discs are formed from cells that belong to the same embryonic thoracic segment, the eye-antennal imaginal disc derives from the fusion of different groups of cells originated in distinct embryonic head segments (Cohen et al., 1993; Younossi-Hartenstein, 1993).

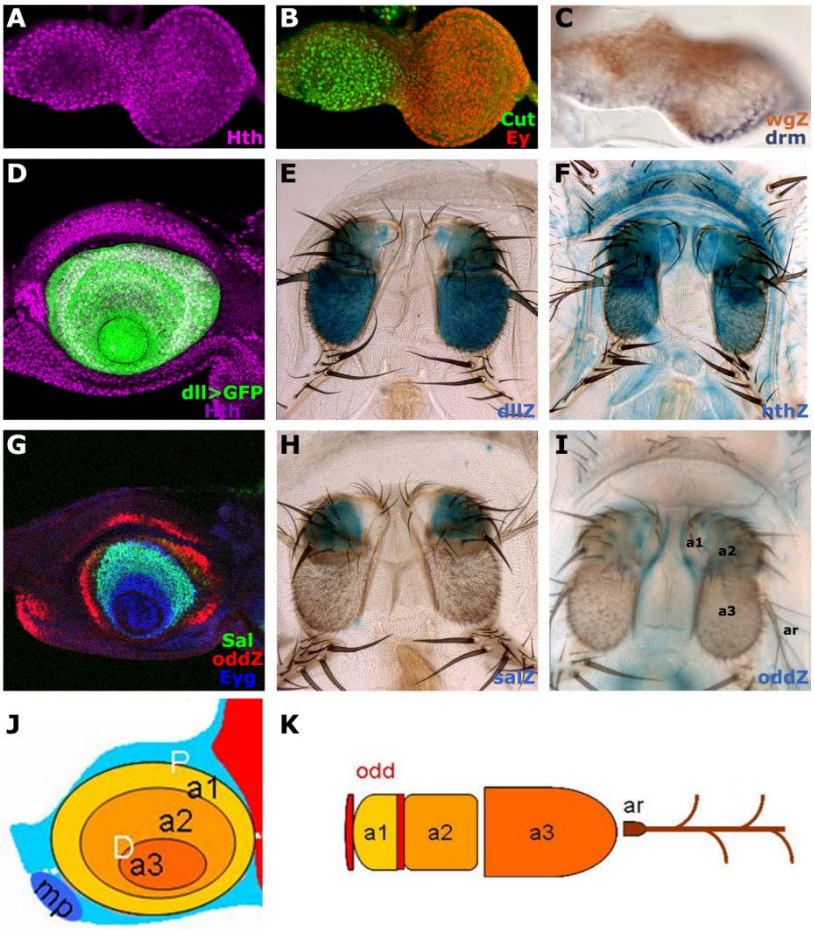

Figure 11. Differential gene expression along the proximo-distal (PD) axis of the antenna. (A, B) During second instar (L2), all cells from the eye-antennal imaginal disc express *homothorax* (*hth*, blue). Two lobes are morphologically and genetically distinguishable: the anterior lobe or antennal disc expresses the gene *cut* (*ct*, green). The posterior lobe, the eye disc, expresses the eye selector gene *eyeless* (*ey*, red). (C) *drumstick* (*drm*) *in situ* hybridization (blue) reveals that, in addition to its expression along the margin, *drm* is also transcribed in the ventral region of the antenna disc, while *wingless* (*wgZ*, orange) has a dorsal expression. (D) After the establishment of the PD axis, the antennal selector gene *hth* (purple) is downregulated in the most distal domain, which corresponds to the arista (ar) in the adult antenna (*hthZ*, F), while the gene required for the specification of all ventral appendages, *Distal-less* (Dll>GFP, green) is expressed in the whole primordium except for its most proximal region, which corresponds to the segment 1 (a1) of the adult antenna (*DllZ*, E). In response to different levels of Wg and Dpp signals, different genes are expressed along the PD axis. *spalt* (*sal*) is expressed specifically in the primordium of the segment 2 (a2) in late third instar (green, G) and in the adult a2 (*salZ*, H). *eyegone* (*eyg*) is expressed in both a2 and a3 (antenna segment 3) (blue, G). *odd-skipped* (*odd*) is expressed in two concentric rings (*oddZ*, red, G) that correspond to the joints between the head capsule (light blue, J) and a1 and between the a1 and a2 (*oddZ*, I, K). In addition, *odd* (*oddZ*, red, G) is expressed in the maxillary palp primordium in the disc (mp, blue, J). (J, K) Schematic representation of a late third instar antennal disc (J) and an adult antenna (K).

The similarity between the actions of the Hh/Dpp/Wg pathways had lead to believe that the PD axes of the antenna and leg as constructed in a similar way.

Nonetheless, genes that are regulated by Dpp and Wg in the developing leg like *Dll*, *hth* and *dac* show different relative patterns in the antennal and the leg discs (Dong et al., 2001).

In the leg, Wg and Dpp opposite gradients initiate the leg PD patterning by: 1) activating *Dll* distally (Lecuit and Cohen, 1997); 2) repressing dac distally (Lecuit and Cohen, 1997); and 3) repressing *hth* in the presumptive distal and medial leg (Abu-Shaar and Mann, 1998). *Dll* is activated in the central part of the disc (the future limb's distal tip), where the *wg* and *dpp* are expressed at high levels and suppresses *hth*/*exd* activity (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998). The activity of *hth*/*exd* remains in the periphery of the disc (the prospective proximal leg), where they block the response to Dpp and Wg target genes (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998), that were activated at lower concentration of Dpp and Wg signals than *Dll* (Lecuit and Cohen, 1997). The mutual antagonism between *hth*/*exd* function and the Hh/Dpp/Wg pathways ensure the maintenance of the two distinct domains (P and D). Hth/Exd downregulate the activity of Wg and Dpp pathways in the proximal region neither by reducing transcription levels (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998; Wu and Cohen, 1999) nor affecting signal diffusion. Indeed, the analysis of the distinct functions of *hth* in leg development has shown that *hth* interferes with the function of Dpp pathway by two different mechanisms (Azpiazu and Morata, 2002). First, *hth* appears to regulate the levels of Mad phosphorylation, which can be used as an indicator of the levels of Dpp transduction (Tanimoto et al., 2000). Second, *hth* may also interfere with the activity of genes secondarily regulated by Dpp. In addition, *dac* and *Dll* repress each other, maintaining medial and distal leg as distinct domains (Dong et al., 2001). *tsh*, a Zn-finger-encoding gene, co-expressed with *hth* in the proximal leg disc, is sufficient to repress *dac* (Dong et al., 2001; Erkner et al., 1999), while *hth* is neither required nor sufficient to repress *dac* (Dong et al., 2001; Wu and Cohen, 1999). The expression of *tsh* is determined by the convergence of Dpp and Wg signals and have no effect on development of distal leg segments (Erkner et al., 1999; Wu and Cohen, 2000). Tsh reinforces the action of both Dpp and Wg to prevent the expansion of *Dll* expression into more proximal regions (Lecuit and Cohen, 1997) and represses *dac* in the developing leg (Erkner et al., 1999; Wu and Cohen, 2000). On the other hand, *dac* is responsible for the maintenance of the distal limit of the *hth*-proximal domain, but does not regulate *tsh* expression (Abu-Shaar and Mann, 1998; Dong et al., 2001). Thus, proximal and medial leg are kept as distinct domains via the repression of *dac* by *tsh* and the repression of *hth* by *dac*, respectively.

In the antenna, *dac* is not responsible for *hth* and *Dll* repression, given that first, *dac* expression pattern coincides with the area of *hth* and *Dll* coexpression (Figure 11 D-F) and second, that *dac* null mutant clones do not de-repress *hth* or *Dll* (Dong et al., 2001). Thus, contrary to what occurs in the leg, the mutual antagonism between these genes is not present in the antenna. Moreover, as reported by Mardon and colleagues, *dac* null flies do not show antennal defects, although have shortened legs, with their intermediate segments missing (Mardon et al., 1994). Accordingly, the expansion of *dac* expression domain in the antenna leads to the differentiation of medial leg structures in a 100% of antennae (Dong et al., 2001). Together these results indicate that *dac* functions in the specification of leg fates and seems to play no role in antenna development. In addition, the analysis of *Dll* null clones indicates that *dac* expression requires *Dll* (Dong et al., 2001).

Dll and *hth* specify antenna fates via multiple genes (Dong et al., 2002). Both are required for the antennal expression of *spineless* (*ss*), *dac*, *ato* and *sal (spalt*, Figure 11G, H*)*, whereas *Dll* is required independently for the activation of *arista-less* (al), *Bar* and *bric-a-brac* (*bab*) (Campbell and Tomlinson, 1998; Chu et al., 2002; Gorfinkiel et al., 1997; Kojima et al., 2000) and *hth* is required independently for the activation of *cut (ct)* (Dong et al., 2002).

distal antenna (dan) and *distal antenna related (danr)* (also known as *hernández* and *fernández)* genes, which encode novel nuclear proteins, are required for distal antenna specification, acting downstream of genes that control the differentiation of distal antenna structures, such as *hth* and *Dll* (Emerald et al., 2003; Suzanne et al., 2003). Indeed, Hth and Dll regulate *dan* and *danr* expression through the regulation of *ss* and *ct*. Inactivation of both genes partially transforms distal antenna into leg and ectopic expression of either of the genes results in transformation of distal leg into antenna. *dan* and *danr* seem to act as effectors of *ss* to specific distal antenna. *ss* encodes bHLH-PAS transcription factor and is the closest homolog of the mammalian dioxin receptor (Duncan et al., 1998). This gene is expressed early in both distal antenna and leg, but persists only in the antenna later in development. Consistent with its expression pattern, loss of function alleles are characterized by transformation of distal antenna to leg and deletion of distal leg structures. In addition, ectopic *ss* expression in distal leg transforms it into distal antenna (Duncan et al., 1998). Thus, *ss* functions in the control of antenna identity, being its regulation dependent on both Dll and Hth activity: removal of either *Dll* or *hth* functions results in the downregulation of *ss* expression (Dong et al., 2002; Duncan et al., 1998). Like *ss*, *ct* is also expressed differentially in leg and antenna. During antennal development *ct* expression is restricted to proximal

domains of the antenna, whereas in the leg *ct* is expressed in small clusters. However, unlike *ss*, *ct* is only regulated by Hth (Dong et al., 2002).

In conclusion, both *hth* and *Dll* are required for antennal identity (Casares and Mann, 1998; Dong et al., 2000; Dong et al., 2001) and function by, for example, activating the antenna-specific transcription of *sal* (Casares and Mann, 1998; Dong et al., 2000; Dong et al., 2001).

6.3. Segmentation: formation of articulations

Segmentation is a developmental mechanism that subdivides a tissue into repeating functional units, which can then be further elaborated during development. In the leg, segmentation must be coordinated with tissue growth and PD axis specification.

The leg anlage is divides in concentric segments along the PD axis (Couso and Bishop, 1998). The articulated joints localize at the boundaries between these segments (Fristrom, 1993).

Notch signaling pathway localizes the presumptive joint areas between segments. Moreover, spatially restricted activation of the Notch pathway is required for joint development, i. e., segment formation depends on localized expression of the Notch ligands Dl and Ser, that activate Notch pathway in the domain where joins will form (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). In this process, in contrast to Dl-N lateral inhibition described for R8 specification, N receives signals from several ligands (Fleming et al., 1997): Serrate (Ser) and Dl. In addition, Fringe (Fng) has been proposed to bind N and to modulate its sensitivity to the ligands (Panin et al., 1997).

Notch activation through a combined Ser and Dl signaling induces joints formation, activating the expression of *E(spl)* complex genes and *disconnected (disco)* in the presumptive joint areas (Bishop et al., 1999). Removal of *Notch* or one of Notch ligands reduces or completely eliminates the joins, whereas ectopic expression of any of these genes provokes the expansion of joints regions or the formation of ectopic joints, with associated *disco* expression activation. *Ser* and *Dl* are expressed in rings, their expression partially overlap near the distal end of each leg segment and induce joint development in the cells immediately distal to their expression domains.

The work by Rauskolb (2001) demonstrates that some PD patterning genes are required to establish the segmental pattern of Notch ligand and *fng* expression. However, these genes act in different ways: *hth* and *dac* positively regulate the segmentation genes, while *Dll* inhibits their expression (Rauskolb, 2001). Regarding the distinct expression patterns of the PD patterning genes, these findings indicate

that each segmental ring of Notch ligand and *fng* expression is independently regulated.

As mentioned, the antenna is dramatically different in its shape, number of segments and size relative to the leg. Although the signals involved in the patterning of leg and antenna appear to be identical (Brook et al., 1996; Campbell, 1995; Lecuit and Cohen, 1997), still it is not completely understood to what extent the mechanisms operating during leg development are the same in the antenna, and if that were not the case, where the differences lie. In addition, while leg discs derive from a single embryonic thoracic segment (Cohen et al., 1993), the eye-antennal disc derives from several embryonic segments (Younossi-Hartenstein, 1993). This more complex developmental origin of the eye-antennal primordia also poses questions as to how these different cell groups coalesce and coordinate their development or how the highly modified structures of the head of dipterans have arisen during evolution.

7. Odd family genes

Odd-skipped family of proteins (Odd in *Drosophila* and Osr in vertebrates) are evolutionarily conserved zinc-finger (Zn-f) transcription factors, although the number of Zn-fs varies among them (Goldstein et al., 2005).

In *Drosophila*, *odd* is the founder gene of this gene family and was first identified as pair-rule segmentation gene (Nusslein-Volhard and Wieschaus, 1980). *odd* together with other pair-rule genes is required to specify the anterior domain of odd-numbered segments (Coulter and Wieschaus, 1988). Further analysis has detected *odd* expression in the heart, CNS and distinct regions of the gut, including the posterior region of the midgut and the proximal Malpighian tubules (Ward and Coulter, 2000), indicating that *odd* could have functions other than embryonic epidermal patterning.

In addition to *odd*, three other genes, *brother of odd with entrails limited* (*bowl*), *drumstick* (*drm*) and *sister of odd and bowl* (*sob*) belong to this family. All four genes are clustered on the left arm of the second chromosome (Figure 12A). Molecular analysis of Odd has revealed that the protein contains four tandem C_2H_2 (Cys-Cys/His-His) Zn-f repeats, suggesting that it could function as a DNA binding protein and transcriptional regulator (Coulter et al., 1990). *sob* and *bowl* were described as *odd* paralogues (Hart et al., 1996; Wang and Coulter, 1996). As expected for paralogous genes, they exhibit high conservation within the Zn-f putative DNA binding regions and diverge appreciably in other regions. In addition, Sob and Bowl have an extra Zn-f, located at the C-terminus. Besides this feature, *sob* shows a similar embryonic expression pattern to *odd* and *drm*, while *bowl* has a

more widespread pattern. Thus, *odd*, *drm* and *sob* may have overlapping functions (Hart et al., 1996). *drm* was first identified in a screen for genes controlling Malphigian tubules and other epithelia morphologies and implicated in fore- and hindgut morphogenesis (Liu et al., 1999). In contrast to the other members of this family, Drm presents only two Zn-fs: one C2H2 and one C2HC (Figure 12B, C) (Green et al., 2002).

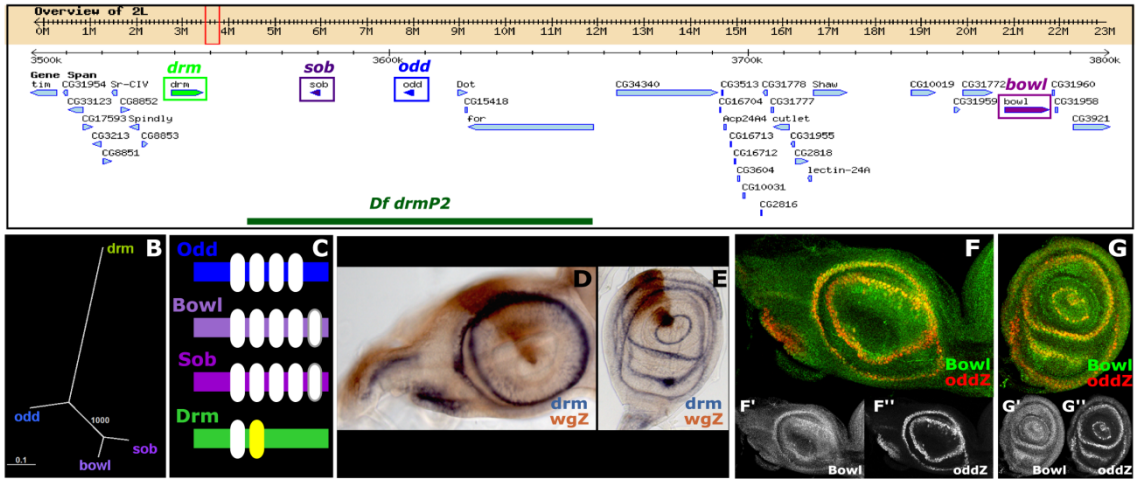

Figure 12. *odd* family genes: comparison between the four elements of the family and their relative expression in the antenna and the leg discs. (A) All four *odd*-family genes map within a region of approximately 250kb on the second chromosome (from Flybase). The green line marks a deficiency (*Df drmP2*) that removes the *sob*, *odd* and *drm* loci, plus a number of other genes (described in Green et al., 2002). (B, D) odd is the founder member of this gene family and encodes a protein that contains four C2H2 Zn-fingers. Bowl and Sob have a fifth Zn-finger domain. The more divergent gene of this family is *drm* that encodes a protein with one C2H2 Zn-finger and a divergent C2HC Zn-finger. Zn-finger domains are represented by white ovals and the divergent Zn-finger domain by a yellow oval (C). *drm* (*drm in situ* hybridization, blue, D), *bowl* (anti-Bowl antibody, green, F, F') and *odd* (*oddZ* reporter, red, F, F'') show two rings of expression in the antenna. In the leg, *drm* (*drm in situ* hybridization, blue, E), *bowl* (anti-Bowl antibody, green, G, G') and *odd* (*oddZ* reporter, red, G, G'') show six rings of expression. *bowl* (green) and *odd* (red) show extensive co-expression in the antenna (yellow, F) and in the leg (yellow, G).

drm and *bowl*, together with *lines* (*lin*) were shown to be required for normal hindgut morphology (Iwaki et al., 2001). Removal of any of these genes causes shorter and wider hindguts that show defects in cell arrangement. However, the distinct intestine domains are differentially affected: *drm* together with *bowl* are required for small intestine development, whereas *lin* represses small intestine fate and favors large intestine and rectum development (Iwaki et al., 2001). Further investigation in hindgut development revealed that *drm* and *lin* interact genetically and that *lin* is epistatic to *drm* (Green et al., 2002). In the dorsal epidermis, Lin seems to act in parallel to the Wg pathway to specify a specific cell type, while interactions with Hh are thought to be responsible for the determination of other

cell type (Bokor and DiNardo, 1996). In addition, Lin was proposed to interact with Wg transducer effectors during dorsal epidermal patterning, being required for late Wg signaling activity (Hatini et al., 2000). Hh signaling seems to regulate *lin* function, since in Hh-reading cells Lin localize in the cytoplasm, whereas in Wg-reading cells it localzes in the nucleus. Based on this, Hatini and colleagues (2000) hypothesize that Lin might act as a transcriptional regulator. Indeed, this seems to be the way *lin* functions in the specification of the small intestine of the hindgut. The proposed model reveals a relief-of-repression mechanism where Drm binds to Lin, through the first zinc finger (C2H2), allowing the expression of genes required for small intestine fate (Green et al., 2002). Nevertheless, a recent study show that *bowl* is epistatic to *lin* in the development of the posterior foregut and anterior hindgut, meaning that these fates are regulated by a Drm-Lin-Bowl genetic hierarchy based on protein-protein interactions (Johansen et al., 2003). Indeed, competitive protein-protein interactions between Drm and Lin and between Lin and Bowl regulate the steady-state accumulation of Bowl (Hatini et al., 2005). This competition leads to the redistribution of Lin to the cytoplasm in the presence of Drm, allowing the accumulation of Bowl. Moreover, this mechanism is affected by Hh and Wg signaling, since Hh promotes *drm* expression, while Wg represses its expression (Hatini et al., 2005). Bowl protein is detected in the nucleus in fore- and hindgut where *drm* is expressed. In *drm* mutant embryos, nuclear Bowl accumulation is barely detected, whereas *lin* mutant embryos look like *drm lin* double mutant embryos, where Bowl accumulates nuclearly throughout the fore- and hindgut primordium. This mechanism seems to be conserved in imaginal discs, where Bowl is detected in the nucleus where *drm* is expressed. Indeed, *lin* mutant clones in leg disc causes the accumulation of Bowl in a cell-autonomous manner (Hatini et al., 2005).

In the leg, *odd* family genes are required downstream of Notch to promote leg segmentation. Their expression is dependent on Notch activity, showing a segmentally repeated pattern in rings (Figure 12E, G) (de Celis Ibeas and Bray, 2003; Hao et al., 2003). Accordingly, *odd* genes may instruct the formation of folds during the process of leg joint development: ectopic expression of *odd*, *sob* or *drm* induces invagination in the leg disc epithelium that in adult leg is revealed as ectopic joins (Hao et al., 2003). *odd* and *drm* have been proposed to act redundantly during leg segmentation, since *odd* or *drm* single mutant clones do not affect leg segmentation (Hao et al., 2003). On the other hand, the removal of *bowl* impedes the development of the joints, without affecting the expression of N ligands (de Celis Ibeas and Bray, 2003; Hao et al., 2003). In the antenna, the expression of those ligands apparently does not seem to correlate with the rings of

odd expression (Casares and Mann, 2001). In the eye disc, the *odd* gene is expressed along the margin and never in eye developing cells (Figure 4A). In addition, *odd* can be detected in phc in adult heads (Figure 4C). This feature raises the question of whether *odd* is required in the posterior margin cells for their function as the firing point for retinal differentiation onset.

In addition, *bowl* seems to have a role in tarsal development, controlling the acquisition of tarsal fate through the regulation of *bab* expression. *bowl* mutant clones induce the expansion of *bab* expression domain and repress *dac* and *BarH1* expression (de Celis Ibeas and Bray, 2003; Hao et al., 2003). Indeed, Bowl helps resolving the pattern of these genes by promoting dAP-2 expression and repressing Nubbin (Nub) expression. Moreover, mutual repression between dAP-2 and Nub further refines the pattern and maintains the subdivision of the field into non-overlapping and adjacent territories (Greenberg, 2007).

In addition, results from the Hatini lab (Hatini, 2007; Kula-Eversole, 2007) revealed that *lin* acts as a tumor suppressor gene, since the removal of *lin* function from the wing disc, that results in *bowl* activation, induces ectopic epithelial growth characterized by cells with increased size and division rate. In addition, ectopic expression of *bowl* or *drm* induces hyperplastic growth that is reverted when *wg* function or JAK/STAT pathway is blocked.

Odd and Bowl proteins present an Engrailed homology 1 (eh1) like domain that recruits the Groucho co-repressor to downregulate target genes during embryonic segmentation (Goldstein et al., 2005). Although Groucho does not bind DNA, it is recruited to target promoters by associating with a large number of DNA-binding transcriptional regulators (Chen and Courey, 2000). Indeed, in zebrafish and in *Xenopus*, both Osr1 and Osr2 genes have been shown to be required for normal kidney development, acting as transcriptional repressors. This function seems to be conserved in *Drosophila*, where *odd* genes are required for proper development of the Malpighian (renal) tubules (Tena et al., 2007).

Two mammalian *odd-skipped* related genes, Osr1 and Osr2, have been described each containing three Zn-fs, except the mammalian OsrA splice variant that contains five (Goldstein et al., 2005). Both genes show a very dynamic expression pattern during mouse embryogenesis that includes expression in kidneys and limbs (Lan et al., 2001; So and Danielian, 1999). In mice, Osr1 seems to function in heart and kidney development (Wang et al., 2005), whereas its paralogue Osr2 is an important regulator of secondary palate development (Lan et al., 2004; Stricker et al., 2006). In chicken, Osr1 and Osr2 are expressed in the developing kidney, heart, gut, eye, branchial arches, the trunk dermis and the limbs (Lan et al., 2004; Stricker et al., 2006). Thus, the domains of expression Osr genes seem to be

conserved between chick and mouse. In *C. elegans*, two *odd* genes have been identified and characterized (Buckley et al., 2004). *odd1* and *odd2* play essential and distinct roles during gut development.

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Chapter I

***Odd-skipped* genes specify the signaling center that triggers retinogenesis in *Drosophila*.**

In this study we tried to identify the gene(s) required for the functional specialization of the posterior margin of the eye disc as retinal firing point. The mechanism of retinal induction upon the activity of *hh*, that, in parallel and through the activation of *dpp*, is responsible for the instruction of the neighbour eye-committed cells to differentiate as eye cells, is well understood. However, less is known about the upstream mechanism that activates *hh* expression specifically at the posterior margin. Here, we show that the *odd* family genes fulfil the three requirements to be considered as 'margin specification genes': all the four genes are expressed along the margin before the trigger of the MF; the Drm-Odd/Lin/Bowl cassette is active in the margin, where Drm together with Odd are responsible for the relief of Lin repression on Bowl, which then activates *hh* expression; and, in addition, when ectopically expressed within the eye field, Drm and Odd are sufficient to induce ectopic eyes (Bras-Pereira et al., 2006).

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Odd-skipped genes specify the signaling center that triggers retinogenesis in *Drosophila*

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Although many of the factors responsible for conferring identity to the eye field in *Drosophila* have been identified, much less is known about how the expression of the retinal 'trigger', the signaling molecule Hedgehog, is controlled. Here, we show that the co-expression of the conserved odd-skipped family genes at the posterior margin of the eye field is required to activate *hedgehog* expression and thereby the onset of retinogenesis. The fly Wnt1 homologue *wingless* represses the odd-skipped genes *drm* and *odd* along the anterior margin and, in this manner, spatially restricts the extent of retinal differentiation within the eye field.

KEY WORDS: Retinal differentiation, Eye, *Drosophila*, *odd* genes, Hedgehog, *wingless*

INTRODUCTION

In *Drosophila*, the eye primordium is specified as a subdomain of the *Pax6*-expressing cells in the center of the eye disc, by the co-expression of a set of retinal determination genes (Bonini et al., 1993; Cheyette et al., 1994; Dominguez and Casares, 2005; Halder et al., 1998; Mardon et al., 1994; Pappu and Mardon, 2004). Then, retinogenesis is triggered by the *hedgehog* (*hh*) and the *hh* target *decapentaplegic* (Dpp/Bmp4) signals that are produced by the surrounding posterior margin cells (Fig. 1A), at the so-called 'firing point' (Treisman and Heberlein, 1998). These margin cells abut the eye primordium and give rise to part of the adult head capsule surrounding the eye (Haynie and Bryant, 1986). Once initiated, retinal differentiation propagates in a posterior-to-anterior wave (Fig. 1B,C), with the differentiation wavefront marked by an epithelial indentation: the morphogenetic furrow (MF) (Treisman and Heberlein, 1998). The gene(s) responsible for this specialization of the posterior margin are unknown.

MATERIALS AND METHODS

Drosophila strains

odd^{5}, *drm*^{6}, *bowl*^{1}, *wg*$^{1\text{-}16}$ (*wg*^{CX3}), *odd*^{rK111} (*oddZ*), *hhP30* (*hhZ*), *dppBS3.0* (*dppZ*), *P{en1}wgen11* (*wgZ*), *P{GAL4}hhGal4* (*hh*-GAL4) are described in FlyBase. Df(2L)*drmP2* (Green et al., 2002; Hao et al., 2003) deletes from *tim* to *odd*, and uncovers ~30 predicted genes, including *drm*, *sob* and *odd*. UAS strains were UAS-*odd(A)* and UAS-*sob(6)* (Hao et al., 2003), UAS-*bowl(1.1)* (de Celis Ibeas and Bray, 2003), UAS-*drm* (on the III) and UAS-*lines* (Green et al., 2002; Hatini et al., 2000), and UAS-*Src-GFP* (Kaltschmidt et al., 2000). *odd*-GAL4 faithfully reproduces *odd* expression (a gift from G. Morata and M. Calleja, CMB, Spain). *drm*^{6} was recombined onto a *FRT40A* chromosome.

Loss-of-function clones:

odd^{5}, *drm*^{6} and *bowl*^{1} mitotic clones were induced between 24 and 48 hours after egg laying (AEL) by a 45 minute 37°C heat-shock in larvae from the crosses of *odd* FRT 40A*/balancer males to *yw* hsFLP 122; *Ubi-GFP FRT40A* females (*odd** represents each of the alleles used). Df*drm*P2 cells do not survive unless given a growth advantage, for which we used the 'Minute technique' (Morata and Ripoll, 1975). Clones were induced between 24 and 72 hours AEL by a 20 minute 37°C heat-shock in larvae from the crosses of *odd* FRT40A* males to *yw*, *hsFLP122*; *M* armZ FRT40A females. In some experiments, we used *yw ey-FLP* as flipase source (Newsome et al., 2000) to maximize the amount of mutant tissue in eye discs. Mutant cells were identified by the absence of β-galactosidase (armZ).

Ectopic-expression ('flip-out') clones of *odd*-family genes and *lines*

These clones were induced between 24 and 48 hours AEL (L1 stage) in larvae from the crosses between UAS-*odd** (where *odd** means *odd*, *drm*, *sob* or *bowl*) or UAS-*lines* males and *y*, hsFLP122, *actinP*>hsCD2>Gal4 females (Basler and Struhl, 1994). Clones were marked negatively by the absence of CD2 (CD2 was induced by a 45 minute 37°C heat-shock, followed by 45 minutes recovery at room temperature). The *hhZ*, *dppZ* or *oddZ* reporters were introduced in the genotypes of some experiments. The overexpression of *drm* in *bowl*$^{-}$ cells was achieved using the MARCM technique (Lee and Luo, 2001). *UAS-drm* was balanced over *TM6B, Tb*, so *drm*-expressing larvae were *Tb*$^{+}$. Clones were marked positively by expression of GFP.

Antibodies

We used rabbit anti-β-gal (Cappel), mouse anti-β-gal (Sigma), rabbit anti-GFP (Molecular Probes), mouse anti-CD2 (Serotec), guinea pig anti-Odd (Kosman et al., 1998) and mouse ant-Ptc (Nakano et al., 1989). Rat anti-Elav, mouse anti-Wg (4D4) and mouse anti-Eya are from the Iowa University Studies Hybridoma Bank. RNA probes for *odd*, *drm*, *sob* and *bowl* were as described previously (Hao et al., 2003). Phalloidin-FITC was used to mark filamentous actin. Appropriate fluorescent secondary antibodies were from Molecular Probes. Anti-mouse-HRP (Sigma) was used for immunoperoxidase staining.

RESULTS AND DISCUSSION

bowl, *odd*, *drm* and *sob* are expressed in the margin-peripodial cells in early eye discs, but their expression patterns differ later on in development

The eye disc is a flat epithelial sac. By early third larval stage (L3), columnar cells in the bottom (disc proper: Dp) layer are separated by a crease from the surrounding rim of cuboidal margin cells. Margin cells continue seamlessly into the upper (peripodial; Pe) layer of squamous cells (Fig. 1C-G). The Dp will differentiate into the eye, while the margin and Pe will form the head capsule (Haynie and Bryant, 1986). In addition, the posterior margin produces retinal-inducing signals (Treisman and Heberlein, 1998).

By examining gene reporters we found that the zinc-finger gene *odd-skipped* (*odd*) is expressed restricted to the posterior margin and Pe of L3 eye discs (Fig. 1). As the odd family members *drumstick*

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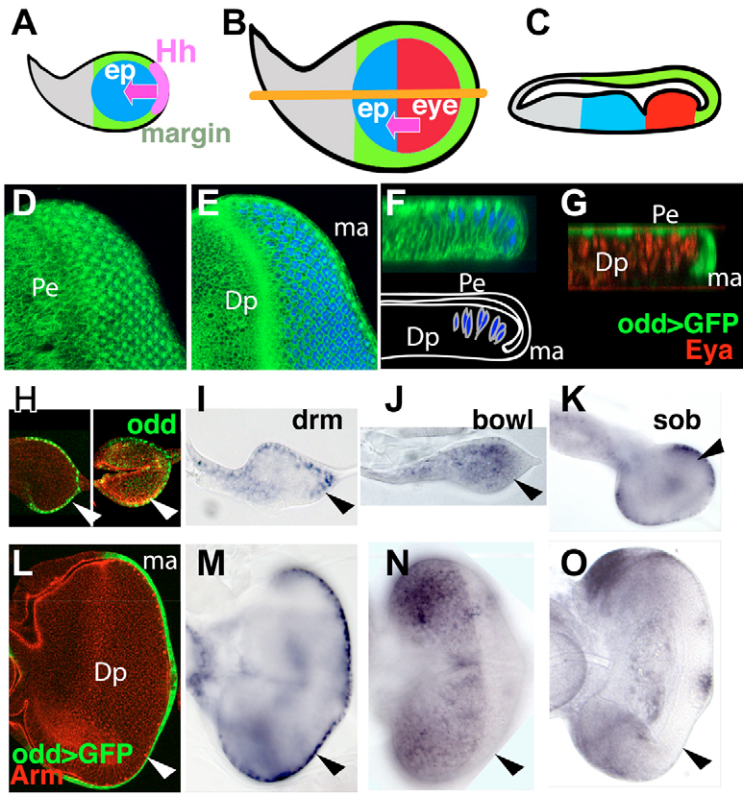

Fig. 1. Expression of the odd-genes is associated to the margin-peripodial cells of the eye disc during development. (**A**,**B**) Schemes of late L2/early L3 (A) and late L3 (B) eye discs. (A) Posterior margin cells trigger retinogenesis in the adjacent eye primordium (ep) by producing Hh. (B) Once triggered, retinal differentiation progresses anteriorly (eye). (**C**) Cross-section through the line in B shows the peripodial and margin cells (green) overlaying the differentiating eye primordium. (**D**,**E**) Confocal images of the posterior region of a third larval stage (L3) disc through the peripodial (Pe, D) and disc proper (Dp, E) layers, stained with phalloidin-FITC and Elav (a photoreceptor marker used in this and following figures). The margin (ma) is a thin strip of cells adjacent to the posterior-most row of photoreceptors. (**F**) Confocal *z*-section through the same disc showing the three cell types (schematized below). (**G**) Confocal *z*-section through the posterior region of a L3 *odd*-GAL4>GFP disc, co-stained with Eya. *odd* is restricted to the Pe and margin. (**H-O**) Patterns of expression of the four odd genes in L2 (H-K) and L3 (L-O). Expression of *odd* is monitored by the *odd-GAL4* reporter (H, left; L) or with an anti-Odd antibody (H, right), and that of *drm* (I,M), *bowl* (J,N) and *sob* (K,O) by RNA in situ hybridization. The patterns of *drm* and *odd* seem identical. (H, left) Propidium iodide marks nuclei. (H, right) Rhodamine-phalloidin stains actin. (L) Arm expression marks cell membranes. Arrowheads indicate the margins. Discs are oriented with posterior towards the right and dorsal upwards.

(*drm*), *brother of odd with entrails limited* (*bowl*) and *sister of odd and bowl* (*sob*) are similarly expressed in leg discs (de Celis Ibeas and Bray, 2003; Hao et al., 2003), we examined them in eye discs. In L2, before retinogenesis has started, *odd* and *drm* are transcribed in the posterior Pe-margin (Fig. 1H,I), and this continues within the posterior margin after MF initiation (Fig. 1L,M). *bowl* is transcribed in all eye disc Pe-margin cells of L2 discs (Fig. 1J), but retracts anteriorly along the margins and Pe after the MF passes (Fig. 1N). In addition, *bowl* is expressed weakly in the Dp anterior to the furrow. *sob* expression in L2 and L3 is mostly seen along the lateral disc margins (Fig. 1K,O). Therefore *drm, odd* and *bowl* are co-expressed at the posterior margin prior to retinal differentiation initiation.

bowl is required for *hedgehog* expression in margin cells and for triggering retinal differentiation

Odd family genes regulate diverse embryonic processes, as well as imaginal leg segmentation (de Celis Ibeas and Bray, 2003; Green et al., 2002; Hao et al., 2003; Hatini et al., 2005; Johansen et al., 2003). Bowl is required for all these processes (Green et al., 2002; Hao et al., 2003). In embryos, the product of the gene *lines* (Bokor and DiNardo, 1996) binds to Bowl and represses its activity, while Drm relieves this repression in *drm*-expressing cells (Hatini et al., 2005). As *drm/odd/bowl* expression coincides along the posterior margin around the time retinal induction is triggered, we asked whether they controlled this triggering. First, we removed *bowl* function in marked cell clones induced in L1. *bowl*$^{-}$ clones spanning the margin, but not those in the DP, cause either a delay in, or the inhibition of, retinal initiation (Fig. 2A,B) and the autonomous loss of *hh*-Z expression (Fig. 2C,E). Correspondingly, there is a reduction in expression of the *hh*-target *patched* (*ptc*) (Fig. 2D). These effects on *hh* and *ptc* are not due to the loss of margin cells, as *drm* is still expressed in the *bowl*$^{-}$ cells (not shown). The requirement of Bowl for *hh* expression is margin specific, as other *hh*-expressing domains within the disc (Royet and Finkelstein, 1997) are not affected by the loss of *bowl* (not shown). As expected from the *bowl*-repressing function of *lines* (Green et al., 2002; Hatini et al., 2005), the overexpression of *lines* along the margin phenocopies the loss of *bowl* (Fig. 2F). Nevertheless, the overexpression of *bowl* in other eye disc regions is not sufficient to induce *hh* (not shown). This suggests that, in regions other than the margin, either the levels of *lines* are too high to be overcome by *bowl* or *bowl* requires other factors to induce *hh*, or both.

drm and *odd* are required for and sufficient to initiate retinogenesis

drm and *odd* are expressed together along the posterior disc margin-Pe (Fig. 1), and *drm* (at least) is required for Bowl stabilization in leg discs (Hatini et al., 2005). Nevertheless, the removal of neither *drm* (Fig. 3A) nor *odd* (not shown) function alone results in retinal defects. *odd* and *drm* may act redundantly during leg segmentation (Hao et al., 2003) and this may also be the case in the eye margin. To test this, we induced clones of Df*drm*P2, a deficiency that deletes *drm*, *sob* and *odd*, plus other genes (Green et al., 2002). When Df*drm*P2 clones affect the margin, the adjacent retina fails to differentiate, suggesting that *drm* and *odd* (and perhaps *sob*, for which no single mutation is available) act redundantly to promote *bowl* activity at the margin (Fig. 3B,C) (although we cannot exclude that other genes uncovered by this deficiency also contribute to the phenotype). To test the function of each of these genes, we expressed *drm*, *odd* and *sob* in cell clones elsewhere in the eye disc. Only the overexpression of *drm* or *odd* induced ectopic retinogenesis (Fig. 3D and not shown), and this was restricted to the region immediately anterior to the MF, which is already eye

committed. Interestingly, *bowl* is also expressed in this region of L3 discs (Fig. 3E). The retina-inducing ability of *drm* requires *bowl,* because retinogenesis is no longer induced in *drm*-expressing clones that simultaneously lack *bowl* function (Fig. 3F). Therefore, it seems that in the eye, *drm* (and very likely also *odd*) also promotes *bowl* function.

The expression of *hh* (Heberlein et al., 1995) or activation of its pathway (Chanut and Heberlein, 1995; Dominguez and Hafen, 1997; Ma and Moses, 1995; Pan and Rubin, 1995; Strutt and Mlodzik, 1995; Wehrli and Tomlinson, 1995) anterior to the furrow is sufficient to generate ectopic retinal differentiation. As (1) *bowl* is required for *hh* expression at the margin, (2) this *hh* expression is

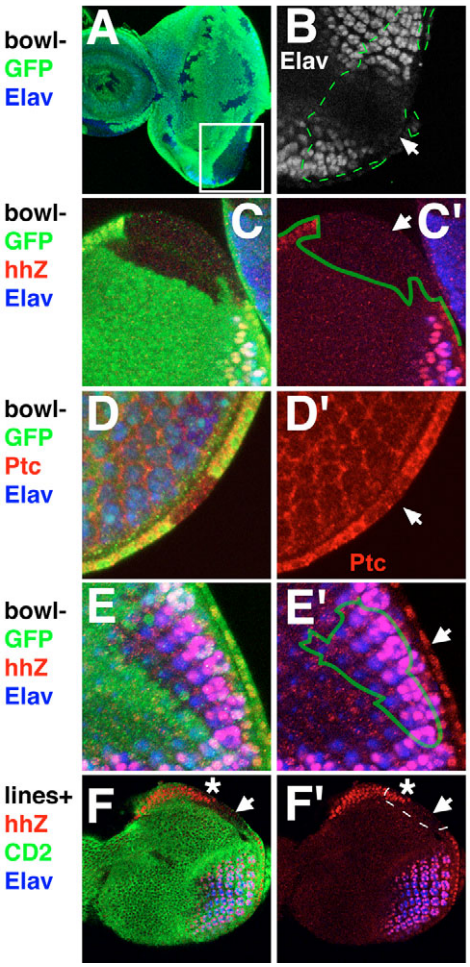

Fig. 2. *bowl* is required specifically at the margin for retinal triggering and *hh* expression. Clones are marked by the absence of GFP (A-E) or CD2 (F). (**A-E**) *bowl*$^{-}$ clones spanning the posterior margin. (A, inset in B) Defective retinal initiation is associated with *bowl*$^{-}$ mutant margin (arrow). Retinal initiation is partially rescued non-autonomously by neighboring tissue (clone outlined in B). (C,C′) *bowl*$^{-}$ clone spanning the margin loses *hh-Z* autonomously (arrow; clone outlined in C′). (D,D′) The expression of Ptc is also reduced in a *bowl*$^{-}$ clone (arrow). (E,E′) Internal *bowl*$^{-}$ clone abutting, but not including, the margin develops retina normally (clone outlined in E′). The *hh-Z* margin expression (arrow) is normal. (**F**,F′) *lines*-expressing clone at the margin resembles loss of *bowl,* causing loss of margin *hh-Z* and retinal failure (arrow). The *hh-Z* ocellar expression is not affected (asterisk). Discs are oriented with posterior towards the right and dorsal upwards.

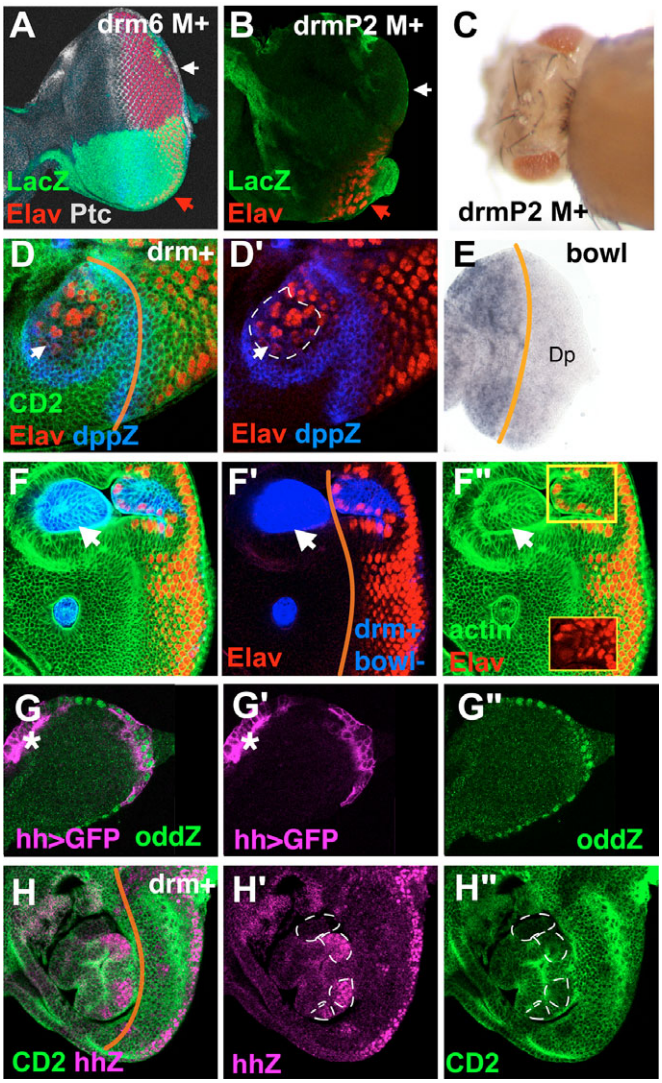

Fig. 3. *drm* and *odd* regulate *hh* expression, probably through enabling *bowl* function. (**A**,**B**) Eye discs containing *M+* clones mutant for (A) *drm*^{6} or (B) Df*drm*P2 (marked by absence of *lacZ*). (A) No effect on retinogenesis or Ptc expression is seen adjacent to *drm*-mutant margin. (Similar results were obtained for *odd*^{5}.) (B) Retinogenesis fails when the adjacent margin is mutant for Df*drm*P2. White and red arrows indicate mutant and wild-type margin, respectively. (**C**) Adult head from the Df*drm*P2, *M+* experiment showing severely reduced eyes. (**D**,D′) *drm*-expressing clone (absence of CD2, and outlined in D′) induces an ectopic furrow (marked by *dpp*-Z) and associated retinogenesis (detected by Elav). The line indicates the position of the endogenous furrow (D). (**E**) Disc proper (Dp) expression of *bowl* mRNA is detected anterior to the furrow (line) in late L3 discs. (**F**-F″) *drm*$^{+}$ *bowl*$^{-}$ clones (blue) do not induce ectopic retinal differentiation anterior to the morphogenetic furrow (arrow; line indicates the furrow). Phalloidin stains actin. A *drm*$^{+}$ *bowl*$^{-}$ clone located immediately after the furrow (boxed) shows Elav-positive neurons (inset). (**G**-G″) L2 eye disc from *oddZ*/UAS-GFP; *hh*-GAL4 larvae shows extensive overlap of *hh* and *odd* at the posterior margin. Asterisk indicates the *hh* ocellar domain, which, at this stage, does not express *odd-Z*. (**H**-H″) Most *drm*-expressing clones (absence of CD2, outlined in H′ and H″) induce *hh-Z* expression just anterior to the morphogenetic furrow (line). Discs are oriented with posterior towards the right and dorsal upwards.

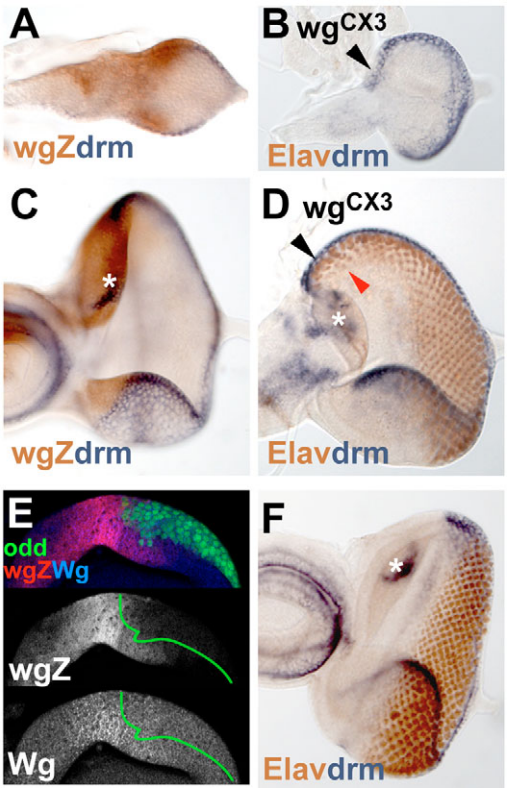

Fig. 4. *wingless* represses *drm* transcription in anterior eye disc margin. (**A**,**B**) Early and (**C-F**) late L3 discs. (A) In *wg*-Z discs (β-galactosidase, orange), prior to the initiation of retinal differentiation, *drm* and *wg* expressions are complementary. (C) In late L3 discs, this complementarity is maintained with the exception of the appearance of a dorsal head *drm*-expressing patch (asterisk in C,D,F). (B) In early L3 *wg*CX3 discs, *drm* transcription extends dorsally to reach the antenna (black arrowhead) before retinogenesis starts. (D) In older discs, ectopic retinogenesis (red arrowhead) can be seen progressing from the *drm*-expressing anterior margin (black arrowhead). (E) Dorsal margin of an *odd*-GAL4/*wg-Z*; UAS-GFP L3 disc. *odd* reporter expression (green and outlined in by the green line in the single channel panels) is complementary to both *wg* transcription (*wg-Z*) and protein expression (Wg). (F) A late wild-type L3 disc stained for *drm* and Elav is shown for comparison. Discs are oriented with posterior towards the right and dorsal upwards.

largely coincident with that of *odd* and *drm* (Fig. 3G), and (3) *drm* (and possibly *odd*) functionally interacts with *bowl*, we checked whether *drm*- and *odd*-expressing clones induced the expression of *hh*. In both types of clones *hh* expression is turned on autonomously, as detected with *hh-Z* (shown for *drm* in Fig. 3H), which would thus be responsible for the ectopic retinogenesis observed. That the normal *drm*/*odd*/*bowl*-expressing margin does not differentiate as eye could be explained if margin cells lack certain eye primordium-specific factors.

wingless represses *drm* transcription along the anterior dorsal eye disc margin

Our results indicate that the expression of *odd* and *drm* defines during L2 the region of the *bowl*-expressing margin that is competent to induce retinogenesis. How is their expression controlled? *wingless* (*wg*) is expressed in the anterior margin, where it prevents the start of retinal differentiation (Ma and Moses, 1995; Treisman and Rubin, 1995). *drm/odd* are complementary to *wg* (monitored by *wgZ*) during early L3, when retinal differentiation is about to start, and also during later stages (Fig. 4A,C,E). In addition, when *wg* expression is reduced during larval life in *wg*CX3 mutants, *drm* transcription is extended all the way anteriorly (Fig. 4B,D). This extension precedes and prefigures the ectopic retinal differentiation that, in these mutants, occurs along the dorsal margin (Fig. 4B,D,F). Therefore, *wg* could repress anterior retinal differentiation by blocking the expression of odd genes in the anterior disc margin, in addition to its known role in repressing *dpp* expression and signaling (Hazelett et al., 1998; Treisman and Rubin, 1995).

Interestingly, the onset of retinogenesis in L3 is delayed relative to the initiation of the expression of *drm/odd* (this work) and *hh* (Cavodeassi et al., 1999; Cho et al., 2000) in L1-2. This delay can be explained in three, not mutually exclusive, ways. First, the relevant margin factors (i.e. *drm/odd*, *hh*) might be in place early, but the eye primordium might become competent to respond to them later. In fact, *wg* expression domain has to retract anteriorly as the eye disc grows, under *Notch* signaling influence, to allow the expression of eye-competence factors (Kenyon et al., 2003). Second, building up a concentration of margin factors sufficient to trigger retinogenesis might require some time. In fact, the activity of the *Notch* pathway along the prospective dorsoventral border is required to reinforce *hh* transcription at the firing point (Cavodeassi et al., 1999). Third, other limiting factors might exist whose activity becomes available only during L3. Such a factor might be the EGF receptor pathway, which is involved in the triggering and reincarnation of the furrow along the margins during L3 (Kumar and Moses, 2001).

In addition to *hh*, other genes are required for retinal triggering, including *dpp* (Burke and Basler, 1996; Pignoni and Zipursky, 1997; Wiersdorff et al., 1996), *eyes absent* (*eya*) (Bonini et al., 1993) and the target of *eya dachshund* (*dac*) (Mardon et al., 1994; Pignoni et al., 1997). These genes are expressed in both the posterior region of the eye primordium and the posterior margin. In addition to their role in eye specification, they might also specify the margin. Although the regulatory relationships between *hh* and *dpp*, or *dpp* and *eya* are obscured by cross-regulatory interactions (Borod and Heberlein, 1998; Chen et al., 1999; Curtiss and Mlodzik, 2000; Hazelett et al., 1998; Pignoni and Zipursky, 1997), recent functional data indicate that *dpp* and *eya* are functionally downstream of *hh* (Pappu et al., 2003). The possibility that the odd genes control the expression or function of *dpp* and *eya* at the margin remains to be tested.

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Chapter II

An antennal-specific role for *bowl* in repressing supernumerary appendage development in *Drosophila*.

Based on the homology between the ventral appendages, legs and antenna, we decided to investigate the function of *odd* family genes in antennal development. These genes were known to play an essential role in leg segmentation. Besides, *bowl* seems to have an additional function in the leg PD patterning. Our results reveal that *odd* family genes, despite their segmented expression pattern, may not be necessary for joint formation in the antenna. However, we describe a role for *bowl* in the establishment of the PD axis. Before PD patterning is established, bowl function is required in the ventral antennal disc to ensure that these cells, along the AP compartment boundary, respond to Hh signal by activating *dpp* instead of *wg* expression (Bras-Pereira and Casares, *submitted*).

An antennal-specific role for *bowl* in repressing supernumerary appendage development in *Drosophila.*

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Keywords: *bowl*, antennal development, proximo-distal axis, *wingless*, *decapentaplegic*, *Drosophila*, evolution.

ABSTRACT

In *Drosophila*, antennae and legs are serially homologous appendages, and yet they develop into organs of very different structure and function. This implies that different genetic mechanisms operate onto a common developmental ground state to produce antennae and legs. Still few such mechanisms have been uncovered. During leg development, *bowl*, a member of the odd-skipped gene family, has been shown to participate in the formation of the leg segmental joints. Here we report that, in the antennal disc, *bowl* has a dramatically different role: *bowl* is expressed in the ventral antennal disc to prevent inappropriate expression of *wg* early during development. The removal of *bowl* function leads to the activation of *wg* in the *dpp*-expressing domain. This ectopic intersection of *wg* and *dpp* results in a new proximo-distal axis that promotes non-autonomous antennal duplications. The role of *bowl* in suppressing a supernumerary PD axis is maintained even when the antennal disc is homeotically transformed into a leg-like appendage. Therefore, *bowl* is part of a genetic program that suppresses the formation of supernumerary appendages specifically in the fly's head.

INTRODUCTION

In *Drosophila*, antennae, mouthparts, legs and genitalia are considered to be serially homologous ventral appendages (Cohen, 1993). This means that despite their very different structure and function, they are thought to develop from a common developmental ground state. It is the segment-specific selector gene expression that, acting upon this ground state, defines their specific morphologies (Casares and Mann, 2001; Duncan et al., 1998; Estrada and Sanchez-Herrero, 2001; Joulia et al., 2006). Of these ventral appendages, the development of leg is best understood (Kojima, 2004; Morata and Sanchez-Herrero, 1999). The leg primordium is set aside as a cluster of epidermal cells, composed of a distal population, that expresses *Distal-less* (*Dll*) and a proximal one, expressing

homothotax (hth), *teashirt* and *escargot* (Kojima, 2004). This early genetic subdivision would correspond to the proximo-distal (PD) telopoditecoxopodite subdivision of the insect appendages proposed by Snodgrass on comparative/evolutionary grounds (Gonzalez-Crespo and Morata, 1996; Snodgrass, 1935). *hedgehog (hh)*, expressed by posterior cells within the leg primordium, triggers the expression of the *decapentaplegic* (*dpp*) and *wingless* (*wg*) signaling molecules in anterior cells (Basler and Struhl, 1994; Campbell et al., 1993; Diaz-Benjumea et al., 1994) which, through mutual repression, become expressed in a dorsal and a ventral wedge, respectively (Brook et al., 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Theisen et al., 1996). *wg* and *dpp* expressions only coincide at the center of the leg disc and it is this confluence of maximal signaling that defines the distal tip of the future leg and triggers growth (Jiang and Struhl, 1996; Lecuit and Cohen, 1997). The larval development of the leg primordium– called leg imaginal disc then progresses by the successive definition of intermediate domains of gene expression and it is through this combinatorial of genes that the segments of the leg (coxa, trocanter, femur, tibia and tarsus) are defined (Kojima, 2004). During late larval life, leg development becomes *wg*/*dpp*-independent, and the distal disc tip becomes a source of EGFR signaling, which is responsible of the further segmentation of the tarsus into the five tarsomeres and the terminal claw (Campbell, 2002; Galindo et al., 2002). Growth and segmentation of the leg also depends on Notch signaling. The activation of Notch by its ligands *Delta (Dl)* and *Serrate (Ser)* is necessary for the disc growth, and the overlapped expressions of *Dl* and *Ser* in concentric rings define the position of the joints of the leg segments as the cells immediately distal to these rings (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). The *odd*-skipped family of genes, *odd-skipped (odd)*, *drumstick* (*drm*) and *sister of odd and bowl* (*sob*) are among the Notch targets in legs. These genes are expressed in concentric rings at the prospective leg joints, just distal to the *Dl*/*Ser* ring domains (de Celis Ibeas and Bray, 2003; Hao et al., 2003). A fourth member of the family, *brother of odd with entrails limited (bowl)*, has a more widespread expression pattern (de Celis Ibeas and Bray, 2003; Hao et al., 2003). Genetic data indicate that *bowl* is required for the segmentation of the leg, and that the localized co-expression of the other family members allows (probably in a redundant fashion) the activation of *bowl* at the prospective joints (de Celis Ibeas and Bray, 2003; Hao et al., 2003). Further molecular and genetic experiments show that, at least during embryogenesis, the product of the gene *lines* blocks *bowl* function by directly binding to Bowl and preventing its nuclear accumulation. Drm and likely Odd are able to competitively displace Lines from Bowl, thus allowing Bowl to become

nuclear and function (Hatini et al., 2005). The distinct antennal development is promoted by the distal maintenance of *hth* expression in the antennal disc, resulting in the co-expression of *hth* and *Dll*. This co-expression selects the antennal fate (Casares and Mann, 1998; Dong et al., 2000). Compared to the leg, the antenna is a much shorter appendage, with four segments (antennal (a) segments 1 to 3, plus a distal arista), and functions in olfaction, through the specialization of its a3 segment (Figure 1). The antennal disc does not develop as an independent disc, like the leg one, but forms part of the eye-antennal disc complex. This disc comprises cells derived from several embryonic head segments and the unsegmented acron (Jurgens and Hartenstein, 1993). All the eye-antennal disc complex cells express the Pax6 genes *eyeless (ey)* and *twin-of-eyeless* during first larval stage (L1), but during L2, only the posterior two-thirds of the complex express Pax6 genes, while the anterior third expresses *cut (ct).* The L2 *ct* and Pax6 domains correspond to the antennal and eye discs, respectively (Kenyon et al., 2003). The smaller size and fewer segments of the adult antenna when compared to the leg correlate with a different expression of the *Dl* and *Ser* ligands in antennal and leg discs (Casares and Mann, 2001). Accordingly, the antennal disc has only two *odd*-expressing rings, instead of the six present in leg discs (Casares and Mann, 2001). The different control of growth and segmentation in the antenna indicates that there must be mechanisms operating differently in antennal and leg discs. The fact that *bowl* has been placed downstream of Notch signal in the elaboration of distal leg patterning (de Celis Ibeas and Bray, 2003; Hao et al., 2003) prompted us to test whether *bowl* had any function during antennal development, and if it did, whether it was similar to its role during leg segmentation. Our results indicate that, during antennal disc development, *bowl* has a dramatically different role: *bowl* is expressed at early stages in the ventral antennal disc, where it prevents inappropriate expression of *wg*. If *bowl* is removed, the activation of *wg* results in non-autonomous antennal duplications. *bowl* is still required to prevent PD axis duplication in homeotically-transformed antennal discs, which indicates that there are genetic differences between head and thorax discs that are selector gene independent. The site of *bowl* expression, the composite nature of the antennal disc and evolutionary considerations lead us to hypothesize that *bowl* might be suppressing the development of appendages from a "silenced" primordium present in the antennal, but not in the leg discs of *Drosophila*.

EXPERIMENTAL PROCEDURES

Drosophila strains and genetic manipulations

bowl^{1} (a null *bowl* allele), *tkv*^{a12}, *Antp*^{73b}, *odd*^{rK111} *(odd-Z)*, *hh*^{P30} *(hh-Z)*, *P{en1}wgen11* (*wgZ)*, *P{dpp-lacZ.Exel.2}3 (dppZ)* and *ey-FLP (III)* are described in FlyBase. A third-chromosome insertion of the *wg2.4-Z* regulatory construct (Pereira et al., 2006) is also used as *wg* reporter. *y*, hsFLP122, *actin* P>hsCD2> Gal4 (Basler and Struhl, 1994) was used in mosaic missexpression. *ey-FLP* (on the III chromosome) (Newsome et al., 2000) drives flip-recombinase in the eye-antennal disc from L1 stage.

Loss-of-function clones:

Bowl^{1} and *tkv*^{a12} mitotic clones were induced between 24-48h after egg laying (AEL) by a 45', 37°C heat shock, in larvae from the crosses of *bowl*^{1} *FRT 40A*/balancer or *tkv*^{a12} *FRT 40A*/balancer males to *yw* hsFLP 122; *Ubi-GFP FRT40A* females (for imaginal disc analysis) or to *yw* hsFLP 122; *y*$^{+}$ *FRT40A* females (for adult cuticle analysis) (Bras-Pereira et al., 2006). In some experiments, appropriate gene reporters were introduced in the genotype. The analysis of the antennal phenotypes caused by loss of *bowl* in an *Antp* gain-of-function genetic background was analyzed in *yw* hsFLP 122; *Ubi-GFP*, *FRT40A/ bowl*^{1} *FRT 40A*; *Antp*^{73b} late third instar larvae subject to a 45',37°C heat shock at 24-48h AEL.

To generate *bowl*-mutant antennae, *bowl*$^{-}$ clones were induced in *f; bowl*^{1} *FRT 40A/M(2L)Z*, *f*$^{+}$, *FRT40A; ey-FLP* larvae. In this genotype, the flip-recombinase is expressed throughout the early eye-antennal disc. These clones are given a growth advantage, as they are generated in a Minute-heterozygous background (Morata and Ripoll, 1975). As a result, most eye-antennal disc-derived tissue is mutant and is marked in the adult by the *forked (f)* bristle marker.

Targeted missexpression was achieved using the UAS/GAL4 system (Brand and Perrimon, 1993). Lines used were: *P{arm.S10} (UAS-arm*^{S10}, a non-degradable, constitutively-active Arm), *P{UAS-lin.H}* (*UAS-lines)*, *drmScer\UAS.cGa (UAS-drm)* (described in FlyBase), *UAS-tkv*^{QD} (a constitutively-active form of the *dpp*-receptor *tkv*) (Nellen et al., 1996), *UAS-bowl(1.1)* (de Celis Ibeas and Bray, 2003), *UAS-Src-GFP* (Kaltschmidt et al., 2000), *dpp*-GAL4 (Staehling-Hampton et al., 1994), *ptc-GAL4* (Speicher et al., 1994).

Mosaic expression of Arm^{S10} and was induced in "flip-out" clones in larvae of the genotype *y*, hsFLP122, *actin P>hsCD2> GAL4/UAS-arm*^{S10}, by a 35,5°C heat shock at 24-48h AEL. Clones were marked negatively by the absence of CD2 (CD2 was induced by a 45' 37°C heat shock, followed by 45' R.T. recovery prior to dissection). Expression of *bowl* or *drm* in *tkv*^{a12} mosaics was induced, using the MARCM technique, in larvae of the genotype *yw hsFLP122*, *tub-GAL4*, *UAS-GFP;*

tkv^{a12} *FRT40A/ tub-GAL80 FRT40A; UAS-bowl (*or *UAS-drm)* by a 37°C, 30' heat shock at 24-48 after egg laying. *tkv*-mutant cells overexpressing either *bowl* or *drm* were marked by GFP expression.

In situ hybridization and Immunostainings

RNA probes for *drm* and *bowl* and in situ hybridization were as in Hao et al., 2003. Antibodies used: Rabbit anti-Bowl (de Celis Ibeas and Bray, 2003), rabbit anti- ß-gal (Cappel), mouse anti- ß-gal (Sigma), mouse anti-CD2 (Serotec), rat anti-Dan (Emerald et al., 2003), mouse anti-Dll (Panganiban et al., 1994), rat anti-Ey (gift from P. Callaerts), rabbit anti-GFP (Molecular Probes), guinea pig anti-Hth (Casares and Mann, 1998), guinea pig anti-Odd (Kosman et al., 1998), rat anti-phosphorylated Mad (Aldaz et al., 2003), mouse ant-Ptc (Nakano et al., 1989). Mouse monoclonals anti Ct, Dac, Eya, and Wg (4D4), and rat monoclonal anti Elav are from the Iowa Univ. Developmental Studies Hybridoma Bank. Guinea pig anti-Eyg (Aldaz et al., 2003) recapitulates the expression of *eyegone (eyg)* in the antenna as analyzed by in situ hybridization or through an *eyg*-GAL4 insertional reporter (Jang et al., 2003). We first detect Eyg expression in mid-L3 antennal discs in a medial-distal domain (not shown). Appropriate fluorescent secondary antibodies were from Molecular Probes. Imaging was performed on a Leica SP2 confocal system.

X-gal histochemical staining of adult cuticle

Pharate adults of the *odd-Z* genotype were dissected and processed as in (Casares and Mann, 2000).

RESULTS

Bowl is expressed in the ventral antennal disc early during development.

We first analyzed the expression pattern of *bowl* by in situ hybridization and protein distribution. (In this work, we use the terms "dorsal" and "ventral" antenna according to the dorsal and ventral territories of the eye disc, adopting the nomenclature most frequently used when referring to the eye-antennal imaginal disc. This results in that the domains of *wg* and *dpp* are apparently inverted along the dorsal/ventral axis in the antennal disc relative to the leg disc). *bowl* transcripts and protein show a similar distribution, and are detected in the ventral antennal discs through L2 to mid-L3 (Figure 1A,B). This domain roughly spans the future maxillary palp, ventral antenna, and the intervening region between these two prospective appendages, extending posteriorly to the ventral limit between the antennal and eye lobes (Figure 1G, H). The ventral expression of *bowl* is similar to the ventral-most expression domain of the signal activated form of Mad (Phosphorylated-Mad: P-Mad) (Figure 1D), the intracellular transducer of the *dpp*

pathway (Newfeld et al., 1997; Wiersdorff et al., 1996). P-Mad, in addition, is expressed in a ventral wedge in the prospective antenna that opposes the dorsal domain of *wg* expression (Figure 1D). The pattern of expression of *odd*, as reported by the *odd-Z* enhancer trap, is coincident with Bowl at early as well as at later stages (Figure 1 C,F). Due to this coincidence, and since in early discs the ßgalactosidase signal of the *odd-Z* reporter is much more robust than that of the anti-Bowl antiserum, we used *odd-Z* as a correlate of *bowl* expression. To check whether *bowl*/*odd-Z* expression overlapped P-Mad, we doubly stained late L2 discs from *odd-Z* larvae. There is extensive overlap between P-Mad and *odd-Z* (and by correlation, with *bowl)*, although *odd-Z* expression extends further anterior (Figure 1E). A corresponding early accumulation of *bowl* in the *dpp* signaling domain is not seen in developing leg discs (de Celis Ibeas and Bray, 2003; Hao et al., 2003). In late L3 larvae *bowl* expression resolves into two antennal rings, plus a stripe in the prospective maxillary palp, that also coincide with *odd-Z* (Figure 1F). Identical expression patterns in the antenna are seen for *drm* (not shown). Histochemical staining of adult heads of the *oddZ* reporter strain shows that these two rings likely map to the head capsule:a1 and a1:a2 joints in the adult antenna (Figure 1G,H).

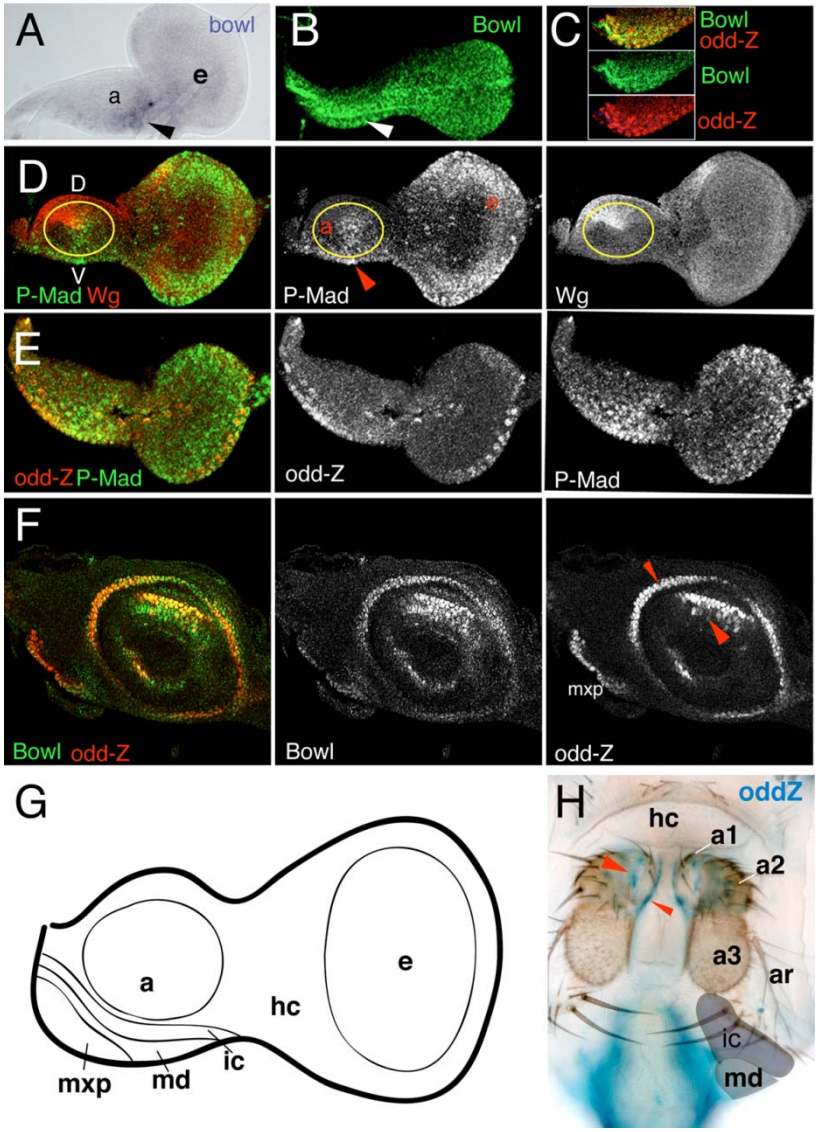

Figure 1. *bowl* is expressed in the ventral antennal disc during early development. Late L2/early L3 (A-E) and late L3 (F) discs. *bowl* expression is detected by in situ hybridization (A; dark blue signal) or with a specific antiserum (B,C,F) while that of *odd* is monitored using an *odd-Z* reporter (C,E,F). *bowl* is expressed along the ventral antennal disc (A,B: arrowhead) and extend to the joint between the antennal and eye disc lobes ("a" and "e", respectively) in late L2/early L3. This ventral expression predates the full specification of a PD axis, as neither Dac nor Eyg, two markers of medial and medial-distal fates, respectively, are not yet activated (not shown). (C) A detail of the ventral region of a late L2 *odd-Z* co-stained with ß-galactosidase and anti-Bowl. Merged and individual signals are shown. There is extensive overlap between *odd-Z* and Bowl expression (visualized as yellow in the merged panel). (D) Immunodetection of Wg and P-Mad in an early L3 disc. Merged and individual signals are shown. In the antennal disc (a, encircled in yellow) Wg is expressed dorsally (D). pMad is detected in the propective antenna and along the ventral (V) antennal rim (arrowhead). In addition, P-Mad signal is present along the posterior margin of the eye disc (e). (E) Late L2/early L3 *odd-Z* disc co-stained with ß-galactosidase and anti-PMad, with merged and individual signals shown. *odd-Z* and P-Mad overlap in the ventral rim of the antennal disc (seen as yellow signal in the merged panel). There is also overlap along the posterior margin of the eye disc, where *bowl* has been shown to be transcribed (Bras-Pereira et al., 2006). (F) Late L3 *odd-Z* antennal disc showing coexpressing of Bowl and *odd-Z* in two concentric rings (arrowheads), plus a stripe in the maxillary palp primordium (mxp). Merged and individual signals are shown. These rings likely map to the joints between the head capsule (hc) and the first antennal segment (a1), and between a1 and a2 in the adult head of *odd-Z* animals stained with Xgal (H; arrowheads). (G) is a schematic representation of the eye antennal disc including the different primordia that form it. "a": antenna; "e": eye; "mxp": maxillary palp; "md": mandibular primordium; "ic": intercalary primordium; "hc": head capsule tissue. "a1-3": antennal segments 1 to 3; "ar": arista. All discs are oriented with posterior to the left and dorsal up in this and following figures. The scheme in (G) and the contribution of the intercalary and mandibular segments to the adult head (in H) have been adapted from Jurgens and Hartenstein, 2003.

Removal of *bowl* function causes non-autonomous duplications of the antenna.

To analyze the role of *bowl* expression during antennal development, we induced mosaic loss of *bowl* during L1 through mitotic recombination (see Materials and Methods). Clones of *bowl*-mutant cells could be recovered throughout the antennal disc, but those located on its ventral half induced antennal duplications (Figure 2), as marked by the generation of new domains of PD genes, such as *ct*, *Dll* or *eyegone (eyg)* (Figure 2A-C). These duplications can also include both anterior and posterior (*hh*-expressing) territories (Figure 2D, E). Very often, these duplications are non-autonomous and comprise mutant and wild type tissue (for example, see Figure 2B, E). These clones result in the duplication of antennal structures, ranging from arista duplications to wholly duplicated antennae (Figure 2F,G; I), including proximal a1 and a2 segments, a3 segment characterized by its high density of olfactory sensillae and arista. Although we cannot mark the adult antennal joints, we performed two experiments to test if *bowl* was required for their formation. First, we induced *y*-marked *bowl*$^{-}$ mosaics and assumed that joints flanked by marked *bowl*-mutant tissue are in most cases also mutant. In all such cases (n>10) the a1:a2 joint always developed normally (Figure 2H). Second, in order to make sure that *bowl*$^{-}$ clones spanned the joints, we generated large *bowl*$^{-}$ clones in *f; bowl*$^{-}$ *FRT40/f*$^{+}$ *M FRT; ey-FLP* individuals (see Materials and Methods). Using this method, adult heads are almost completely mutant for *bowl*, as marked by *forked (f)* bristles (not shown). These flies often showed antennal duplications, but joints were always normal (Figure 2I). This indicates that in the antennal disc, and in contrast with its role in the legs, *bowl* is not required for the formation of joints, despite its joint associated expression during mid to late L3.

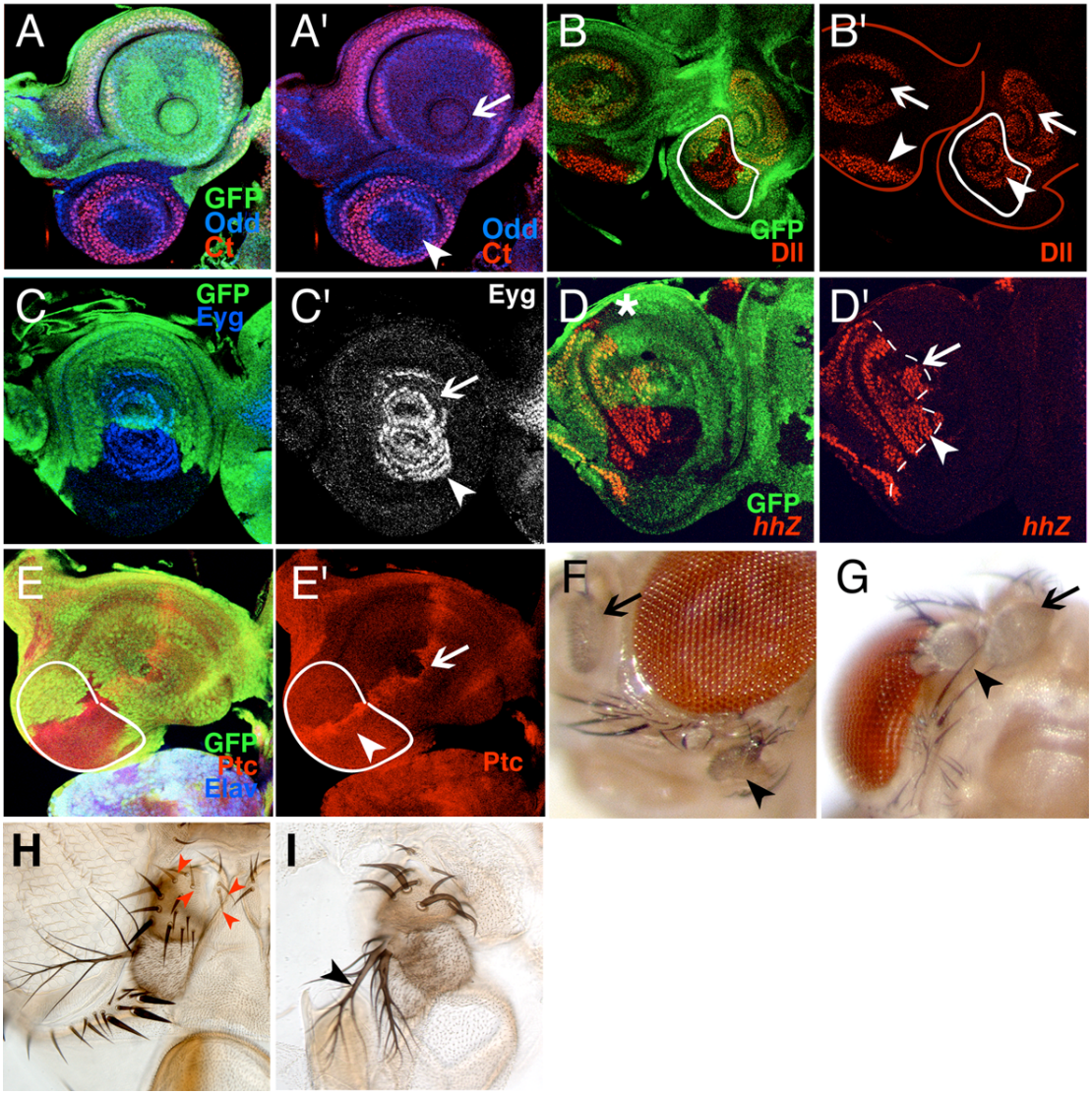

Figure 2. *bowl* mutant cells induce non-autonomous ventral antennal duplications. In this figure, the normal disc or antenna is marked by an arrow, while the duplicated structure is marked by an arrowhead. (A-E) Late L3 antennal discs containing *bowl*^{1} mutant clones, marked by the absence of GFP (green) signal. Only ventral clones cause duplicated PD axes. (A, A') The clone expresses the proximal PD axis marker *ct* and includes two novel *odd* rings, as detected with a specific antibody. (B, B') Two sibling discs containing *bowl*^{1} clones showing either a separate (left) or a bifurcated (right) domain of *Dll* expression. Ectopic *Dll* expression is seen both within and outside the *bowl*-mutant tissue, indicating a non-autonomous induction. (C, C') *bowl*^{1} clone showing a partial antennal duplication and the concomitant duplication of the *eyg* expression domain. (D,D') Ventral *bowl*^{1} clone spanning the *hh-Z* expression domain. The clone partially duplicates the *hh-Z* domain (outlined with the dashed line). A dorsal clone (asterisk) is normal. (E) *bowl*-mutant cells in the disc's anterior compartment (that includes the anterior stripe of *hh*-induced *ptc* expression) induces a non-autonomous antennal duplication involving also posterior cells. In (B, B') and (E,E') the white line marks the antennal duplication as detected morphologically to highlight its non-autonomous nature. (F-G) Adult heads containing *bowl*^{1} clones (unmarked) showing duplicated antennae. (H) Antenna containing *bowl*^{1}-mutant tissue on both sides of a normal a1-a2 joint. The *bowl*^{1}-mutant territory is marked by the *yellow* bristles (red arrowheads). (I) Antenna from a whole *bowl*-mutant head. All antennal bristles are marked with *f.* All joints are normal. In addition, there is a duplication of the a3 segment (one a3 is partly covered by the other a3 in this micrograph) and the arista (arrowhead).

The role of *bowl* in preventing the development of antennal supernumerary appendages is independent of homeotic information.

We have seen that *bowl* plays different functions in antennal and leg discs. If all developmental differences between antennae and legs were solely due to their differential expression of selector genes, we would then expect *bowl* to no longer be required to repress supernumerary antennal development if the antenna has been homeotically transformed into a leg-like appendage. Such a transformation can be achieved in gain-of-function mutations of *Antp* (Jorgensen and Garber, 1987; Schneuwly et al., 1987), in which the ectopic expression of *Antp* in the antennal disc results in the repression of one of the antennal selector genes, *hth* (Casares and Mann, 1998; Yao et al., 1999). Surprisingly, this is not the case. When ventral *bowl*$^{-}$ clones are induced in an *Antp*^{73b} background, which normally results in a variable, but usually close to complete, antenna-to-leg transformation (Kaufman et al., 1980; Schneuwly et al., 1987) we still observe supernumerary appendages (Figure 3A, B). In late L2 discs from *Antp*^{73b} we still detect Bowl expression in the ventral antennal disc (not shown), ruling out a direct regulation of *Antp* on *bowl.* Therefore, the antennal function of *bowl* reveals a cephalic-specific program that operates in the antennal disc independently of the final appendage identity assigned by homeotic gene expression. Interestingly, *eyg*, which is normally expressed in antennal but not in leg discs, remains to be expressed in *Antp*^{73b} antennal discs that are homeotically transformed into legs (Figure 3A). This is not due to low *Antp* expressivity, since the antennal-specific marker *dan* (Emerald et al., 2003; Suzanne et al., 2003) is repressed in sibling *Antp*^{73b} discs (not shown).

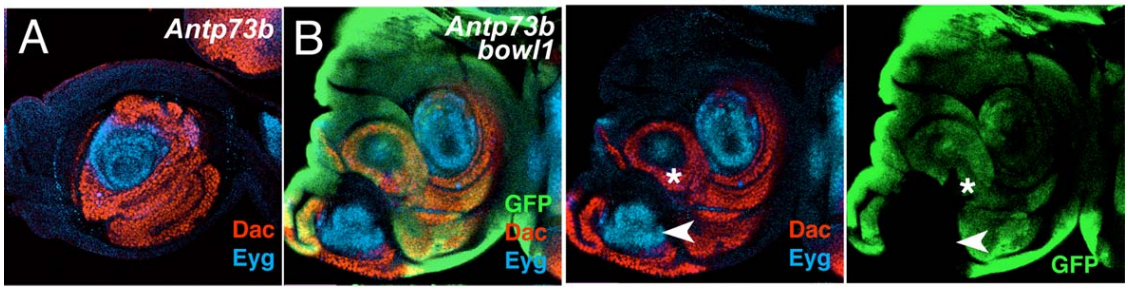

Figure 3. *bowl* clones result in antennal duplications in homeotically transformed *Antp* discs. (A,B) *Antp*^{73b} antennal discs stained for Dac and Eyg. The disc in (B) contains a *bowl*^{1} ventral clone (absence of GFP, green) that results in an ectopic ventral appendage (arrowhead) characterized by a medial domain of Dac (red) and a distal one of Eyg (blue). Strong and expanded Dac expression resembles Dac expression in legs. A second partial duplication can be seen in this disc (asterisk).

***bowl* is required to repress *wg* expression in the ventral antennal disc.**

The region where *bowl* clones produce antennal duplications, and which corresponds with *bowl*'s expression domain in early antennal discs, correlates with the realm of *dpp* signaling (Figures 1D,E and 4A, B). If the establishment of the PD axis in the antenna required maximal levels of both *wg* and *dpp* signaling, as it has been shown to happen in the leg disc (Lecuit and Cohen, 1997), then the duplications caused by *bowl*-mutant cells could be explained by the derepression of *wg* in the *dpp*-signaling region. We tested this prediction by analyzing *wg* expression, using either a reporter of *wg* transcription or an anti-Wg antibody, in *bowl*-mutant cell clones. All bona fide *wg* transcriptional reporters, such as wg^{en11} or *wg*-GAL4, are insertional enhancer traps that are, in addition, mutant for *wg* (Fly Base). We then used the *wg2.4-* regulatory construct reporter, which recapitulates most of the *wg* expression domain (Pereira et al., 2006) (compare Figure 4B and C). $bowl^{-}$ clones in the ventral antenna derepress *wg* transcription and protein production (Figure 4C, D). Wg signal can be detected in some wild type cells surrounding the *bowl-* clones, likely due to the diffusible nature of the Wg protein. Dorsally-located clones (that is, in the *wg* territory) do not have any effect. The cause of the duplications associated with *bowl*-mutant clones seems to be the derepression of *wg*, as the sole expression of a constitutively active form of the *wg* signal transducer Armadillo (Arm^{S10}) in the ventral antenna, either in cell clones or driven by a *dpp*-GAL4 line, causes similar antennal duplications (Figure 4E,F). These results indicate that, during normal development, the ventral expression of *bowl* is required for repressing the establishment of a supernumerary appendage in the antennal disc by preventing the ventral misexpression of *wg*. In contrast, $bowl^{-}$ clones induced simultaneously in the leg discs do not result in *wg* derepression, but cause abnormal disc folds (not shown), which prefigure the joint defects reported earlier (de Celis Ibeas and Bray, 2003; Hao et al., 2003).

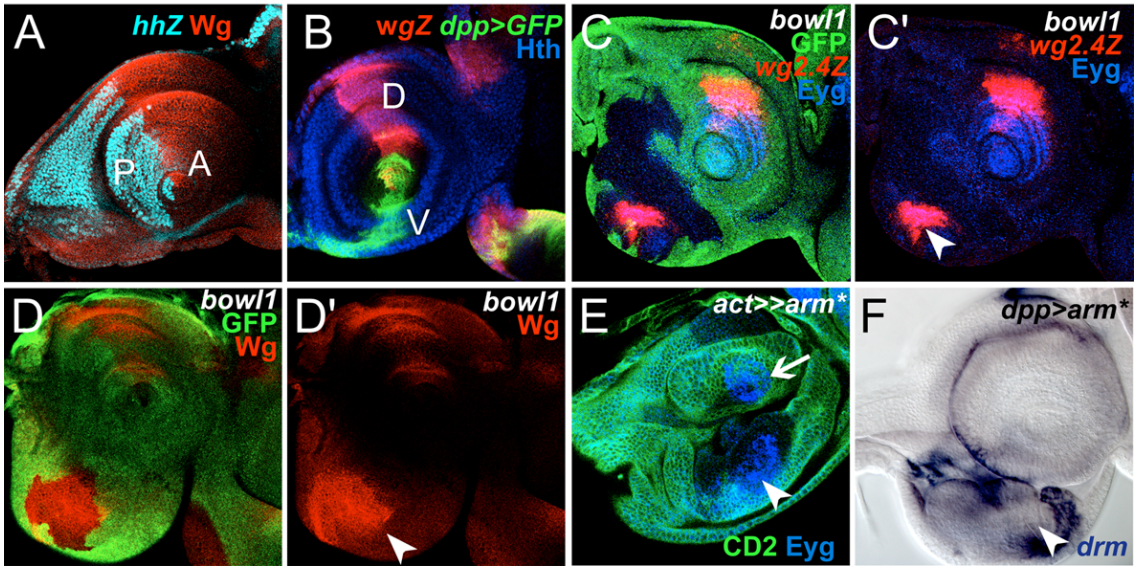

Figure 4. *bowl* mutant cells derepress *wg* expression in the ventral antenna. Late L3 antennal discs are shown. (A) *hh-Z* eye antennal disc. *hh* is expressed in posterior cells ("P", blue), and Wg protein (red) is detected in a dorsal wedge in anterior ("A") cells. (B) *wgZ; dpp*-GAL4/UAS-GFP antennal disc. *wg* and *dpp* are transcribed in a dorsal ("D") and a ventral ("V") anterior stripe, respectively, overlapping in the disc's center, which corresponds to the distal-most tip of the prospective antenna (yellow signal). Hth marks the proximal and medial PD axis territories. In the antennal disc the *wg* expression domain is designated "dorsal" (D) and the *dpp*-expressing one, "ventral" (V), to make it concordant with the D and V territories of the eye disc. This nomenclature results in an apparent inversion of the DV domains relative to the leg, where the *wg* and *dpp* domains are assigned V and D identity, respectively. (C, D) Antennal discs containing *bowl1*-mutant clones (marked by the absence of GFP, green). Both *wg* transcription, monitored by the *wg* reporter *wg2.4Z* (C, C'), and Wg protein (D, D') are derepressed in ventral clones (arrowheads). These clones contain duplicated medialdistal structures (marked by Eyg: C, C'). (E) Flip-out clones expressing Arm^{S10} (arm*) (marked by the lack of CD2, green) induce antennal duplications in the ventral disc (arrowhead). Eyg (blue) marks antennal medial-distal domains. The normal antennal primordium is marked by an arrow. (F) *dpp*-GAL4>Arm^{S10} L3 antennal disc. *drm* is detected by in situ hybridization. A new set of *drm* rings is observed (arrow) in a morphologically duplicated antenna (arrowhead).

bowl is insufficient to prevent *wg* misexpression in the absence of *dpp* signaling.

It has been previously shown for leg discs that the establishment of the opposing wedges of *wg* and *dpp* expression depends on their mutual repression (Brook et al., 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Theisen et al., 1996). We verified that this paradigm holds true during antennal disc development, because the loss of *dpp* signaling in ventral clones mutant for the *dpp* receptor *thick veins (tkv)* result also in *wg* derepression (Figure 5A). Therefore, one possible mechanism to explain the *wg* derepression caused by loss of *bowl* is if *dpp* expression and/or signaling were dependent on *bowl* function. We tested this point by inducing *bowl*$^-$ clones in a *dpp*-Z background. In *bowl*$^-$ clones that cause

antennal duplication, as monitored by ectopic domains of the distal antennal marker *eyg*, *dpp*-Z expression is still detected within the mutant tissue (Figure 5B), suggesting that *bowl* is not required for *dpp* expression. Still, it might be that, even though *dpp* continues to be expressed, the Dpp signal is not properly transduced in *bowl*-mutant cells. This seems not to be the case, as *bowl*- cells express phosphorylated-Mad at similar levels as their wild type neighboring cells do (Figure 5C). The fact that the domains of *bowl* transcription and P-Mad expression are similar (Figure 1) might indicate that the *dpp* signaling pathway controls *bowl* expression. In order to test this point, we ectopically activated the *dpp* pathway by driving a constitutively active form of *tkv* with *ptc-GAL4.* In these discs we detect ectopic *bowl* transcription in the dorsal antenna (Fig. 5D). Therefore, these results place *bowl* transcription downstream of *dpp.* Since the *dpp* pathway represses *wg* transcription and *bowl* expression seemed to lie downstream of *dpp,* we asked next if *bowl* was sufficient to repress *wg*, even in the absence of *dpp* signaling. We performed two experiments to answer this question. First, using the *ptc-GAL4* line, we drove *bowl* expression in the *wg* domain. In these discs, the ectopic Bowl protein was detected in the nuclei and at high levels, and yet *wg* expression remained unaltered (Fig. 5E). Second, we induced *bowl* expression in cell clones simultaneously mutant for the *dpp* receptor *tkv.* In ventral $bowl^{+}$ tkv^{-} clones *wg* was derepressed as in tkv^{-} clones (Fig. 5A, F). Therefore, both experiments indicated that *bowl,* albeit required, is not sufficient to repress *wg* in the antennal disc. One possibility to explain this insufficiency was that the experimentally induced *bowl* levels were not enough to overcome the inhibitory function of endogenous *Lines.* To counteract this possible inhibition, we drove *drm,* which is capable of inducing high levels of nuclear Bowl when expressed ectopically in antennal discs and elsewhere (not shown) and outcompetes Lines in binding to Bowl (Hatini et al., 2005), in tkv^{-} clones. In these drm^{+} tkv^{-} cells, *wg* was still derepressed (Fig. 5G). These experiments rule out the lack of nuclear localization of Bowl or its functional inhibition by Lines as simple explanations for the insufficiency of *bowl* to repress *wg* in the absence of *dpp* signaling.

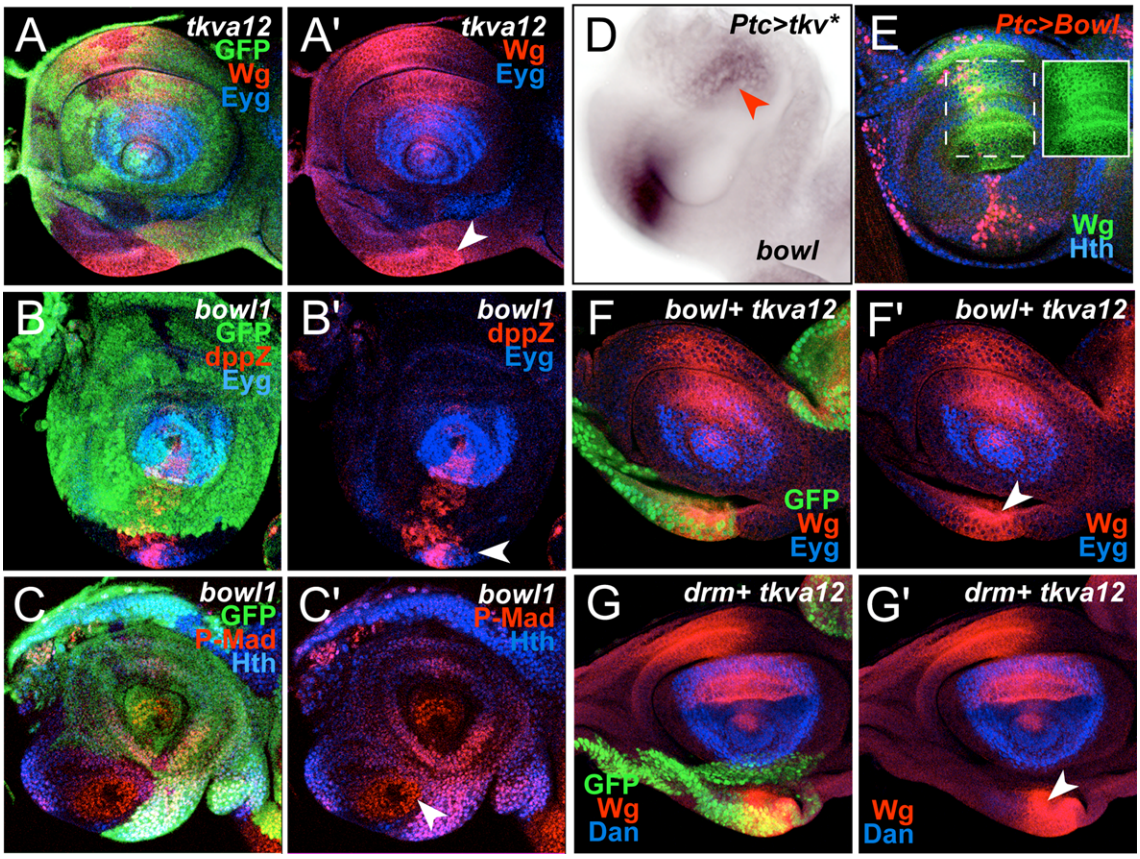

Figure 5. *bowl* acts downstream of the *dpp* signaling pathway to repress *wg*. (A,A') *tkv*^{a12} clones (marked by the absence of GFP, green). In ventral (arrowhead), but not dorsal, clones Wg becomes derepressed, similarly as in *bowl* clones (Figure 4). (B-C) *bowl*^{1} clones (marked by the absence of GFP, green). In (B,B'), *dpp* expression, monitored by a *dppZ* reporter, is still detected within the clone (arrowhead), which is forming a new PD axis [as indicated by a new domain of *eyg* expression (blue)]. (C, C') In a similar clone, the active form of the Dpp signal transducer Mad (P-Mad) is detected at normal levels (arrowhead). Hth is included as a counter stain. (D) Antennal disc from a *ptc-GAL4; UAS-tkv*^{QD} *(tkv*)* late L3 larva, hybridized with a *bowl* anti-sense RNA probe. Ectopic *bowl* expression is detected in the dorsal antenna (arrowhead). (E) *ptc-GAL4; UAS-bowl* antennal disc. Bowl protein (red) is strongly detected in the nuclei of cells along the AP axis. In the dorsal disc, Bowl overlaps the Wg domain (green). The inset shows the Wg expression of the region boxed with dashed lines, which is not affected. Hth is included as a counterstain. (F, F') Ventral antenna *bowl*-expressing *tkv*^{a12} clone (GFP). Wg (red) is derepressed within the clone. (G, G') Ventral antenna *drm*-expressing *tkv*^{a12} clone (GFP). Wg (red) is derepressed within the clone. In F and G *dan* (blue) marks the distal antenna.

Bowl is also required for the autonomous repression of eye fate in a small domain of the eye antennal disc.

Some *bowl*$^{-}$ clones result in the autonomous de-repression of the retinal determination gene *eyes absent* (Bonini et al., 1993; Voas and Rebay, 2004) and the differentiation of the mutant patch as *elav*-positive photoreceptors (Figure 6B). Frequently, adults carrying *bowl*-clones show ventral eyelets (Figure 6C), which likely derive from the *eya*, *elav*-positive patches we observe in the discs. This effect is seen only when the clones affect the ventral-posterior rim of the antennal disc

lobe (Figure 6), close to the stem that joins the antennal to the eye disc. We analyzed this "eye-sensitized" region in detail and found that it co-expresses both the antennal marker *ct* (Kenyon et al., 2003) as well as the eye selector gene *ey* (Halder et al., 1998) (Figure 6A).

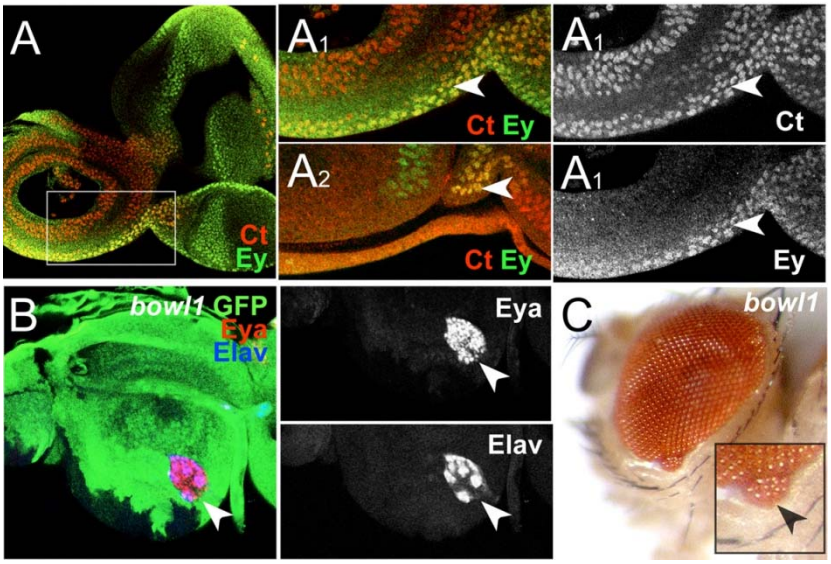

Figure 6. *bowl* silences eye development autonomously in a small ventral domain of the eye-antennal disc. (A) Late L3 eye-antennal disc co-stained for Ct and Ey. The confocal section is focused on the basal side of the disc that corresponds to the most proximal segments of the antennal disc. Ey signal can be detected in the anterior domain of the eye disc, although not as strongly as in more apical sections. (A1) and (A2) are two confocal sections corresponding to the area outlined in (A). Ct and Ey coexpression is seen in yellow (arrowheads). Unmerged channels are also shown. (B) Antennal disc containing *bowl*^{1} clones. Mutant cells in the posterior clone (arrowhead) autonomously derepress *eya* expression and differentiate as Elav-expressing photoreceptors. (C) Adult head of an individual carrying *bowl*^{1} clones. An eyelet develops ventral and adjacent to the normal eye, shown at higher magnification in the inset (arrowhead).

In this region, *bowl* is required autonomously to repress eye identity and to allow ventral head capsule development instead. Interestingly, when the *bowl* inhibitor Lines (Hatini et al., 2005) is expressed in the ventral antennal disc, using the *dpp-GAL4* driver, either of two phenotypes could be detected, both in discs and in pharate adults: antennal duplication (Fig. 7A,C) or formation of ventral eyelets in the head capsule (Fig. 7B,D). These results reinforce the idea of a specific requirement of *bowl* in the ventral antennal disc.

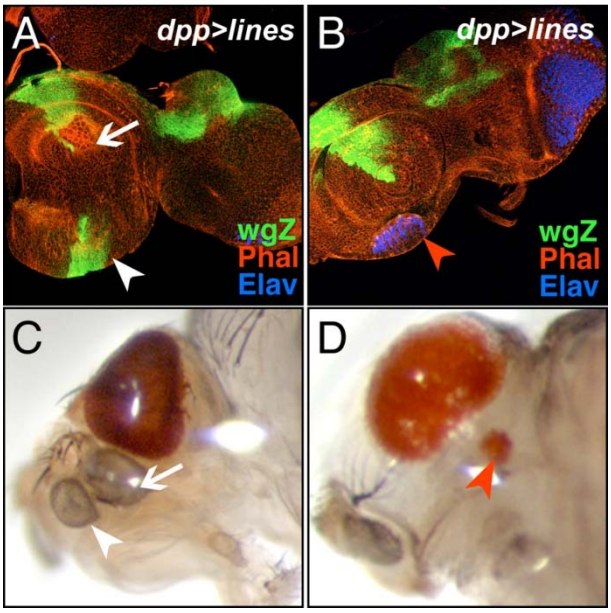

Figure 7. Ventral expression of *Lines* causes either antennal duplication or ventral ectopic eyelets. Eye-antennal imaginal discs (A, B) or adult heads (C,D) of *dpp-GAL4; UAS-lines* individuals. The *wg-Z* transgene has been introduced in the genotype to monitor *wg* transcription. (A) Duplicated antennal disc, detected morphologically (arrowhead), with an ectopic *wg-Z* wedge. The endogenous antennal disc is marked with an arrow. (B) Disc of the same genotype showing a ventral cluster of Elav-positive photoreceptors (red arrowhead). The discs are counterstained with rhodaminephalloidin that marks cell outlines. *dpp-GAL4; UAS-lines* flies show either duplicated antennae (C, arrowhead; arrow marks the normal appendage) or ventral eyelet (D, red arrowhead).

DISCUSSION

During the development of the antennal disc, *bowl* has two phases of expression: an early expression in the ventral disc, required to maintain *wg* repressed, and a later one in concentric rings. Both phases have antennal-specific properties. The early *bowl* expression and function is unique to the antenna. And the expression in rings associated to prospective joints, and which recapitulates the ring expression in leg discs, does not seem required for joint formation in the antenna, contrary to the legs. In addition, *bowl* is still required to repress a ventral supernumerary PD axis even if the antenna has been homeotically transformed into a leg-like appendage by overexpression of the leg selector *Antp*. All these results indicate that the development of the head structures deriving from the antennal disc depends not only on the activity of selector genes, but also on a cephalic-specific genetic program. Supporting this claim, we find that the expression of *eyg*, an antennal-specific marker, is maintained in homeotically transformed antennal discs.

These cephalic vs. thoracic differences might reflect the very different developmental histories of antennal and leg discs. While each leg disc primordium is formed from cells derived from just two adjacent parasegments (or one embryonic segment), the antennal disc is part of a composite disc, the eye-antennal disc, which forms by the fusion of imaginal primordia derived from several embryonic head segments [the labial, antennal, intercalary, mandibular and maxillary segments plus the unsegmented acron (Cohen, 1993; Jurgens and Hartenstein, 1993)]. The origin of the ectopic ventral antennae that form in the

absence of *bowl* can be explained in two, non-mutually exclusive, ways. First, the new antennal primordium could result from the bifurcations of the normal one. We have noticed that the more proximal the clone, the more complete the duplicated appendage, as detected by PD markers such as *eyg*, *dac* or *odd.* This observation agrees with a model in which the developing appendage re-specifies more or fewer positional values depending on whether the ectopic source of *wg* is generated farther or closer, respectively, to the endogenous domain (Bryant, 1993). A second possibility, though, is that in some cases the ectopic antenna derives from a "silent" primordium contained within the disc and normally suppressed by *bowl,* especially in cases when the ectopic antenna is clearly spatially separated from the normal one, as in (Figure 2E). The hypothesis that the ectopic antenna might derive from a non-antennal primordium is supported by a number of facts: first, *bowl*- clones give rise in many instances not to a bifurcated antenna, but to a fully duplicated antennae, leaving the endogenous one intact. In these cases, the maxillary palp is also normal. Therefore, the new antenna does not develop at the expense of neither of these appendage types; second, the duplicated antennae originate from the proximal-most region of the ventral antennal disc. This region might contain, in addition to antennal segment derivatives, imaginal cells coming from the mandibular and intercalary segments (Jurgens and Hartenstein, 1993). In fact, the intercalary segment, very reduced in insects, is thought to be homologous to a second antennal-bearing segment, present in extant crustacea and likely ancestral in the hypothetical mandibulate ancestor (Abzhanov and Kaufman, 1999; Telford and Thomas, 1998). In this scenario, loss of *bowl* would result in the expression of *wg* and the generation of a new PD axis from one of these reduced, appendage-less, head primordia. That the resulting appendage is an "antenna" could be the result of the selector information available –i.e. *ct* and *hth* expression- on top of the de novo induction of PD axis determinants, such as *Dll*. A similar situation has been described in the case of labial palps which, if devoid of *pb* and *Scr* Hox selector information, develop as antennae (Joulia et al., 2006).

The putative "silencing" function of *bowl* might extend to other parts of the eye-antennal disc. *bowl*$^{-}$ clones in the ventral region of the stem that connects the antennal and eye disc lobes develop autonomously into eye tissue. In contrast to the antennal suppressing function, *bowl* is required autonomously to repress eye development. This autonomy indicates that either the signals normally operating to spread retinal differentiation in the normal eye (Treisman and Heberlein, 1998) are not produced in these ectopic retinal patches, or that the wild type tissue is refractory to these signals. At present, we cannot favor either of the two hypotheses. We have noticed, however, that the overexpression of the *bowl*

inhibitor Lines driven by the *dpp*-GAL4 driver leads to two phenotypic outcomes: antennal duplication or ectopic ventral eyelet. Interestingly, only in one case out of more than 20 discs analyzed these two phenotypes co-occurred. This suggests that the cells in the sensitive region adopt collectively only one of either fate, antenna or eye, and that deciding upon one excludes the other. In addition, we note that this *ct, ey*-expressing region is particularly prone to develop into eye upon genetic perturbations. For example, it is this region that is preferentially transformed into eye when *hth* function is removed (Pai et al., 1998; Pichaud and Casares, 2000) or when *tsh* is ectopically expressed (Bessa and Casares, 2005; Pan and Rubin, 1998). Perhaps, the unique fact that this region co-expresses antennal and eye determinants makes its fate more ambiguous. In the absence of *bowl, hth* might tilt the equilibrium towards head capsule or antennal development, while the opposite fate –eye- would be adopted in the presence of *tsh* and *ey.* It will be interesting to determine whether functional relationships between *bowl* and these factors exist to determine specific fates within the eye disc.

Mechanistically, *bowl* function seems to lie downstream of *hh* and *dpp*. In *bowl*- cells associated with an antennal duplication, *hh* is still expressed (Figure 3D) and the Hh coreceptor *patched* is normally up-regulated in anterior cells abutting the *hh*-expressing domain (Figure 3E), which indicates correct *hh*-signaling (Capdevila et al., 1994; Ingham et al., 1991). Accordingly, *wg* derepression in *bowl*- cells occurs closest to the P cells, as expected for a *hh* target gene. In the embryo, *bowl* has also been placed downstream of *hh* during the process of epidermal differentiation (Hatini et al., 2005).

In the antenna, as in the leg disc, the *dpp* and *wg* signaling pathways repress each other to establish two opposing wedges of *dpp* and *wg* expression (Johnston and Schubiger, 1996; Theisen et al., 1996). In *bowl*-clones, though, *dpp* expression, monitored by a *lacZ*-expressing reporter, is not turned off. Although this might be due to the perdurance of the LacZ product, *bowl*- cells accumulate normal levels of phosphorylated-Mad. This indicates that *bowl*-mutant cells transduce the *dpp* signal, regardless of whether *dpp* is expressed or not. Therefore, it seems that in the antennal disc, *bowl* is required to prevent the inappropriate expression of *wg* in the *dpp* domain even if the *dpp* pathway is still active. Nevertheless, *bowl* is not sufficient to repress *wg.* We have ruled out simple explanations for this fact, such as low levels of the induced Bowl protein, or its retention in the cytoplasm. We also show that this insufficiency is not due to the inhibition by Lines, because even in the presence of Drm, which prevents Lines from binding to Bowl, this latter is still unable to repress *wg.* Although further work is required to identify which other factor or factors collaborate with *bowl* during ventral antennal disc development,

one possibility is that this factor may be the *dpp* signaling itself. This is because *bowl* cannot block the ectopic *wg* expression in ventral antennal cells devoid of *dpp* signal. We have shown that *bowl* transcription roughly coincides with the domain of maximal *dpp*-signaling is the antennal disc, as visualized by P-Mad, and that *dpp* signaling can activate *bowl* transcription in this disc. These results suggest that high levels of *dpp* induce *bowl* which, in turn, is required to prevent inappropriate expression of *wg* in the antennal disc together with the *dpp* pathway. Two are the likely sources of Dpp: the wedge of *dpp* that can be visualized using the *dpp*-disc enhancer reporters (Blackman et al., 1991) in the antenna, and a ventral disc expression that is controlled by a separate enhancer (Stultz et al., 2006). This enhancer drives *dpp* expression in the prospective ventral head region (Stultz et al., 2006), close to the region where *bowl* is transcribed in early discs (not shown).

bowl and the related genes *odd* and *drm* show a late pattern of expression in rings, similar to the one deployed in leg discs. But contrary to their requirement for leg segmentation, *bowl* seems to be dispensable for antennal segmentation. A similar situation has been described for the gene *dachshund* (*dac*). *dac* is expressed in the medial segment of both leg and antennal discs, but while loss of *dac* in the leg leads to the loss of intermediate adult leg structures, the antenna develops normally (Dong et al., 2001; Mardon et al., 1994). These results might reflect the fact that, although antennal and leg discs have specific developmental programs, the mechanisms for generating the PD axis are shared by both appendages. This mechanism would call a similar battery of genes, even if only a subset of them is effectively used for the development of each appendage.

In summary, our results show that the zinc-finger encoding gene *bowl* is part of a cephalic-specific program that represses appendage formation in the ventral eye-antennal disc. Here, *bowl* is required to repress *wg*, downstream of *dpp*, to prevent the generation of supernumerary antennae. These extra appendages might arise from some silenced primordium in the proximal part of the antenna, which would be normally fated to become part of the head capsule. In addition, *bowl* also silences the development into eye of another cell population of the prospective head that presents mixed expression of antenna and eye selector genes. The silencing of appendage development might have been essential for the coalescence of cells deriving from several different embryonic cephalic segments into a single imaginal disc, as well as for the formation of the head structures of adult cyclorraphan flies, such as *Drosophila.*

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General discussion

In this thesis, through the study of the functions of the *odd* family genes, I have investigated processes such as tissue specification, signaling and patterning. Pattern organ formation depends on the correct coordination between proliferation and differentiation. If any of these parameters is affected the resulting pattern could result aberrant. Thus, the development of an organ can be disrupted if cells proliferate more or less than normal, if cells are wrongly specified and as a consequence differentiate incorrectly, or if both of these aspects are impaired. The different studies we performed reflect how important the precise control of these aspects is in order to ensure normal organ development:

1- The initiation of retinal development in *Drosophila* is an inductive process, in which a specialized signalling center, the *posterior margin*, secretes the inducer molecules, most notably *hh,* to trigger retinal differentiation and patterning in the adjacent eye-competent cells. In this thesis I show that the differential expression and activity of *odd* family genes is required to specify this signalling center. *odd* genes function through a genetic cassette that is deployed in several other developmental contexts during *Drosophila* development. Key to the coordination of retinal differentiation is the spatial restriction of the signalling center through the regulated expression of *odd* genes. Here I show that *wg* is required for the anterior repression of *odd* genes along the eye disc margin. (Chapter I).

2- The antenna is a highly derived ventral limb. As such, it is believed that it shares most basic patterning mechanisms with other limbs, such as walking legs, despite their anatomical and functional differences. One of these mechanisms is proximodistal (PD) axis establishment, which is responsible for the patterning and growth of limbs. Hox selector genes would impinge upon the shared generalized limb-forming genetic program to confer each limb type its specific traits. Here I show that *bowl* is required for repressing *wg* specifically in the ventral antennal disc. If *bowl* is removed, the *wg* derepression is likely the cause of the formation of an ectopic PD axis and the development of an extranumerary antenna. I also argue that this antennal specific role of *bowl* might be indicative of basic differences between cephalic and thoracic limb developmental programs. I further discuss that the extranumerary antennae formed in the absence of *bowl* might have two origins within the antennal disc: a new *wg* maximum in the *dpp*-expressing domain and, thus, a new PD axis, or the "derepression" of a

normally silent limb primordium that would be part of the ventral antennal disc, possibly an intercalary segment. (Chapter II).

Margin cells behave as a signaling center, being responsible of the expression of the diffusive signals required for the induction of retinal differentiation within the eye field (Treisman and Heberlein, 1998). Which are the gene(s) required for the functional specification of the margin as a signaling center?

In Chapter I, we present a genetic mechanism taking place along the margin that drives the key expression of *hh*. *odd* genes are specifically expressed in the PE and margin cells and act as 'margin specification genes' that are responsible for *hh* expression. Margin cells are specialized PE cells that give rise to the adult head capsule that surrounds the eye, the posterior head capsule (Figure 4, Introduction). In addition, we demonstrate genetically that they work in a cassette Drm/Lin/Bowl that was before described to function in the patterning of the embryo's epidermis and gut (Hatini et al., 2005; Johansen et al., 2003). In the presence of Drm or Odd, which seem to act redundantly, Lin, Bowl´s inhibitor, is blocked and Bowl can regulate the transcription of downstream genes. In the eye margin, we propose that *bowl* is required for the activation of *hh* expression. Clearly, *wg* controls the functional specification of the margin that is under the control of *odd* family genes. *wg* is required to prevent premature expression of *drm* (and presumably of *odd)* in anterior lateral margins of the disc, which otherwise correlates with premature retinal differentiation triggered from lateral and anterior regions. *lin* could be, together with *wg,* involved in restricting the ability of the margin to signal, since it has been proposed that Lin acts in parallel or downstream of the Wg signaling pathway (Hatini et al., 2000). Moreover, we have observed that ectopic expression of *lin*, using a margin driver, represses *odd* and *drm* transcription and as a consequence the eye does not develop (Figure 1A and B). This suggests that *lin* is involved in restricting the domain of expression of *odd* genes.

As mentioned before, the fact that *wg* and *drm/odd* expression patterns are mutually exclusive, and the removal of *wg* from the margin leads to anterior expansion of *drm,* points to *wg* as *drm/odd* repressor (Figure 4, Chapter I). However, ectopic expression of a constitutively activated form of the Wg effector, *armadillo* (*arm**), in clones is not sufficient to repress *drm/odd* expression (Figure 1D and E). Still, when driven with a margin driver (*dpp* driver), *arm** causes the downregulation of *odd* genes, but not their complete repression (Figure 1C). This raises several possibilities: that *wg* represses *odd* genes through non-canonical pathways or that other factors or signals yet unidentified are required to repress *odd/drm* along the margin. On the other hand, ectopic expression of *drm* or *odd*

ahead of the MF causes the activation, not the repression, of *wg* in the eye (Figure 1F and G) and wing discs, which results in flies with ectopic bristles in the head and in the notum. The results indicate that, the regulatory relationship between *wg* and *odd* genes still needs further investigation.

Once the expression of *hh* is activated, a positive feedback loop is established, where *hh* is responsible for the maintenance of *odd* expression. Indeed, ectopic expression of a form of *ptc* that acts as a constitutive repressor of the *hh* pathway *($ptc^{\Delta loop2}$) (Briscoe et al., 2001)* downregulates *odd* expression (Figure 1I). Supporting this idea, in embryonic epidermis, it was described that Hh promotes *odd* genes expression (Hatini et al., 2005).

Probably Odd and Bowl, that harbour an eh1 domain that recruits the transcriptional co-repressor Groucho (Goldstein et al., 2005), regulate *hh* expression indirectly by repressing a *hh* inhibitor.

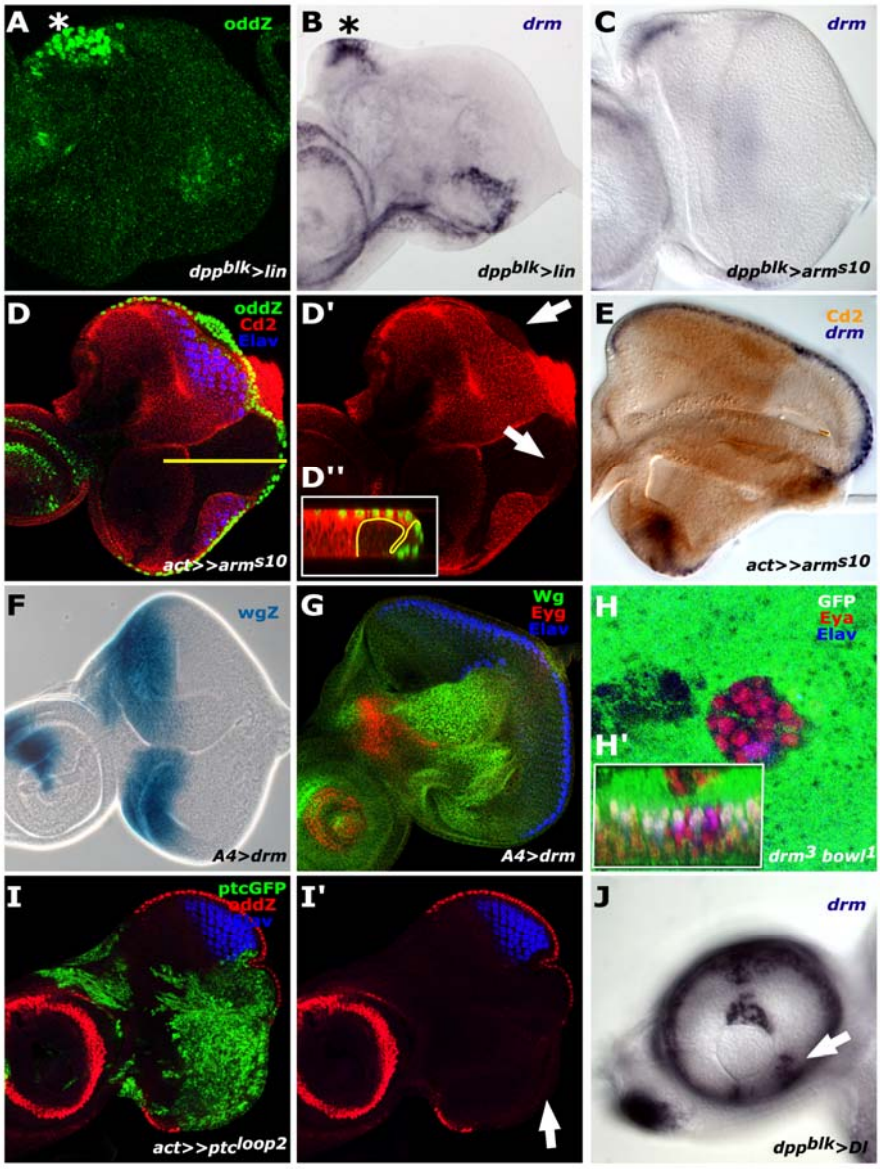

Figure 1. Supplementary data. *odd* and *drm* transcriptions are detected using a lacZ reporter or by *in situ* hybridization, respectively. (A, B) Ectopic expression of *lin*, using the dpp^{blk} driver, blocks both *odd* (*oddZ* reporter, A) and *drm* (*in situ* hybridization, B) transcriptions along the posterior margin. Their late expression in the ocelli region (asterisk) functions as an internal control, since the dpp^{blk} driver is not expressed in this region. (C) Ectopic expression of a constitutively activated form of

the Wg effector, *arm* (*arm**), driven by dpp^{blk} downregulates the levels of *drm* transcription. (D, E) In clones, the constitutive activation of *wg* pathway is not sufficient to regulate *odd* and *drm* transcription. (D) Ectopic expression of *arm** (negative cells for Cd2) blocks retinogenesis in the ME, as expected, but not *odd* transcription along the margin. Mutant posterior margin still expresses *odd* (arrows, D'). A cross-section along the yellow line in D (D'') shows an *arm** clone that comprises the margin and that does not affect *odd* expression. (E) Ectopic expression of *arm** (Cd2negative cells) does not affect *drm* transcription along the margin. (F, G) *drm* ectopic expression ahead of the MF causes the formation of folds and activates wg transcription (X-Gal staining using a wgZ reporter, F), which results in the accumulation of Wg protein (green, G). (H) Removal of *drm* and *bowl*, which is equivalent to only *bowl* loss of function, from the PE causes the activation of *eya* (red) with concomitant development of photoreceptors (blue, neuronal marker, Elav). Mutant cells are negative for GFP. A cross-section along the clone (H') reveals the change of morphology of the upper PE within the *eya*-positive mutant clone. Similar results were obtained in *bowl*$^{-}$ clones. (I) Ectopic expression of an Hh-insensitive form of *ptc* (GFP-positive cells) blocks photoreceptor differentiation (blue, Elav) and inhibits *odd* transcription in the margin (arrow, I'). (J) Ectopic expression of the Notch ligand, *Dl*, in the ventral antennal disc (dpp^{blk} driver) activates *drm* expression (arrow).

One important aspect of organogenesis is the separation of "labor" between cell populations. In the eye disc, PE and margin cells express *odd* genes and in this manner, they become competent to trigger eye development in the adjacent discs cells, although they are refractory themselves to those signals. Are *odd* genes somehow involved in conferring PE cells that refractoriness? *tsh* is known to coordinate eye development and to be sufficient to induce ectopic eyes in the PE (Bessa and Casares, 2005). *tsh* expression starts during L2 and restricted to the ME, although no gene has been described to regulate its expression. Curiously, removal of *bowl* from the PE results in the expression of the RDGN gene, *eya*, with the subsequent development of photoreceptor cells (Figure 1H). Therefore, the initiation of *tsh* expression during L2 could be restricted to the ME due to the presence of *odd* genes in the PE. In this way, *odd* genes could be indirectly responsible of the specification of the eye domain. If *odd* genes were involved, directly or indirectly, in restricting where within the eye disc *tsh* expression starts, this would be an eye-specific function, because, for example, in the wing disc *odd, drm* and *tsh* are all co-expressed in the PE of the wing disc (Wu and Cohen, 2002) (data not shown). Still a repressor function of *odd/drm/bowl* on *tsh* in the PE has to be demonstrated.

The cuboidal morphology of margin cells allows the transition between PE squamous and ME columnar epithelia. *odd* genes that are expressed in the PE (PE itself and the specialized PE, the margin) might be involved in the control of cell morphology or organization. This function of *drm* could be independent of their action on *bowl: drm*$^{+}$ *bowl*$^{-}$ clones in the eye disc, although incapable of inducing eyes, induce dramatic changes in the morphology of the epithelium with the

formation of folds (Figure 3F, Chapter I). Indeed, in leg discs and in the gut, *odd* genes have been implicated in cell morphology control. Ectopic expression of *odd*, *sob* and *drm* induce invaginations in leg discs, which in adult legs results in the development of ectopic joints (de Celis Ibeas and Bray, 2003; Hao et al., 2003). Moreover, removal of *drm* or *bowl* causes shorter and wider hindguts, due to defects in cell arrangement (Iwaki et al., 2001).

Despite the fact that leg and antenna are homologous ventral appendages, both are dramatically different in structure and function. The antenna is composed of three segments and the arista, and serves as olfaction and audition organ, while the leg is formed by ten segments, and has a locomotory function. Are the mechanisms operating during antennal development the same of in the leg?

The signals involved in the patterning of leg and antenna appear to be identical (Brook and Cohen, 1996; Campbell, 1995; Lecuit and Cohen, 1997). The shared use of the Hh/Dpp/Wg pathways suggests that the PD axis is constructed in a very similarly way. However, there are significant differences in the way their PD axes are subdivided. Genes regulated by Dpp and Wg like *Dll*, *hth* and *dac* show different relative patterns in the antennal and leg discs (Dong et al., 2001). Our results reveal a molecular mechanism involved in antennal patterning that has been also shown to operate in the segmentation of the leg (de Celis Ibeas and Bray, 2003; Hao et al., 2003). In chapter II, we showed that the molecular machinery involved in the establishment of the DV subdivision differ from antenna to leg. Our work demonstrated that, in the antenna, *bowl* is required for the correct DV patterning and subsequently the proper establishment of a single PD axis. Accordingly, *bowl* and the other members of the family are expressed early in the ventral region of the antennal disc and seem to be required to guarantee the repression of *wg* in this domain. *bowl* mutant clones de-repress *wg* but do not affect the levels of phosphorylated Mad (pMad). Thus, the de-repression of *wg* creates a new point where the high levels of Wg and Dpp signaling originate an ectopic PD axis. Moreover, our work shows that Dpp signal activates the *odd* family genes: ectopic expression of a constitutively activated form of the Dpp receptor, *thickveins* (*tkv*), induces *drm* expression. Our data fits with a model where the *odd* genes are activated by Dpp signaling and, at same time, are required downstream this pathway to avoid the misexpression of *wg* in the dorsal antenna.

Furthermore, axis duplication caused by removal of *bowl* is independent of the homeotic transformation of the antenna by *Antp,* known to block the acquisition of the antennal fate by repressing genes essential for its identity, like *hth*. In *Antp*-expressing antennae, that develop as leg–like appendages, the same duplication of

appendage occurs if *bowl* function is removed, meaning that the role of *bowl* is independent of the homeotic genes-induced context. In addition, the *Antp*-transformed antenna retains the expression of antennal specific genes, like *eyg* and *dan* (Figure 3A, Chapter II). Altogether, this suggests that there are a number of gene regulatory steps in the antennal disc that are Hox selector independent and whose control seems to rely on a cephalic specific genetic program.

Another crucial difference between leg and antennal discs are their developmental origin. The leg disc derives from a single embryonic thoracic segment (Cohen et al., 1993), while the eye-antennal disc complex derives from several embryonic head segments (Younossi-Hartenstein, 1993). This begs the question of how these different cell groups coalesce and coordinate their development in the eye-antennal imaginal disc? The composite nature of the disc implies fusion of many segments. But in *Drosophila*, as characteristic of Diptera, the head is very simplified. Other insects have a more complex head structure, in which the embryonic primordia contribute with conspicuous structures– many harbouring appendages- to the adult head (Snodgrass, 1935). Nevertheless, these insects usually are more direct developers (not using imaginal primordia as developmental intermediaries between embryo and adult) and therefore were not "faced" with the necessity to fuse their several head primordia in a single imaginal disc. Multiple primordia within a single epithelial sac are, in principle, exposed to a milieu of signals and yet, they must retain certain independence. Perhaps, this integrated mode of development required some primordia to be suppressed and thus avoid developmental interference. Based on this argument, another possibility arises to explain our results. Taking in account the described role for *bowl* in repressing eye fate in the 'neck' region of the imaginal disc, characterized by the coexpression of *ey* and *ct* (Figure 6, Chapter II), at least some of the ectopic antennae originated by the removal of *bowl* could represent the development of a new appendage instead of a duplication of the antenna. In this hypothesis, *bowl* would function by maintaining silenced the development of other appendages in those cells coming from embryonic segments that do not result in conspicuous structures.

In the leg, *odd* family genes were already known to be required downstream of Notch function in segmentation. Notch activates *odd* family genes in cells adjacent to those where the joint is going to be formed (de Celis Ibeas and Bray, 2003; Hao et al., 2003). This raises the question of whether, as in the case of leg segmentation, *odd* genes are also required for antennal segmentation in addition to their role in early antennal development? We showed that despite the fact that *odd* genes are expressed in rings in the disc and fate map to some of the antennal joints (Figure 11G and I, Introduction) they are not required for antennal

segmentation. The relationship to the Notch pathway is not clear either. While in the legs *Serrate* (*Ser*) and *Delta* (*Dl*) are coexpressed in rings abutting the *odd*-expressing prospective joints, *Ser* and *Dl* are not co-expressed in the antennal disc (Casares and Mann, 2001). Nevertheless, the fact that *Dl* expression is sufficient to induce *odd/drm* in antenna (Figure 1J) might indicate that *Dl/Ser* co-expression is not absolutely necessary for Notch signaling and the concomitant activation of *odd* genes' expression. Still, the fact that joint-associated *odd/drm/bowl* expression is not involved in segmentation could be a reflection of the shared developmental program with the leg discs.

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Appendix

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Odd-skipped genes encode repressors that control kidney development

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Abstract

Odd-skipped family of proteins (Odd in *Drosophila* and *Osr* in vertebrates) are evolutionarily conserved zinc finger transcription factors. Two *Osr* genes are present in mammalian genomes, and it was recently reported that *Osr1*, but not *Osr2*, is required for murine kidney development. Here, we show that in *Xenopus* and zebrafish both *Osr1* and *Osr2* are necessary and sufficient for the development of the pronephros. *Osr* genes are expressed in early prospective pronephric territories, and morphants for either of the two genes show severely impaired kidney development. Conversely, overexpression of *Osr* genes promotes formation of ectopic kidney tissue. Molecularly, Osr proteins function as transcriptional repressors during kidney formation. We also show that *Drosophila Odd* induces kidney tissue in *Xenopus*. This might be accomplished through recruitment of Groucho-like co-repressors. *Odd* genes may also be required for proper development of the Malpighian tubules, the *Drosophila* renal organs. Our results highlight the evolutionary conserved involvement of *Odd-skipped* transcription factors in the development of kidneys.

Keywords: *Drosophila*; *Odd-skipped*; Kidney; Repressor; *Xenopus*; Zebrafish

Introduction

During vertebrate development, three renal structures of increasing complexity form successively from the intermediate mesoderm: pronephros, mesonephros and metanephros (Saxén, 1987). Each of these develops by an inductive process mediated by the previous structure. In mammals, the pronephros is not functional but is required for mesonephros formation, which will execute renal functions in the embryo. Later in development, the mesonephros will be replaced by the metanephros, the adult functional kidney. In fish and amphibians, the pronephros is the functional embryonic kidney, being replaced in the adults by the mesonephros. In these organisms, a metanephros does not develop. The three kidney types differ in their organization, but share the same structural unit, the nephron. The number of nephrons varies from 1 to 50 in simple kidneys to a million in the mammalian ones. The nephron is divided in three basic segments: the corpuscle, the tubules and the duct. The corpuscle or glomerulus filters the blood, the tubular epithelium is the site of selective re-absorption and secretion and the duct collects and excretes the urine (Brandli, 1999; Burns, 1955; Saxén, 1987; Vize et al., 1997).

In *Xenopus* and in zebrafish (*D. rerio*), the pronephros is formed by a pair of unique non-integrated nephrons, symmetrically localized in the embryo (Brandli, 1999; Burns, 1955; Saxén, 1987; Vize et al., 1997). Most of the genes necessary for the formation of the *Xenopus* and zebrafish pronephros are also crucial for the formation of the more complex mammalian kidneys (reviewed in Carroll et al., 1999; Ryffel, 2003). These similarities at the molecular level correlate with physiological homologies. Thus, the tubules of all nephrons have similar

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subdivisions along the anterior–posterior axis with an analogous distribution of transporters of small molecules and ions along this axis (Zhou and Vize, 2004).

In *Xenopus*, the transcription factors *XPax8* and *Xlim1* are the earliest known genes to be expressed in the pronephric primordium. Their expression in the intermediate mesoderm at early neurulation stage precedes any morphological indication of pronephros formation (Carroll and Vize, 1999; Heller and Brandli, 1999). Both genes are essential for tubule and duct formation (Carroll and Vize, 1999; Chan et al., 2000). Moreover, only the combined overexpression of *XPax8* (or the partially redundant *XPax2*) and *Xlim1* efficiently forms ectopic renal tissue (Carroll and Vize, 1999). Early expression of *Lim1* and *Pax2/8* in the pronephric territory and functional requirement for at least *Pax2* have been reported in zebrafish (Majumdar et al., 2000; Pfeffer et al., 1998; Toyama and Dawid, 1997). Consistently with these results, mice lacking *Lim1* or *Pax2/8* have severe kidney malformations (Bouchard et al., 2002; Porteous et al., 2000; Shawlot and Behringer, 1995; Torres et al., 1995).

In *Drosophila*, the renal (or Malpighian) tubules are the major excretory and osmoregulatory organs. They originate from the embryonic proctodeum, a posterior region of the ectoderm that gives rise to the hindgut. After specification, they proliferate and evaginate from the gut epithelium as four buds, which later extend by cell rearrangement to form the four slim renal tubules (Jung et al., 2005). In addition, cells from the caudal visceral mesoderm migrate into the tubules to give rise to the stellate cells (Denholm et al., 2003). Stellate cells transport water and chloride anions, while the rest of the tubule's cells (so-called 'principal cells') transport organic solutes and cations. The transcription factors Kruppel (Kr) and Cut mark the renal tubules primordium within the proctodeum, and both are required for normal proliferation and eversion of the renal tubules. Still, *Kr* and *cut* mutant embryos form uric acid excreting cells – therefore with renal tubules characteristics – on the hindgut wall (reviewed in Ainsworth et al., 2000). This suggests the existence of other genes involved in renal tubule specification in *Drosophila*.

Despite major differences in embryonic origin, general organization and physiology, vertebrate kidneys and *Drosophila* renal tubules share certain developmental and genetic aspects. For instance, in *Drosophila*, renal tubules arise from the hindgut primordium, which expresses *brachyenteron* (Singer et al., 1996). Its vertebrate homologue, *Brachyury*, is required to specify mesoderm and is thus necessary for kidney development (Technau, 2001). The *Kr* and *cut* homologues *Glis2* and *Cux-1*, respectively, also play a role in kidney formation in mammals (Sharma et al., 2004; Vanden Heuvel et al., 1996; Zhang et al., 2002). The *Wnt* pathway is required for the specification and proliferation of the renal tubules (Wan et al., 2000), while *Wnt-4* knock-out mice develop small and dysgenic kidneys (Stark et al., 1994). Moreover, *hibris*, a fly homologue of vertebrate *nephrin* (Kestila et al., 1998), is expressed in prospective stellate cells and is required for their colonizing of the tubules (Denholm et al., 2003).

In *Drosophila*, the *odd-skipped* (*Odd*) family of genes comprises four transcription factors with high homology in their zinc finger domains: Odd, Sob, Drm and Bowl (Hart et al., 1996; Iwaki et al., 2001). *odd*, *sob* and *drm* are similarly expressed in the segment of the gut where midgut–hindgut join, and in the ureters of the mature tubules (Ward and Coulter, 2000), while *bowl* is expressed along the hindgut (Hart et al., 1996; Iwaki et al., 2001; Ward and Coulter, 2000). No renal tubules phenotype has been described for *odd*-family mutants. Two mammalian *odd-skipped related* genes, *Osr1* and *Osr2*, have been described (Lan et al., 2001; So and Danielian, 1999). In the mouse, *Osr1* expression starts early (E8.0) in the intermediate mesoderm, from where renal structures derive (So and Danielian, 1999), and is maintained until kidney organogenesis occurs. *Osr2* is activated at stage E9.25 in the mesonephros, and later (stage E14.5) in the mesenchyme that surrounds the ducts of the mesonephros and metanephros (Lan et al., 2001). *Osr1* knock-outs lack renal structures (Wang et al., 2005; James et al., 2006), while *Osr2* mutants have normal kidney development (Lan et al., 2004).

Here we report that both Osr1 and Osr2 function as transcriptional repressors required for pronephros development in *Xenopus* and zebrafish. When overexpressed, both lead to formation of ectopic renal tissue. Moreover, *Drosophila Odd* genes may be also necessary for renal tubule formation and can generate renal tissue when overexpressed in *Xenopus*. Therefore, *Odd/Osr* genes are utilized to generate filtration organs in both insects and vertebrates.

Materials and methods

Plasmid constructions

The following cDNA clones were obtained from the I.M.A.G.E. Lawrence Livermore National Laboratory Consortium: *XOsr2* (IMAGE 4405046), *zOsr1* (IMAGE 7226990) and *zOsr2* (IMAGE 7406070). The *XOsr1* cDNA clone (Mochii XL211m14) was a kind gift from N. Ueno and the NIBB/NIG *Xenopus laevis* EST project. The pCS2-*XOsr1* construct was generated by inserting the full-length cDNA into *Eco*RI site of pCS2+ (Turner and Weintraub, 1994). The pCS2-*XOsr2* construct was generated by inserting the full-length cDNA into *Eco*RI and *Xho*I sites of pCS2+. To generate the pCS2-*zOsr1* and pCS2-*zOsr2* constructs, we cloned the corresponding cDNAs into *Eco*RI and *Xba*I sites of pCS2+. To generate the *MT-Osr* and *Osr-MT* constructs, we PCR-amplified the corresponding *Osr* coding regions with the following pairs of primers: 5′-**GAATTC**GATGGGGAGCAAGACGCTTCC-3′ and 5′-**CTCGAG**GCATTTGATTTTGGAAGGCTTGAGTTC-3′ for *XOsr1*; 5′-**GAATTC**GATGGGCAGCAAAGCTCTTCCAG-3′ and 5′-**CTCGAG**AATCGCAATTTCTCCGGAAAACTTTTC-3′ for *XOsr2*; 5′-**GAATTC**GGAATTAGTCATGGGTAGTAAGACG-3′ and 5′-**CTCGAG**CTTTATCTTGGCTGGCTTGAG-3′ for *zOsr1*; 5′-**GAATTC**TGCACCGGGAATGG-3′ and 5′-**CTCGAG**GACTGTGGCGCCGC-3′ for *zOsr2*. The corresponding *Eco*RI and *Xho*I sites are shown in bold. The different PCR fragments were subcloned in pGEMT-Easy (Promega) and sequenced. For generating the *MT-Osr* or the *Osr-MT* constructs, we cloned the PCR fragments between the *Eco*RI and *Xho*I sites of pCS2 MT or the pCS2p+MTC2, respectively. These vectors were kindly provided by D. Turner. To generate the *MT-XOsr-EnR* and *MT-XOsr-E1A* constructs, we removed a *Xho*I and *Sac*II fragment containing SV40 polyA region from the *MT-XOsr* construct and replaced it with a *Xho*I and *Sac*II fragment containing the EnR or E1A and the SV40 polyA region. These fragments were obtained from the pCS2-MT-NLS-EnR and pCS2-MT-NLS-E1A plasmids kindly donated by N. Papalopulu. The complete open reading

frame (ORF) from the *Drosophila drm* gene was excised with *Xho*I and *Xba*I from the *drm* cDNA and cloned into the *Xho*I and *Xba*I sites from pCS2+ vector to generate the *drm* construct. To generate the *odd* construct, we amplified the ORF from the *Drosophila odd* gene with the following primers: 5′-**GAATTC**AATGTCTTCCACATCGGCCTC-3′ and 5′-**TCTAGA**-TATCTGCTCATGATCTCATCGATG-3′. The PCR fragment was subcloned into pGEMT-Easy (Promega), sequenced and then transferred to pCS2 MT between the *Eco*RI and *Xba*I sites. The *oddΔeh1* construct was generated by subcloning an *Eco*RI–*Xho*I fragment from the *Drosophila odd* gene into the *Eco*RI and *Xho*I sites from the pCS2+ vector. This fragment encodes a truncated Odd protein that lacks the last 19 amino acids (SSEKPKRMLGFTI-DEIMSR), which include the eh1 domain (underlined).

DNA sequencing

DNA sequencing was performed with ABI chemistry in an automatic DNA sequencer using T3 and T7 oligonucleotides. Custom synthesized oligonucleotides were obtained from Sigma.

Xenopus, zebrafish and Drosophila in situ hybridization, X-Gal and antibody staining

Antisense RNA probes were prepared from cDNAs using digoxigenin or fluorescein (Boehringer Mannheim) as labels. *Xenopus*, zebrafish and *Drosophila* specimens were prepared, hybridized and stained as described (Hao et al., 2003; Harland, 1991; Jowett and Lettice, 1994). *Xenopus* and *Drosophila* X-Gal staining was performed according to Coffman et al. (1993). *Xenopus*, zebrafish and *Drosophila* antibody staining was performed as described (Gómez-Skarmeta et al., 2001; Hernandez et al., 2005; Sanchez-Herrero, 1991). Antibodies used in this study were rabbit anti-β-galactosidase (Cappel), rabbit anti-GFP (Molecular Probes) and guinea pig anti-Odd (Kosman et al., 1998). The monoclonal antibody 3G8 were kindly provided by E. Jones. The monoclonal antibodies 12/101 and 2B10 (developed by J. P. Brockes) and Mouse anti-Cut (developed by I. Rebay, G. Dailey, K. Lopardo and G. Rubin) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Science, Iowa City, IA 52242.

In vitro RNA synthesis and microinjection of mRNA and morpholinos

All DNAs were linearized and transcribed as described (Harland and Weintraub, 1985) with a GTP cap analog (New England Biolabs), using SP6, T3 or T7 RNA polymerases. After DNAse treatment, RNA was extracted with phenol-chloroform, column purified and precipitated with ethanol. mRNAs for injection were resuspended in water. *X. laevis* and *Xenopus tropicalis* embryos were injected in the marginal region at the 2-cell stage using a volume of 10 or 2–5 nl, respectively. V2.2 blastomeres of *X. tropicalis* 8–16 cell stage embryos were injected with 1–2 nl of morpholino solution. In these experiments, embryos were co-injected with Dextran-Fluorescein (10,000 MW, Molecular probes). Embryos showing fluorescence in the prospective kidney domain but not in the somites were selected under a fluorescent dissecting scope and further processed for *in situ* hybridization. The localization of Fluorescein was later determined with anti-Fluorescein antibody coupled to alkaline phosphatase (Roche). The following morpholinos were used in this study: MOXOsr1: 5′-TGCTGGAAGGGTCTTGCTCCCCATC-3′, MOXOsr2: 5′-GGCTGGAAGAGCTTTGCTGCCCATT-3′, MOzOsr1: 5′-GCGTCTTACTACCCATGACTAATTC-3′ and MOzOsr2: 5′-AGAGTCT-TACTGCCCATTCCCGGT-3′. The *Xenopus* morpholinos were designed to target *Osr1* or *Osr2* genes from both *X. laevis* and *X. tropicalis*. *X. tropicalis* embryos were injected with 10–20 ng of morpholinos at the two cell stage and with 2 ng at the 8–16 cell stage. Zebrafish embryos were injected in the yolk at 1–2 cell stage with 10–20 ng of morpholinos.

Drosophila strains and genetic manipulations

The following mutant alleles are described in Flybase (http://flybase.org/): *odd5*, $bowl^{l}$, drm^{6}. Deficiency drm^{P2} (Green et al., 2002) deletes from *tim* to *odd* and uncovers approximately 30 predicted genes, including *drm*, *sob* and *odd*. Mutant chromosomes were balanced over the 2nd marked balancer chromosomes *CyO*, *Kr*-GFP; homozygous mutant embryos were detected as GFP-negative. To trace the lineage of *odd*-expressing cells in the RTs, we crossed *odd-Gal4* (a Gal4 insertion in *odd* that faithfully recapitulates its expression, gift from M. Calleja and G. Morata, CBM, Madrid) into *UAS-flip; act>Draf>LacZ* (Campbell and Tomlinson, 1998). In the resulting *odd-Gal4/UAS-flip; act>Draf>LacZ* cells derived from *odd*-expressing cells are constitutively marked by the expression of *lacZ*. The expression of the *odd* lineage (*odd*>lineage) was compared to the actual expression of *UAS-lacZ; odd-Gal4* larvae.

To overexpress *Odd*-family genes in the RT primordial and hindgut, we used a *brachyenteron (byn)-Gal4* driver (Iwaki et al., 2001). *byn-Gal4/TM3*, *ftz-Z* females were crossed to homozygous *UAS-bowl* (de Celis Ibeas and Bray, 2003), *UAS-sob* or *UAS-odd/TM6B* (Hao et al., 2003) or *UAS-drm* (Green et al., 2002) males. Embryos carrying *byn-Gal4* were detected as *LacZ* (β-galactosidase)-negative. Those expressing *odd* were detected using an anti-Odd specific antibody. *UAS-src-GFP* is described in Kaltschmidt et al. (2000).

Results

Osr genes are expressed in the renal primordium of Xenopus and zebrafish

A search in databanks identified two *X. laevis* and two zebrafish EST clones that correspond to genes encoding orthologues of human and mouse *Osr1* and *Osr2* (Supplementary Fig. 1). We named these genes *XOsr1*, *zOsr1*, *XOsr2* and *zOsr2*. No additional *Osr* genes were detected by Blast searches in these species, suggesting that, as in mammals, they have two *Osr* genes. Both *XOsr* genes are initially detected during early gastrulation in the involuting mesoderm and endoderm (Figs. 1A, E). At the end of gastrulation, *XOsr2* is detected in the intermediate mesoderm (inset in Fig. 1F) preceding the activation of the early pronephric markers *XPax8* and *Xlim1* (not shown and inset in Fig. 1I). During neurulation, this expression resolves in a broad domain largely overlapping that of *Xlim1* and *XPax8* (Figs. 1F, G, I–K, M–O) (Carroll and Vize, 1999). *XOsr1* is similarly expressed although at lower levels (Figs. 1B, C). During tailbud (stage 35), *XOsr1* is expressed in the rectal diverticulum and in the ducts (Fig. 1D). At this stage, *XOsr2* mRNA is also expressed in the duct but in a broader domain. In addition, *XOsr2* is also expressed in the tubules (Fig. 1H). See for comparison the expression of *Xlim1* and *XPax8* in the tubules and duct at this stage (Figs. 1L, P).

In zebrafish, *zOsr1* also precede the expression of the early pronephros marker *zlim1* and *zPax2*, while the expression of *zOsr2* appears at the 8-somite stage, once *zlim1* and *zPax2* are transcribed but prior to any sign of pronephros histogenesis (see Fig. 2 for a full description of *zOsr1* and *zOsr2* expression patterns). The staggered expression of *Osr1* and *Osr2* we observe in zebrafish is similar to that recently described in mice and chicken (So and Danielian, 1999; Lan et al., 2001; Wang et al., 2005; James et al., 2006; Stricker et al., 2006). This situation is reversed in *Xenopus*, where *Osr2* expression in the prospective renal territory seems to precede that of *Osr1*, even if both genes are co-expressed by the time the early renal markers *XPax8* and *Xlim1* begin to be expressed.

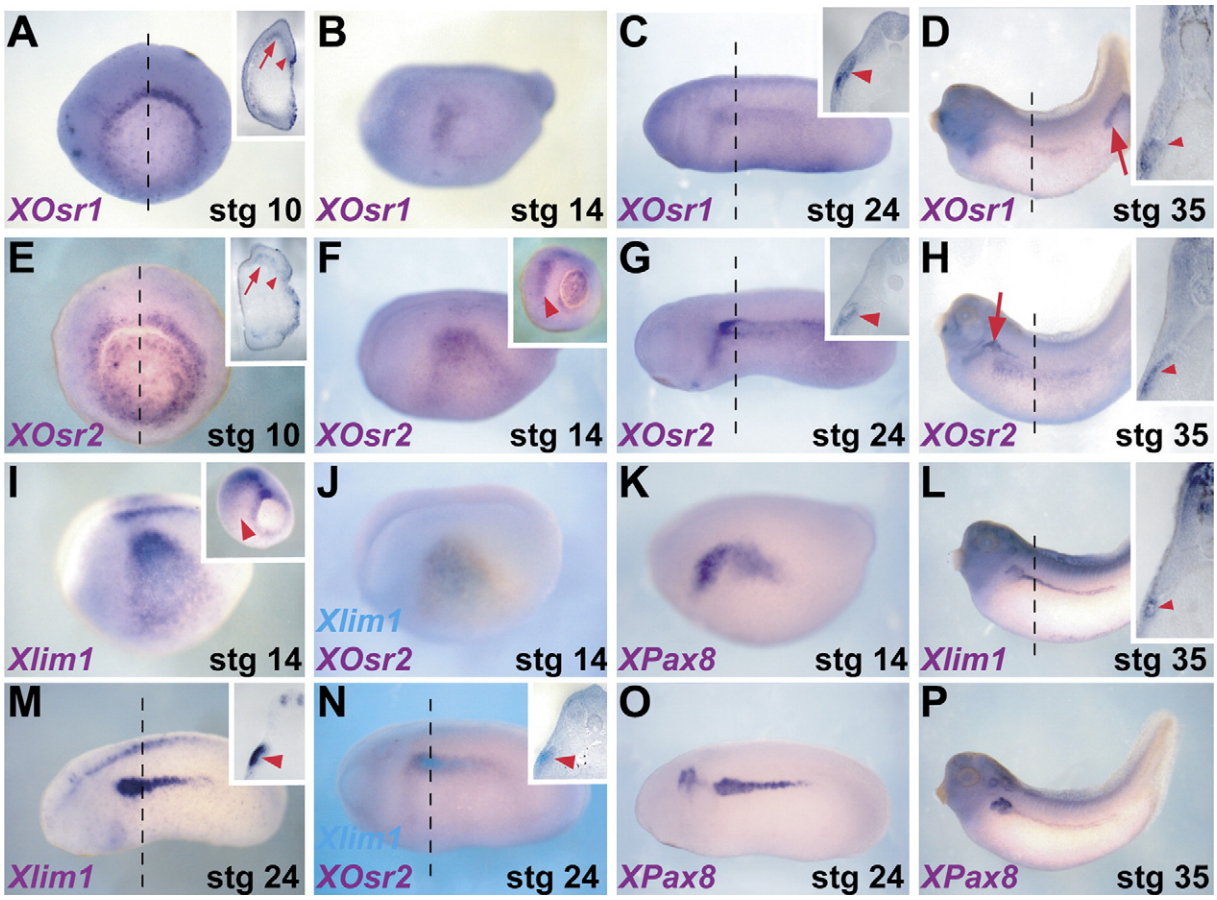

Fig. 1. Expression pattern of *XOsr* genes. Panels A, E are vegetal and panels B–D, F–P are lateral views. Insets in panels A, C, D, E, G, H, L, M and N are transverse vibratome sections through the dashed lines in the main panels. (A) Early gastrula stage (stg). *XOsr1* is expressed in the involuting mesoderm and endoderm (arrowhead and arrow in inset, respectively). (B, C) During neurulation, *XOsr1* mRNA is detected in the pronephric territory. (D) At tailbud, *XOsr1* is expressed in the ducts (arrowhead in inset) and in the rectal diverticulum (arrow). (E–G) Expression of *XOsr2* is similar, but stronger. In the prospective kidney territory, *XOsr2* is detected earlier than *XOsr1* (stage 11.5–12, inset in panel F; arrowhead marks the prospective kidney domain), and earlier than other pronephric markers (see inset in panel I for the expression of *Xlim1* at this stage; arrowhead marks the prospective kidney domain). (H) At tailbud, *XOsr2* is expressed in the tubules (arrow) and in a broad domain adjacent to the ducts (arrowhead in inset). (I, J, M, N) During neurula, expressions of *XOsr2* and *Xlim1* (I, M) largely overlap in the pronephric region (double *in situ* hybridization, J, N). (K, O) During neurula, *XPax8* is also detected in the pronephric territory. (L, P) At tailbud, *Xlim1* and *XPax8* are expressed in the tubules and ducts. Inset in panel L show *Xlim1* expression in the duct (arrowhead).

Morpholino knockdown of Osr1 and Osr2 impairs renal development in Xenopus and zebrafish

In mouse, *Osr1*, but not *Osr2*, is essential for kidney development (Lan et al., 2004; Wang et al., 2005). We have examined whether *Osr* genes are required for pronephric development in *X. tropicalis* and zebrafish by blocking the translation of *Osr1* and *Osr2* mRNAs with specific morpholinos (MOs) (Supplementary Fig. 2A).

X. tropicalis embryos injected with 10–20 ng of *XOsr1* or *XOsr2* MOs show similar downregulation of the early pronephric territory markers *Xlim1* and *XPax8* (84% and 71%, $n=68$ and 66, respectively; Figs. 3A–I, M). This downregulation was not associated with an expansion of muscle tissue as determined by the muscle specific antibody 12/101 (Figs. 3A–I, M). Indeed, in some cases, muscle size was reduced (see Fig. 3M). Moreover, a strong defect in, or the disappearance of, the differentiated embryonic kidneys was observed, as determined by the pronephros monoclonal antibody 3G8 (Vize et al., 1995) (92 and 78% with reduced kidneys, $n=175$ and 166, respectively; Figs. 3J–L, N–P and not shown). To avoid possible kidney defects caused by altered muscle development, we co-injected the *XOsr* MOs with Dextran-Fluorescein in a single blastomere (V2.2) of 8–16 cell stage embryos, and analyzed tailbud-stage embryos showing Fluorescein signal restricted to the kidney territory, but not in the somites. In these embryos, injection of *XOsr1* or *XOsr2* MOs promoted a clear downregulation of *XSGLT1K* and *XNKCC2* (Figs. 3Q–T), two genes encoding pronephric epithelial transporters that are specifically expressed in the proximal and distal tubule, respectively (Zhou and Vize, 2004), without any visible effect on somites formation.

In zebrafish, MOs targeting *zOsr1* or *zOsr2* genes caused downregulation of the early pronephric markers *zlim1* (76% and 46%, $n=85$ and 93, respectively; Figs. 4A, E, I) or *zPax2.1* (73% and 38%, $n=81$ and 77, respectively; Figs. 4B, F, J) and induced defects in the differentiated renal structures (Figs. 4C, G, K). The observed downregulation of *zlim1* at 4-somite stage, though, was more pronounced in MO*zOsr1* morphant embryos (compare Figs. 4E and I). In addition, at 72 hpf these morphant

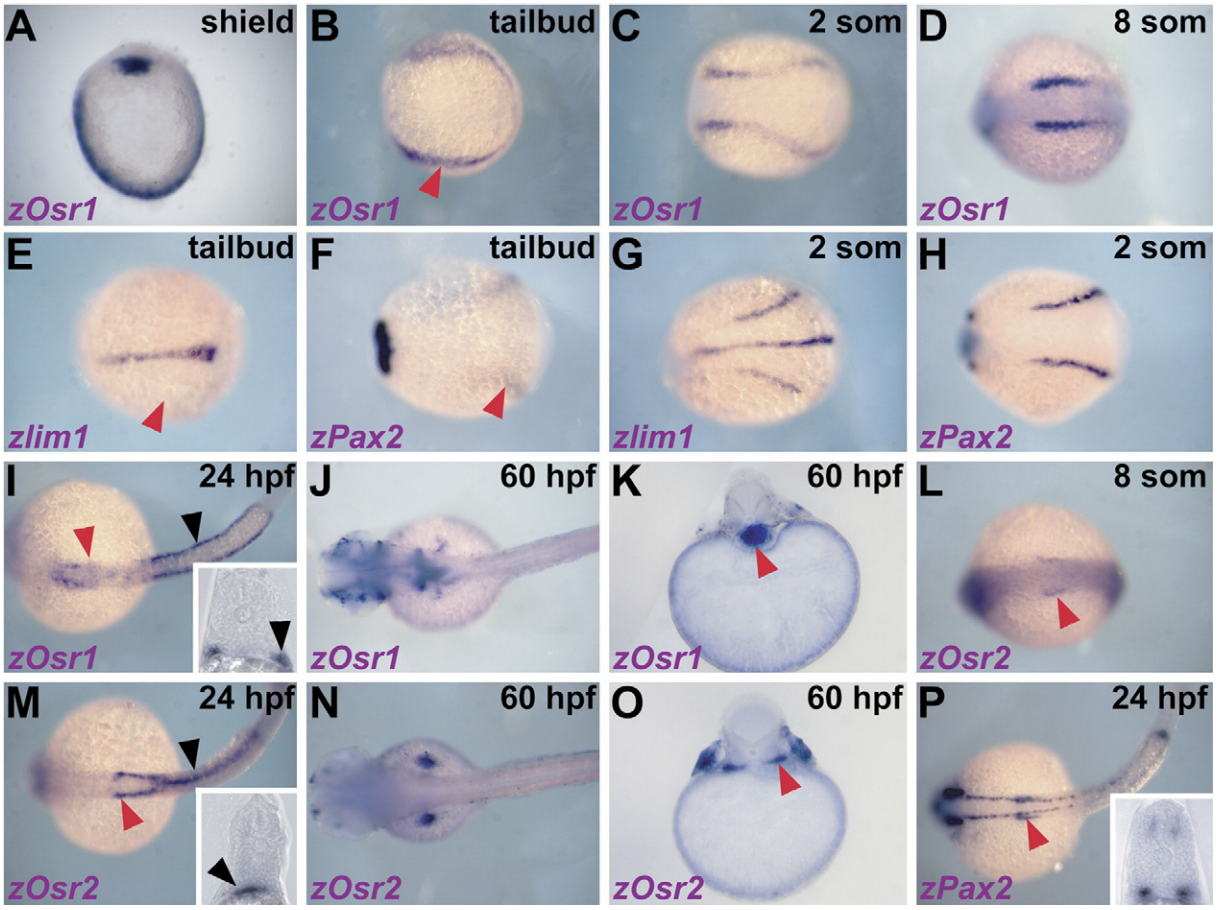

Fig. 2. Expression pattern of *zOsr* genes. Dorsal views are shown, except (A), a vegetal view and (K, O) and insets and (I, M, P), transverse sections through the pectoral fin buds or the posterior spinal cord, respectively. (A) At shield stage, *zOsr1* mRNA is detected in the shield and in a ventro-lateral ring. (B, E, F) At tailbud, *zOsr1* (B) is expressed in the pronephric territory (arrowhead), preceding the expression of *zlim1* (E) and *zPax2.1* (F) (arrowheads mark the prospective pronephric territory at this stage). (C, G, H) During early somitogenesis, *zOsr1* (C), *zlim1* (G) and *zPax2.1* (H) show similar expression domains within the pronephros territories, although *zOsr1* seems to extend more rostrally. (D, L) At the 8-somite stage, both *zOsr1* (D) and *zOsr2* (L) mRNAs are expressed in the pronephros, *zOsr2* being a transcription domain weaker and shorter. In addition, *zOsr2* also shows a weak generalized expression. (I, M) At 24 hpf, the expression of *zOsr1* in the pronephros starts to be downregulated (I, red arrowhead). At this stage, *zOsr2* is detected in the tubules and in the anterior duct (M, red arrowhead). In addition, *zOsr1* is expressed in two rows that run parallel to the pronephros (I, black arrowheads and inset) while *zOsr2* is found in the gut (M, black arrowheads and inset). (J, K, N, O) Expression of *zOsr1* (J, K) and *zOsr2* (N, O) at 60 hpf. *zOsr1* is detected in the glomerulus, in some patches in the eye and brain, and weakly in the pectoral fin buds (J). The expression in the glomerulus is clearly visible in a transverse section (arrowhead in panel K). *zOsr2* is expressed in the tubules and the pectoral fin buds (N). The expression in the tubules is more evident in a transverse section (arrowhead in panel O). (P) Expression of *zPax2* at 24 hpf. The expression in the pronephros is pointed at by an arrowhead and can be visualized in a transverse section in the inset.

embryos showed pericardial edemas and kidney cysts (Figs. 4D, H, L), defects characteristic of renal failure (Drummond et al., 1998; Hostetter et al., 2003).

In zebrafish and *Xenopus*, we have compared the effect of targeting both *Osr* genes at the same time (with half the dose of each MO) with reducing individual *Osr* gene function. No synergistic effect was observed by reducing *Osr1* and *Osr2* function simultaneously (not shown). Thus, in contrast to mice (Lan et al., 2004; Wang et al., 2005; James et al., 2006), in *Xenopus* and zebrafish, both *Osr* genes seem to be required for development of kidney structures.

Osr1 and Osr2 gain of function promotes ectopic renal tissue

We next examined the effects of overexpressing *Osr* genes on *Xenopus* kidney development. Either wild-type or Myc-tagged versions of either *Xenopus* or zebrafish *Osr1* and *Osr2* mRNAs yielded similar results. Many of the *Xenopus Osr*-injected embryos showed gastrulation defects that were the more severe the higher the doses of mRNA. However, in embryos injected with 100 pg of mRNA, about 30% showed no gastrulation defects. In most of these (75%, $n>200$; Figs. 5A–F), *Xlim1* and *XPax8* were expressed in ectopic patches of cells. Other pronephric markers such as *XNHF1β* or *Gata3* were similarly ectopically expressed, but not the glomerulus marker *XWt1* (not shown). We also examined the effect of *Osr* overexpression on genes encoding pronephric epithelial transporters. Late neurula injected embryos showed ectopic patches of *XSGLT1* and *XNKCC2* expression at similar frequencies (Figs. 5G, H). These patches differentiate as pronephric structures later, as determined by the tubule-specific monoclonal antibody 3G8 (Figs. 5I, J). Morpholino-insensitive

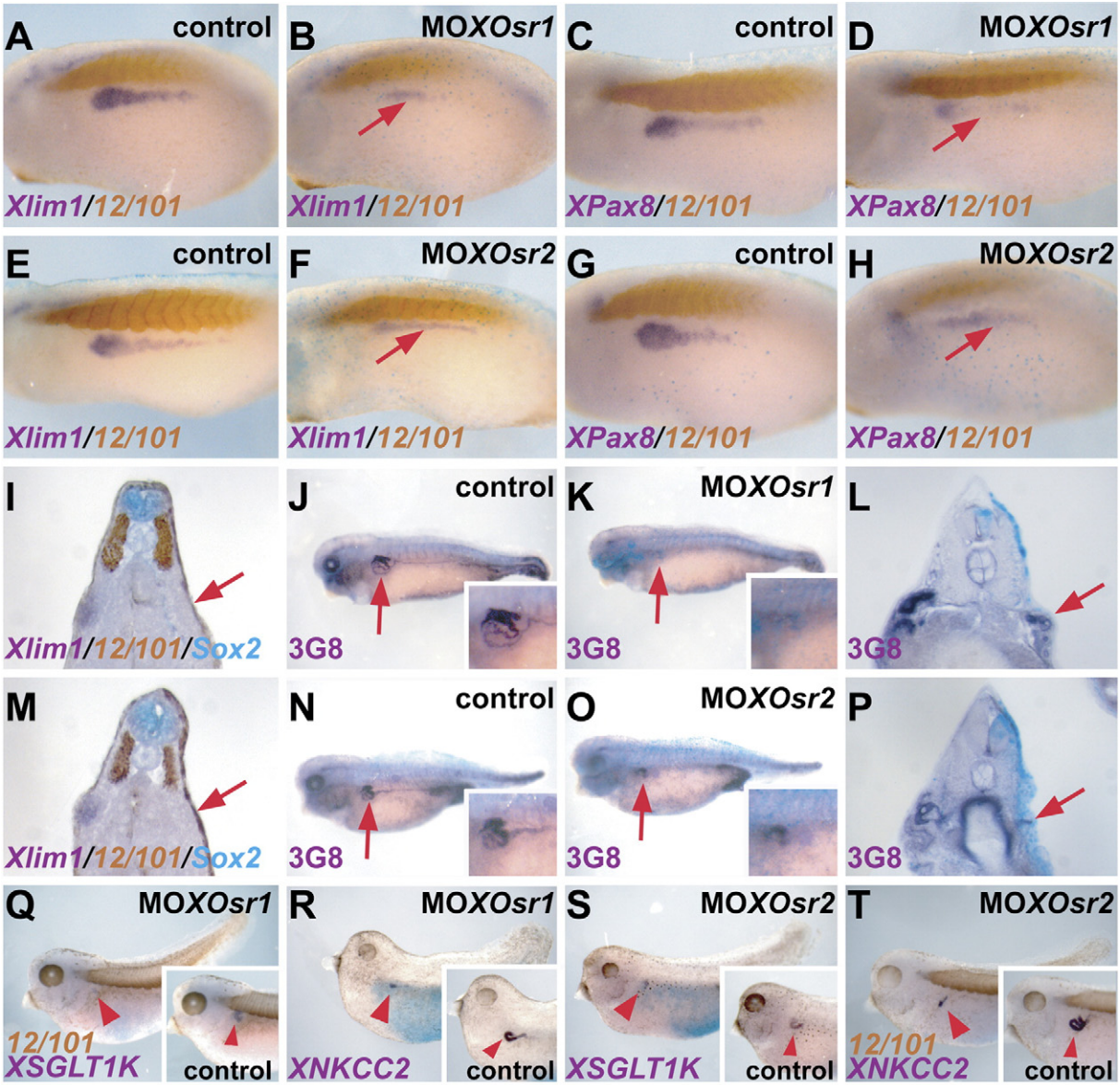

Fig. 3. *Xenopus Osr* morphant embryos have severely impaired kidneys. (A–H) Lateral views of stage 25 *Xenopus tropicalis* embryos injected with 20 ng of MO*XOsr1* (A–D) or 20 ng of MO*XOsr2* (E–H) and 300 pg of *LacZ* mRNA to determine the injected side. Purple staining shows the expression of *Xlim1* (A, B, E, F) or *XPax8* (C, D, G, H), and brown staining the somitic muscles, labeled with the monoclonal antibody 12/101. The MO injected embryos show a reduced expression of the kidney markers on the injected sides (arrows in panels B, D, F and H; compare with the control sides shown in panels A, C, E and G). (I, M) Transverse section of stage 25 MO*XOsr1* (I) or MO*XOsr2* (M) injected embryos triple labeled for *Xlim1* (pronephros, purple), muscles (brown) and *Sox2* (neural tissue, cyan). Note the strong reduction of the pronephric tissue in the injected sides (arrows). (J–L and N–P) Stage 37 *Xenopus tropicalis* embryos injected with MO*XOsr1* (J–L) or MO*XOsr2* (N–P) and stained with the monoclonal antibody 3G8. Note the strong reduction of the kidney tissue in the injected sides (arrows in panels K, L, O, P). Insets are closer views. This reduction is clearly visible in transverse sections (arrow in panels L and P). (Q–T) Lateral views of stage 35 *Xenopus tropicalis* embryos co-injected with MO*XOsr1* (Q, R) or MO*XOsr2* (S–T) and Dextran-Fluorescein in the V2.2 blastomere at the 8–16 cell stage. The expression of *XSGLT1K* (Q, S; purple) and *XNKCC2* (R, T; purple) is impaired in the injected side (Fluorescein distribution is visible in cyan). Brown staining in panels Q and T shows the somitic muscles labeled with the monoclonal antibody 12/101. Insets show the control un-injected side. (U) Target sequences for *Xenopus Osr* Morpholinos (MOs). In all sequences, the first methionine of the corresponding gene is underlined. Identical bases are in blue and mismatches in red. Note that the MOs for each *Xenopus Osr* gene have one mismatch with the corresponding *Xenopus laevis* and *Xenopus tropicalis* target sequences. In contrast, the MO against one of the paralogues has five or more mismatches with the sequence of the other gene. MOs with only one mismatch can efficiently block translation while five or more mismatches make an MO inactive.

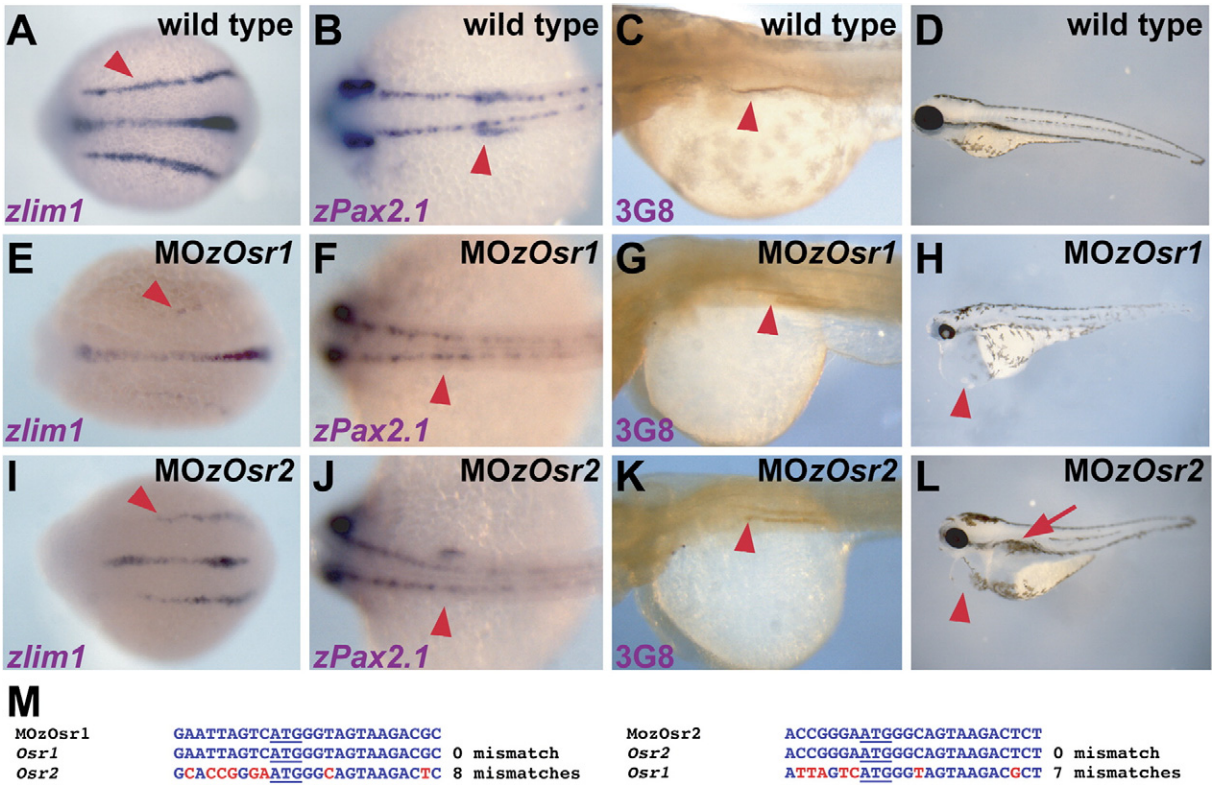

Fig. 4. Zebrafish *Osr* morphant embryos have severely impaired kidneys. (A–D) Wild-type zebrafish embryos. Embryos injected with 20 ng of MO*zOsr1* (E–H) or 20 ng of MO*zOsr2* (I–L). These injected embryos show a reduction of the pronephric markers *zlim1* at the 4-somite stage (A, E, I; arrowheads), *zPax8* at 24 hpf (B, F, J; arrowheads) and reduced kidney tissue at 48 hpf, as determined by the staining with the monoclonal antibody 3G8, which labels tubules and anterior ducts (C, G, K; arrowheads). At 70 hpf, pericardial edemas (arrowheads) and kidney cysts (arrow) are visible (D, H). These are characteristic of renal failure. (M) Target sequences for zebrafish *Osr* Morpholinos (MOs). As in Fig. 3 in all sequences, the first methionine of the corresponding gene is underlined. Identical bases are in blue and mismatches in red.

MT-XOsr1 and *MT-XOsr2* could also rescue the impaired development of renal tissue of embryos injected with *XOsr1* and *XOsr2* MOs, respectively (50% or 37% with rescued kidneys, Figs. 5K, L). We also examined the effect of overexpressing *Osr* genes in transverse sections of stage 22–25 embryos triply stained for pronephros, somitic muscle and neural ectoderm. The ectopic renal tissue was always found close to the neural tube, which in some cases was strikingly enlarged in the direction of the ectopic pronephros. The somitic muscles were normal or slightly reduced (Figs. 5M, N). At stage 38, we also observed a clear enlargement of the endogenous renal tissue and ectopic pronephric structures in the proximity of the spinal cord (Figs. 5O, P).

In zebrafish embryos, both *Osr* mRNAs promoted enlargement of the pronephric domains of *zlim1* and *zPax2.1* markers (Figs. 5Q–S and not shown). At later stages, the differentiated kidney tissue was also expanded (Fig. 5T). In addition, some embryos displayed ectopic renal tissue (Fig. 5T).

Osr proteins function as transcriptional repressors during renal development

Two *Drosophila* Odd proteins, Odd and Bowl, harbor an eh1-like motif that helps recruiting the Groucho co-repressor to downregulate target genes during embryonic segmentation (Goldstein et al., 2005). Therefore, in this context, Odd proteins work as repressors. In contrast, the molecular function of mammalian Osr proteins is unclear. *Osr2* mRNA generates two protein splicing variants, one containing three zinc fingers and the other five, that function as activator and repressor, respectively, in cell culture assays (Kawai et al., 2005). To further investigate this question, we injected *X. laevis* embryos with mRNAs (100 pg) encoding Osr proteins fused to either the Engrailed repressor domain (EnR) or the E1A activation domain. Similarly to wild-type *Osr* mRNAs, *XOsr1-EnR* or *XOsr2-EnR* mRNAs induced patches of ectopic expression of *Xlim1* and *XPax8* (Figs. 6A, B and not shown) that differentiated into renal tissue (Fig. 6C). In contrast, *XOsr1-E1A* or *XOsr2-E1A* mRNAs (500 pg) downregulated *Xlim1* and *XPax8* and strongly reduced differentiated kidney structures (Figs. 6D–F, and not shown). Thus, vertebrate Osr proteins appear to act as transcriptional repressors during kidney development.

The zinc fingers of *Drosophila* and vertebrate Odd/Osr proteins are largely identical in sequence, although the number of zinc fingers varies among them. Vertebrate Osr proteins contain three (except the mammalian Osr2A splice variant that contains five), while *Drosophila* Drm contains two, Odd four

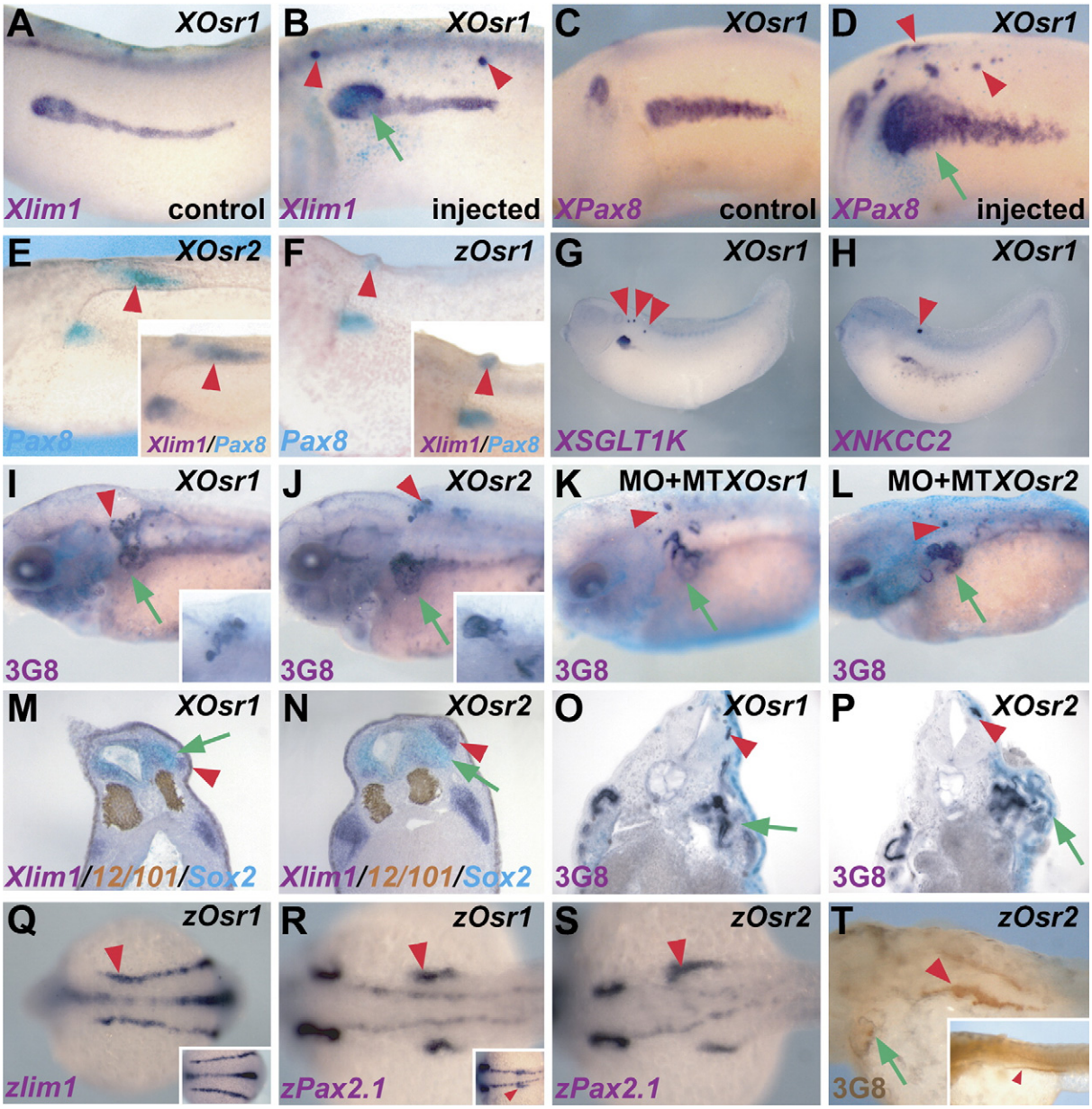

Fig. 5. Overexpression of *Osr* genes promotes ectopic kidney development. (A–L) Lateral views of stage 25 (A–F), stage 30 (G, H) or stage 37 (I–L) *Xenopus* embryos, or 48 hpf zebrafish embryo (T). (M–P) Transverse sections of stage 25 (M, N) or stage 37 (O, P) *Xenopus* embryos. (Q–T) Dorsal views of four somites (Q) or 24 hpf (R, S) zebrafish embryos. Embryos were injected with 50–100 pg of *Xenopus* or zebrafish *Osr* mRNAs. *Xenopus* embryos were co-injected with 300 pg of *LacZ* mRNA as a lineage tracer. (A–D) Embryos injected with *XOsr1* mRNA showed ectopic patches of *Xlim1* (A, B) or *XPax8* (C, D) expression in the injected sides (arrowheads in panels B, D). In addition, many embryos have enlarged pronephros (arrows in panels B and D; compare with control sides in panels A and C). (E, F) Stage 25 *Xenopus* embryos injected with *XOsr2* (E) or *zOsr1* (F) mRNAs and doubly hybridized for *Xlim1* and *XPax8*. The first chromogenic reaction, to detect *Xlim1* expression, is shown in the main panels (cyan), and the second chromogenic reaction, to detect *XPax8*, in the insets (purple). Note that the same cells express ectopically both markers (arrowheads). (G, H) Embryos injected with 100 pg of *Xenopus Osr1* mRNA showed ectopic patches of *XSGLT1K* (G, arrowheads) and *XNKCC2* (H, arrowhead). Note that these embryos have gastrulated properly. (I, J) Enlarged (arrow) and ectopic (arrowhead) kidney tissue, as determined by 3G8 staining, in stage 37 *Xenopus* embryos injected with *XOsr1* (I) or *XOsr2* (J) mRNAs. Insets show magnification of ectopic renal tissue in other injected embryos. (K, L) Stage 37 *Xenopus* embryos co-injected with MO*XOsr1* and *MTXOsr1* mRNA (K) or MO*XOsr2* and *MTXOsr2* mRNA (L) and stained for 3G8 monoclonal antibody. Note that these MO insensitive mRNAs rescue the MO-induced kidney marker reduction (arrow) (see panels K and O in Fig. 3 for comparison) and promote ectopic renal tissue (arrowhead). (M–P) Transverse sections on stage 25 (M, N) or stage 37 (O, P) *Xenopus* embryos injected with *XOsr1* (M, O) or *XOsr2* (N, P) mRNAs. The embryos in panels M and N show a triple staining for *Xlim1* (pronephros, purple), monoclonal antibody 12/101(somitic muscles, brown) and *Sox2* (neural tube, magenta). The embryos in panels O, P show differentiated kidneys labeled with the monoclonal antibody 3G8. Note that the ectopic renal tissue is always found close to the neural tube (arrowhead). In addition, these embryos show a clear enlargement of the neural tube and the endogenous pronephros (arrows). (Q–T) Zebrafish embryos injected with *zOsr1* (Q, R) or *zOsr2* (S, T) mRNAs showing *zlim1* expression at 4-somite stage (Q), *zPax2.1* expression at 24 hpf (R, S) and differentiated renal structures, as determined by 3G8 monoclonal antibody staining (T). Note the enlarged pronephros (arrowheads) and the ectopic renal tissue (T, arrow). Insets in panels Q, R and T show control non-injected embryos.

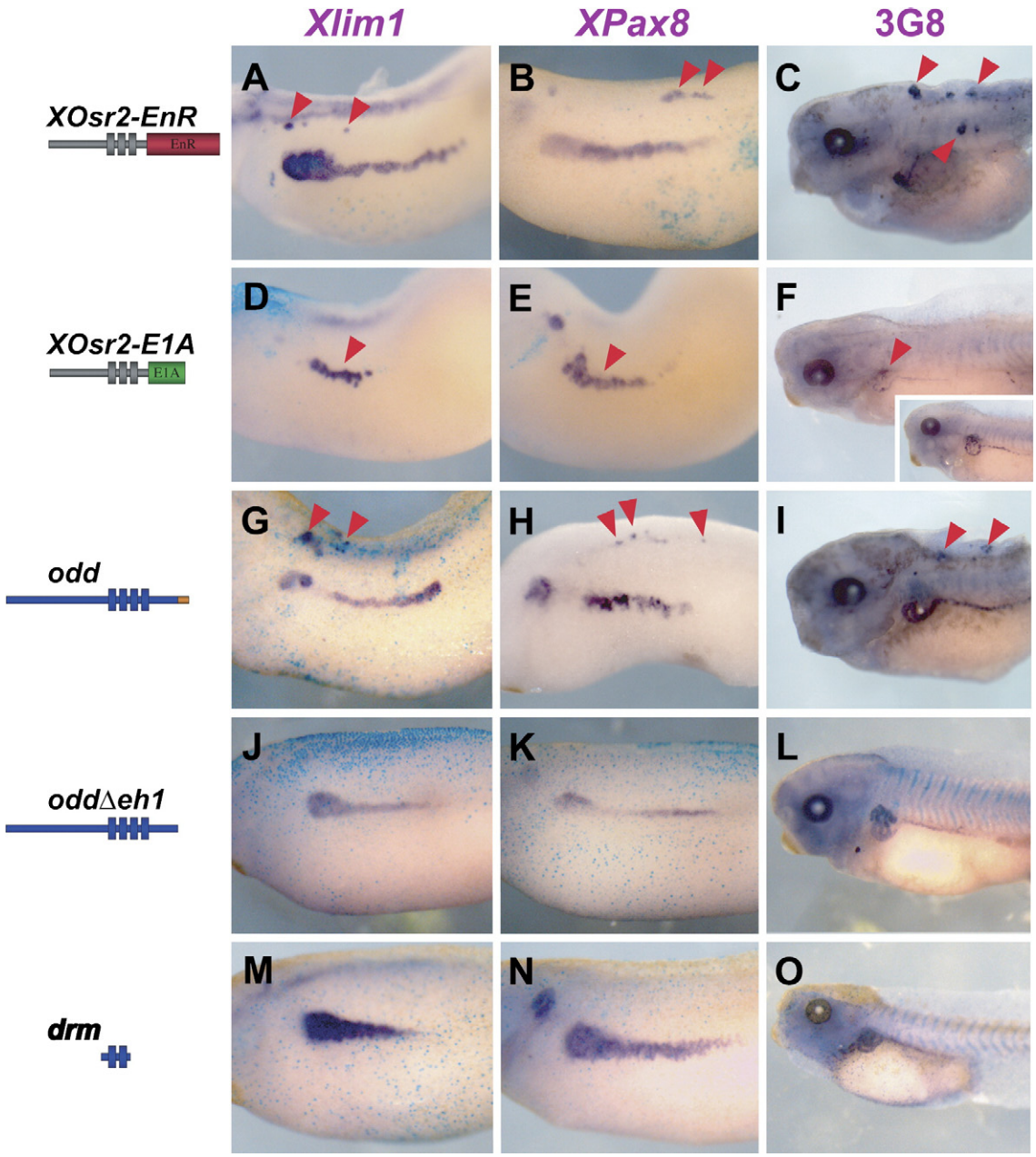

Fig. 6. Osr proteins function as repressors during kidney development. All panels show lateral views of late neurula (left and middle panels) or tailbud (right panels) *Xenopus* embryos. The left, middle and right panels show *Xlim1*, *XPax8* and 3G8 staining, respectively. Cartoons on the left indicate the proteins encoded by the injected mRNAs. (A–C) Injection of 100 pg of *XOsr2-EnR* mRNA promotes ectopic pronephros (arrowheads). In contrast, injection of 500 pg of *XOsr2-E1A* mRNA downregulates pronephric markers (D–F). Inset in panel F shows the control non-injected side. (G–I) Overexpression of *Drosophila odd* mRNA (500 pg) promotes ectopic renal tissue (arrowheads). This activity depends on its eh1 domain (orange) as the deletion of this motif (*oddΔeh1)* impairs its ability to activate renal markers (J–L). (M–O) *Drosophila drm* mRNA (1 ng) is unable to promote kidneys when overexpressed in *Xenopus*.

and Bowl and Sob five. In addition, *Drosophila* Odd and Bowl function in some contexts as repressors by recruiting Groucho, but Sob and Drm do not bind this co-repressor (Goldstein et al., 2005). We examined whether *Drosophila* Odd proteins promote ectopic kidney differentiation in *Xenopus*. *odd*, but not *drm* mRNA, promoted ectopic renal tissue (Figs. 6G–I, M–O). This ability depended on the eh1 domain as its removal abolished it (Figs. 6J–L). These data strongly suggest that, to function in renal development, vertebrate Osr proteins may also require a Groucho-like co-repressor.

Drosophila Odd genes are expressed during RT formation and may be required for their formation

The ability of *odd* to promote renal tissue in *Xenopus* prompted us to determine whether this family of genes is required for renal tubules formation in *Drosophila*. We re-examined the expression of the different *Odd* genes in embryogenesis. The similar expression of *odd*, *drm* and *sob* in the gut suggested that the three genes might also be expressed in the renal tubules ureters. This was the case, as

detected by coexpression with Cut along the proximal ureteric tubes (Figs. 7A–C, I). This expression was detected at least from embryonic stage 12 as a stripe of cells at the base of the budding RT primordia (not shown). We did not detect *bowl* transcription at significant levels in the Cut-expressing cells at any stage.

The expression pattern of *drm*, *sob* and *odd* argues against a role in early stages of renal tubules specification, but suggests a redundant function later in renal tubules development. To test this hypothesis, we examined the renal tubules in *odd*, *drm* and *bowl* single mutants, and in embryos homozygous for a large deficiency (Df*drmP2*), that uncover at least 30 predicted genes, including *drm*, *sob* and *odd* (Green et al., 2002). (No *sob* single mutation is available, which prevented analysis of its individual mutant phenotype.) None of the three individual mutants affected renal tubules specification, growth or extension. Nevertheless, in Df*drmP2* embryos, renal tubules were singled out as Cut-expressing buds, but failed to grow or extend further (Fig. 7D). The secretory activity of the remaining rudiments in these mutant embryos, monitored by the production of uric acid, was greatly reduced when compared with normal tubules (Figs. 7E, F).

The general defective development of renal tubules was not anticipated by the localized expression of *odd* genes in just the ureters primordia. If *odd*, *sob* and *drm* genes were indeed responsible for the phenotype observed in Df*drmP2* embryos, this might be explained if *odd* genes were controlling the production of non-autonomous growth signals. In addition, the *odd*-expressing cells could contribute to the tubules themselves. We tested the second possibility by following the lineage of *odd*-expressing cells by using a lineage tracing system (see Materials and methods). While in *odd>lacZ* larvae, X-gal positive cells were confined to the ureters, in *odd*>lineage-*lacZ* embryos, positive cells were found along the distal tubules, indicating that *drm/sob/odd*-positive ureteric cells give rise to tubule cells that lose expression of *odd* genes (Figs. 7G, H).

When overexpressed, none of the four *odd* genes induced or expanded the renal tissue. Only the overexpression of *odd* (Figs. 7I–K), and to a lesser extent that of *sob* (not shown), resulted in a widening and shortening of the tubules and larger ureters, consistent with an alteration of tubule extension. Our results suggest that *drm*, *odd* and *sob* may be required for proper renal tubules development. This requirement is likely

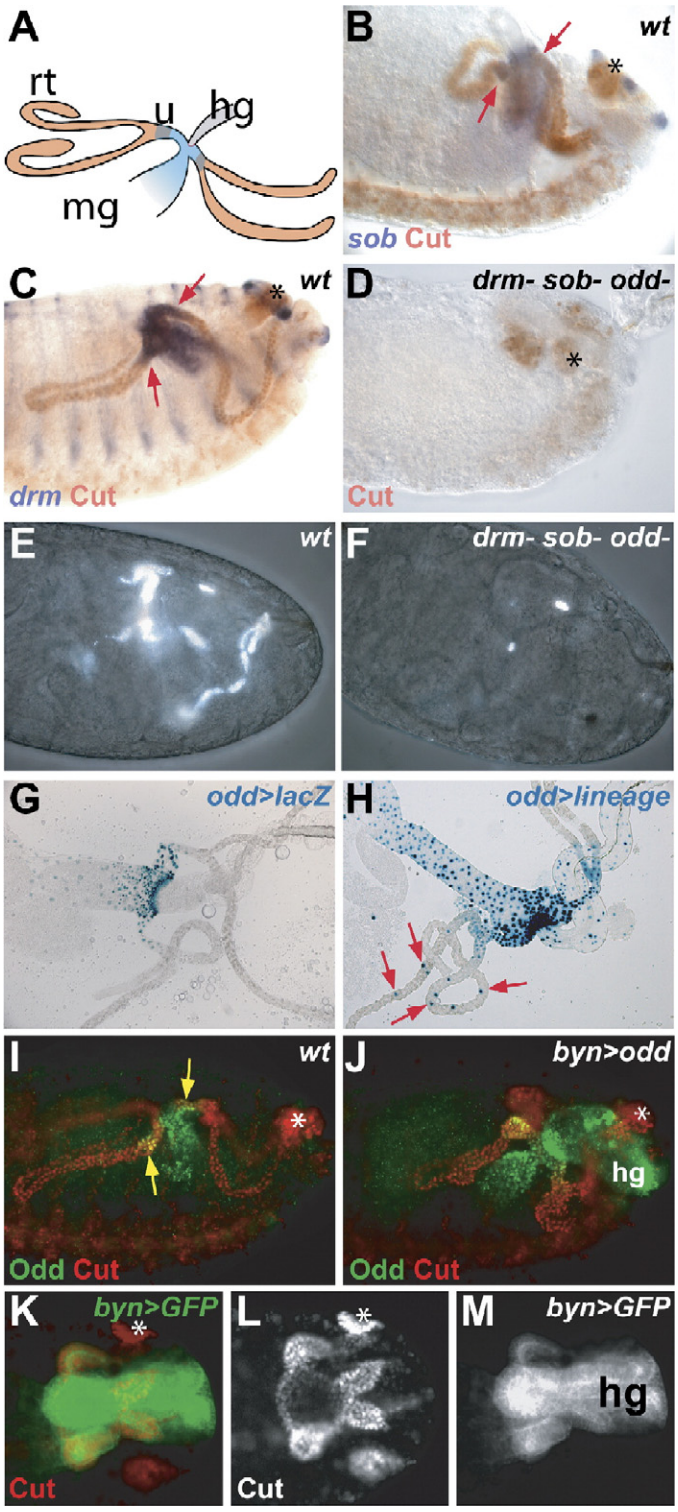

Fig. 7. *Odd* genes expression and requirement for renal tubule development in *Drosophila*. (A) Schematic representation of the late embryonic RTs. The domains of expression of *cut* (orange) and of *odd*, *sob* and *drm* (blue) are shown. *cut* and *drm/sob/odd* overlap in the ureters, shown in gray. Mg: midgut; hg: hindgut; rt: renal tubules; u: ureter. (B, C, I) Expressions of *sob* (B), *drm* (C) and *odd* (I) are similar and co-localize with Cut in the ureters (arrows). (B, C) *sob* and *drm* expression is detected by *in situ* hybridization (purple) and that of *cut* by immunohistochemistry (orange). Overlap is seen as dark gray. (I) Odd (green) and Cut (red) expression is detected by immunofluorescence. Overlap is seen in yellow. Ureters are marked by arrows. (D) Df*drmP2* mutant embryo (labeled as drm^{-} sob^{-} odd^{-}), showing rudimentary Cut-expressing tubules. (E, F) Uric acid excretion in wild-type (E) and Df*drmP2* (F) late embryos, observed under phase contrast optics. (G, H) Histochemical X-Gal staining of RTs of *odd>LacZ* (G) and *odd*>lineage (H; see Materials and methods) L3 larvae. Nuclei of X-Gal positive cells (blue) are seen along the distal tubules in *odd*>lineage (arrows; H) but not in *odd>lacZ* tubules. (J) *byn>odd* late embryo, co-stained for Odd (green) and Cut (red). Tubules (red) and ureters (yellow) are wider, and tubules are shorter. (K, L, M) Early stage 13 *byn>srcGFP* (green) embryo, co-stained for Cut (red). The Cut-positive RT buds are included within the *byn* domain. (*) marks the Cut-expressing posterior spiracles in all panels.

to occur after the Malpighian tubule primordia have been specified.

Discussion

Osr1 and 2 genes function at the top of the genetic hierarchy controlling pronephric development

Here we show that the two paralogues *Osr1* and *Osr2* are expressed at early stages in the intermediate mesoderm. *Osr1* in zebrafish and *Osr2* in *Xenopus* are first detected before the earliest markers of kidney development. This is similar to what was described for mouse *Osr1* (So and Danielian, 1999; Wang et al., 2005; James et al., 2006). However, in contrast to the situation found in mammals, where *Osr2* seems dispensable for kidney development (Lan et al., 2004), our morpholino experiments indicate that both *Osr1* and *Osr2* are required for proper pronephros development in *Xenopus* and zebrafish. In *Xenopus*, both genes are coexpressed just at the time the pronephros territory is being defined, as determined by the expression of *Xlim1* and *Pax8*. This is consistent with both genes being required for the early specification of the kidney anlage. The fact that we do not detect synergistic defects when impairing simultaneously both genes indicates that *Osr1* and *Osr2* are required additively for this specification. In contrast, in zebrafish, *Osr1* precedes the activation of early kidney markers while the onset of *Osr2* expression is delayed until the 8-somite stage, when the early kidney markers are already activated but still there is no histological sign of kidney tissue (Drummond et al., 1998). In mice, the onset of *Osr2* is further delayed, only appearing at the 18-somite stage, when mesonephros are already differentiating (Lan et al., 2001). The degree of delay in the activation of *Osr2* expression correlates with the functional requirement of these genes: while in zebrafish knockdown of *Osr2* mildly affects the expression of early pronephric markers, but severely impairs differentiation of the kidney, the knock-out of *Osr2* in mice seems not to have any effect (Lan et al., 2004). Recent experiments show that the overexpression of *Osr1* is able to induce ectopic kidney markers in chicken (James et al., 2006). It will be interesting to assay if overexpression of *Osr2* can also promote kidney formation in chick to determine whether the *Osr2* gene of higher vertebrates retains the functional capabilities of its paralogue *Osr1*.

In both *Xenopus* and zebrafish, the expression of both genes diverges during pronephros formation, one paralogue being expressed in more proximal segments than the other. Thus, *Osr* genes may provide distinct late functions during pronephric organogenesis. This functional diversification seems to have proceeded further in the lineage leading to mammals as *Osr1* has an additional role in heart development (Lan et al., 2004).

The knockdown of *Osr1* and *Osr2* results in the loss of all pronephric structures, including the glomerulus. However, their ectopic expression activates several early and late markers, but not the glomerulus-specific marker *Wt1* (not shown). Hence, this structure seems to be missing in the ectopic renal tissue. Osr proteins activate *Pax2/8*, which can downregulate *Wt1* (Majumdar et al., 2000). Therefore, strategies devised at inducing functional renal tissue by making use of *Osr* expression should overcome this problem. A transient *Osr* expression might solve it as it would allow early specification of the whole pronephric primordium, and not interfere with the later formation of the glomerulus.

Our results showing that *Osr* genes can drive the development of ectopic pronephros, together with the expression and functional data, suggest that they lay atop the kidney genetic program. Nevertheless, only the dorsal region of the embryo was competent to develop ectopic renal tissue upon *Osr* mRNA injection. Similar results were found with *Xlim1* and *Pax8* co-injection experiments (Carroll and Vize, 1999). In chick embryos, a gradient of BMP activity patterns mesodermal fates with highest signaling levels at the lateral mesoderm inhibiting intermediate fates, including *Osr1* expression and renal development (James and Schultheiss, 2005). Intermediate levels would allow acquisition of intermediate mesoderm fates indirectly, through the relief of a transcriptional repressor activity on intermediate mesoderm genes, such as *Osr1* (James and Schultheiss, 2005). Therefore, intermediate and medial (dorsal) regions would be competent to develop renal tissue. This coincides with the regions in *Xenopus* where *Osr* mRNA injection widens the endogenous pronephros or induces ectopic pronephric tissue. There was no correlation between ectopic pronephros and muscle loss (a derivative of dorsal mesoderm), arguing against a muscle-to-kidney transformation. In zebrafish, injected *zOsr* mRNAs enlarged the pronephros, but only occasionally induced ectopic tissue. This suggests strong restrictions in the competence of the dorsal mesoderm in zebrafish.

The ectopic renal tissue was patchy, while the distribution of ectopic Osr protein was broader and continuous (not shown). Possibly, a lateral inhibition process prevents subsets of Osr expressing cells from differentiating as kidney tissue. Evidently, some sort of signaling, of unknown nature, occurs between the ectopic developing pronephros and the neighboring cells, as shown by the neural tube overgrowths associated with the ectopic renal tissue.

Osr proteins act as transcriptional repressors during kidney development

Native XOsr proteins and the constitutive repressors XOsr-EnR similarly induced ectopic kidney tissue in *Xenopus*, which indicates that vertebrate Osr proteins function as transcriptional repressor *in vivo*. *Drosophila* Odd also acts as a transcriptional repressor during embryonic segmentation by directly binding to the Groucho co-repressor (Goldstein et al., 2005). This interaction occurs through, and requires the C-terminal "engrailed homology 1" (eh1) motif. In *Xenopus*, we show that *odd* mRNA also induces ectopic nephrogenesis, an ability that depends on the eh1 domain. This suggests that some member(s) of the vertebrate Transducin-like Enhancer of Split (TLS) family of Groucho homologues (Chen and Courey, 2000) is recruited by Odd. Moreover, the repressor activity of vertebrate Osr products

may similarly require interaction with TLS co-repressors. Indeed, we identified a putative eh1 motif in vertebrate Osr1 and Osr2 (Supplementary Fig. 1) that is located N-terminal to the zinc fingers, instead of at the C-terminal end as in Odd.

That the activation of the kidney genes *Pax8* and *lim1* requires the repressor activity of Osr proteins implies the existence of at least one additional intermediate repressor in the cascade. Foxc1 and/or Foxc2 are possible candidates. These transcription factors are required for somites development (Topczewska et al., 2001) and are necessary and sufficient to repress intermediate mesoderm markers, such as *Osr1* and *lim1* (Wilm et al., 2004). Still, we do not favor this hypothesis as *Osr1* and *Osr2* morphants did not show expanded somites associated to the loss of pronephros.

Odd genes are expressed in the renal organs of Drosophila and may be required for their development

We find that *Odd* genes may also be required for the development of the renal organs of *Drosophila*. *drm*, *odd* and *sob* are expressed in the ureters of the Malpighian tubules, and embryos homozygous for a deficiency that removes at least 30 predicted genes, including *drm*, *odd* and *sob*, form rudimentary renal structures with impaired excretory activity, a phenotype reminiscent of that seen in *Kr* and *cut* mutants (Harbecke and Janning, 1989). While this may reflect a requirement for these genes in *Drosophila* renal development, other genes within this deficiency may also contribute to the phenotype. We have found that cells born in the *drm/sob/odd* expression domain are incorporated into the tubules. Nevertheless, a failure in this cell contribution does not seem to explain the dramatic reduction of Cut cells in the Df*drmP2* mutants and therefore it is likely that, in these embryos, an additional cell non-autonomous growth signal is defective.

Renal organs, in charge of nitric waste excretion and osmoregulation, are pervasive among metazoans. Although it is conceivable that a kidney precursor existed in the common ancestor of both insects and vertebrates, embryological studies indicated otherwise. Vertebrate kidneys have a mesodermal origin, while insect renal tubules are formed mostly as an ectodermal derivative. Nevertheless, recent work raises again the subject of homology. Cells of mesodermal origin undergo a mesenchymal to epithelial transition and then give rise to the stellate cells (Denholm et al., 2003). Mesenchymal to epithelial transition is also characteristic of mesoderm mesenchymal cells while forming the vertebrate kidney. In addition, several fly renal tubules genes such as *Kr*, *cut* and *hibris* have vertebrate homologues (*Glis2*, *Cux-1* and *nephrin*, respectively) either expressed or having a role in kidney development (Sharma et al., 2004; Vanden Heuvel et al., 1996; Zhang et al., 2002). However, these genes are expressed too late to play a role in the specification of vertebrate renal organs. Therefore, *Odd/Osr* genes are the first ones that seem to participate during early stages of renal development in both vertebrates and invertebrates. This molecular conservation might underlie a deep evolutionary homology between different kidney types. Alternatively, *Odd/Osr* genes might be used in a conserved molecular cassette engaged in forming and/or patterning tubular organs, as they do during foregut, hindgut (reviewed in Lengyel and Iwaki, 2002) and renal tubules (this work) development in *Drosophila*, or nephron formation in vertebrate kidneys.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.063.

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Abbreviations

A	Anterior
A(1,2,3)	Antenna segment (1,2,3)
AP	Antero-posterior
aa	amino acid
AEL	After egg laying
Antp	Antennapidea
Ar	Arista
Arm	Armadillo
Ato	Atonal
bHLH	Basic Helix-Loop-Helix
BMP	Bone Morphogenetic Protein
Bowl	Brother of odd-skipped with entrails limited
C2H2	Cysteine-Cysteine/Histidine-Histidine
C2HC	Cysteine-Cysteine/Histidine/Cysteine
CC	Cone cell
Cdk	Cyclin-dependent kinase
Ci	Cuibitus interruptus
Cl	Claw
Ct	Cut
Cx	Coxa
Cyc	Cyclin
D	Dorsal
DV	Dorso-ventral
Dac	Dacshund
Dan	Distal antenna
Danr	Distal antenna related
DD	Dach Domain
Di	Distal
Disco	Disconnected
Dl	Delta
Dlg	Disc-large
Dll	Distal-less
DNA	Deoxyribonucleic acid
Dpp	Decapentaplegic
Drm	Drumstick
E(spl)	Enhancer of split
ED	Eya Domain
EGFR	Epithelial Growth Factor Receptor
Emc	Extramacroachaetea
En	Engrailed
Exd	Extradenticle
Ey	Eyeless

Eya	Eyes absent
Eyg	Eyegone
F	Forked
Fe	Femur
FGF	Fibroblast Growth Factor
FLP	Flipase
FMW	First Mitotic Wave
FRT	Flipase Recombination Target
GFP	Green Fluorescent Protein
GMR	Glass Multimer Reporter
H	Hairy
HD	Homeodomain
Hh	Hedgehog
Hth	Homothorax
IG	Intermediate Group
JO	Johnston's Organ
kb	Kilo base
L	Instar larvae
Lin	Lines
M	Minute
Mad	Mothers against decapentaplegic
MAPK	Mitogen-Activated Protein Kinase
MARCM	Mosaic Analysis with a Repressive Cell Marker
ME	Main Epithelium
Med	Medea
MF	Morphogenetic Furrow
Mp	Maxilary Palps
N	Notch
Odd	Odd-skipped
Opt	Optix
P	Posterior
PD	Proximo-distal
PC	Pigment cell
PE	Peripodila Epithelium
pH3	phosphoHistone 3
Pka	cyclic AMP-dependent protein kinase A
pMad	phosphorylated Mothers against decapentaplegic
Pnt	Punt
PPN	Preproneural
Pr	Proximal
Ptc	Patched
R	Photoreceptor cell

RDG	Retinal Determination Genes
RDGN	Retinal Determination Genes Network
RNA	Ribonucleic Acid
Rux	Roughex
Sal	Spalt
Sens	Senseless
Ser	Serrate
Smo	Smoothened
SMW	Second Mitotic Wave
So	Sine oculis
Sob	Sister of odd-skipped and drumstick
Spi	Spitz
Ss	Spineless
Stg	String
Su(H)	Suppressor of hairless
Ta	Tarsus
TGF	Transforming Growth Factor
Ti	Tibia
Tkv	Thickveins
Toy	Twin of eyeless
Tr	Trochanter
ts	Temperature-sensitive
Tsh	Teashirt
UAS	Upstream Activating Sequence
V	Ventral
W	White
Wg	Wingless
Y	Yellow
Zn-f	Zinc-finger

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Graças a esse tempo de formação, tive a oportunidade de entrar em contacto com a Biologia de Desenvolvimento. Recordo que foi no primeiro módulo do programa que, de imediato, tive que preparar um artigo sobre o desenvolvimento de olho de *Drosophila melanogaster*. Um bom presságio, diria eu! A minha decisão de entrar nesta área acabou por ser reforçada num Congresso de Bioquímica, onde assisti a uma apresentação interessante e entusiasta do Dr. Fernando Casares, como mais tarde verifiquei ser seu apanágio.

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Com o Zé e com a Sofia, mudei-me para Sevilha. Os primeiros meses de adaptação não foram fáceis, mas realmente o apoio dos dois ajudou a superar a falta da família e dos amigos. Além do lab, a Sofia teve o 'azar' de compartir casa comigo. Gostaria de agradecer o apoio e a paciência, dentro e fora do lab. Muito obrigado! No lab, tive a sorte de partilhar bancada com o Zé e absorver diariamente os seus conhecimentos e valiosos conselhos. O Zé é dos companheiros que toda a gente quer ter a seu lado. Verdadeiramente altruísta, não hesitava em deixar o seu trabalho para ajudar e, com uma paciência imensurável, explicava e voltava a explicar o que quer que fosse. Obrigado pelo apoio e incentivo, pelas discussões científicas através da nossa 'barreira clonal' e pelos conhecimentos transmitidos! Muito obrigado!

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Mais recentemente, a chegada de um novo elemento ao lab trouxe animação e *guasa*. Obrigado Cris pela simpatia e boa disposição! Boa sorte para o teu PhD!

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Catarina

(Vó, estou de volta a casa!)