

## Functional analysis of the eye genetic network in planarian regeneration

DAVID PINEDA, JAVIER GONZALEZ-LINARES, MARIA MARSAL and EMILI SALÓ\*

Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Spain

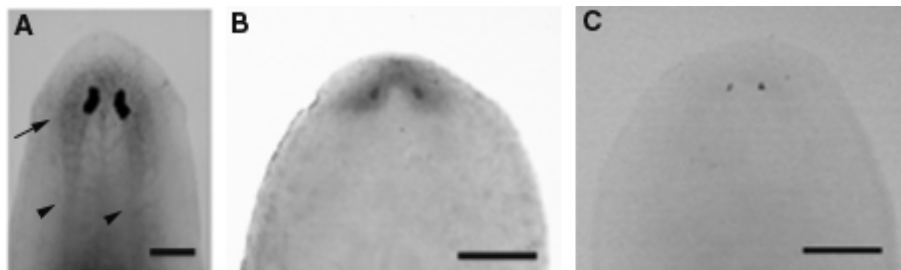
**ABSTRACT** Metazoan eyes are specified by the concerted action of several conserved nuclear factors that act co-ordinately in a genetic network. *Pax6*, a member of the paired-box family and *sine oculis*, a homeobox containing gene, are some of the players in that conservative genetic cascade that we have characterized in Platyhelminthes. Freshwater planarians detect light intensity through their eyespots. Although being very simple, they contain two cell types: photoreceptor and pigment cells. The photoreceptor cells are bipolar nerve cells that connect directly to the cephalic ganglia. They also differentiate into a microvilli extent from the dendrite, the rhabdome, where the opsin photopigment accumulates. The pigmented cells aggregate to form a cup shape in the dorsal head region. In our search for the planarian eye genetic network we have characterized two *Pax6* genes and two *sine oculis* genes. Functional analysis by loss of function using RNA interference (RNAi) approaches shows that the two *Pax6* genes are partially dispensable, while the *sine oculis* gene homologous to the Six-1 family is essential for eye formation during head regeneration and for functional eye maintenance in adult planarians.

In the last 10 years, developmental genetic analysis has shown that numerous regulatory proteins are conserved across metazoan phyla. Such conservation was also observed for genes involved in the establishment of body plans, and even further the conservation of some genetic relationship between individual genes, so that similar genetic networks pattern analogous anatomical structures in a large diversity of organisms phylogenetically distant. One of those examples are the eyes and the *Pax6* network (Wawersik and Maas, 2000). *Pax6*, initially described in vertebrates and *Drosophila*, is a member of the paired-box gene family of transcription factors, and is critical for eye determination and differentiation in most metazoa. Such conserved function is not exclusive of *Pax6*, since some other downstream genes like *sine oculis*, eyes absent and

*dachshund* gene families seem to be conserved, defining an eye genetic cascade specified by seven genes in *Drosophila*. More recently, a new class of signalling molecules, EGF receptor and Notch appears to be acting upstream of that eye specification network (Kumar and Moses, 2001).

Planarians (Platyhelminthes; Turbellaria; Tricladida) belong to the Platyhelminthes phyla being phylogenetically located at the base of the lophotrochozoa protostomate clade (Carranza et al., 1997). They are well known for their regenerative capacity, being the only phyla where the adult organisms contain totipotent proliferative cells, neoblasts, which are responsible of such morphological plasticity. Another interesting point is the presence of simple eyespots that detect light intensity and are very useful for their photophobic behaviour. The planarian eyespots consist of two cell types: a bipolar nerve cell with a rhabdome as a photoreceptive structure and a cup-shaped structure composed of pigment cells. The bipolar cell connects directly to the cephalic ganglia by a nerve fibre that partially crosses to the opposed ganglia and produces a partial optic chiasma (Sakai et al., 2000). Such simple eyes have been considered very close to the prototypic eyes suggested by T.H. Morgan (Gehring and Ikeo, 1999). During head regeneration, new eyespots are formed from precursor cells located close to the new regenerated cephalic ganglia that probably differentiate into both cell types in a restricted area of the newly regenerated tissue or blastema.

We initiated the isolation of the planarian eye genetic network by the use of PCR amplification techniques using degenerated oligonucleotides of *Pax6* conserved regions; such strategy allowed us to isolate the first *Pax6* gene in the planarian *Girardia tigrina* that we call *GtPax6B*. *In situ* hybridization on paraffin sections and electronic microscopy showed a weak and continuous expression in both eye cell types of adult planarians and during regeneration as well. In addition RT-PCR axial studies showed an ubiquitous

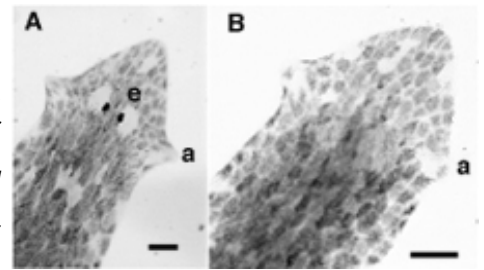


**Fig. 1. *GtPax6A* expression by whole mount *in situ* hybridization.** Planarian heads are shown from the dorsal surface. **(A)** Adult planarian; arrow indicates the expression in the cephalic ganglia, while the arrowheads indicate the expression in the ventral nerve cords. **(B)** 7 days head regenerating planarian; eye spots are already differentiated. **(C)** 7 days head regenerating dsRNA injected planarian; no expression can be observed in the head blastema, although the eyes start to differentiate. Bars, 0.5 mm.

axial expression indicating that it should be expressed in regions other than just the eye cells (Callaerts *et al.*, 1999). Later on we isolated a second Pax6 gene more canonical to the Pax6 consensus sequences that we call *GtPax6A*. Comparative sequence analysis confirms that both planarian genes are *bona fide* Pax6, conserving specific residues of Pax6 family in the paired and home domain regions. Such duplication is not homologous to the Pax6 duplication observed in insects, and can be considered a specific duplication in the Platyhelminthes phyla. *In situ* hybridization studies showed an ubiquitous expression of *GtPax6A* in the whole central nervous system (Fig. 1A). During head regeneration Pax6 is activated in the new cephalic ganglia (Fig. 1B). Electron microscopy *in situ* hybridization also showed expression in the eye cells (work in progress). Loss of function studies were performed injecting dsRNA into the head region of the adult organisms or in the region close to the blastema in the head regenerating planarians. The Pax6 inhibition was checked by whole mount *in situ* hybridization (Fig. 1C) and by RT-PCR with specific oligonucleotides located in different exons to avoid amplification by genomic DNA contamination. In head regenerating organisms we can observe the eye induction (Fig. 1C), implying that loss of the two Pax6 genes does not inhibit the eye regeneration and consequently indicates that Pax6 can be substituted by some other genes of the eye network. In *Drosophila*, the ectopic expression of *optix*, a Six-3 homologous gene, leads to the formation of ectopic eyes in the antennal disc independently of *eyeless*, the Pax6 homologous gene (Seimiya and Gehring, 2000). Both results support the existence of other *eyeless* independent genetic pathways to induce eyes.

A second group of eye genetic network components that we have isolated and analyzed were the planarian *sine oculis* genes. We will discuss the results obtained with the first gene *Gtsix-1*, which belongs to the six-1 family of *Drosophila* and vertebrates. *Gtsix-1* is expressed in the precursor eye cells at the first head regeneration stages and is continuously maintained through the whole regenerative process, even in the fully differentiated photoreceptor eye cells, indicating again that *Gtsix-1* is necessary for eye induction and maintenance. Loss of function by RNAi in adult planarians produces a loss of *Gtsix-1* mRNA in 24 hours and, as a consequence, the opsin transcript level decreases (Pineda *et al.*, 2001). The injected organisms lost the photophobic response due to the light detection incapacity. In head regenerating planarians loss of function of *Gtsix-1* produces a non-eye phenotype with a completely differentiated head with a new cephalic ganglia and functional sense organs, but with no differentiated eyes (Fig. 2)

**Fig. 2. Inhibition of eye-regenerative capacity by *Gtsix-1* dsRNA injection.** Bright-field images of dorsal view after 31 days of regeneration. (A) control. (B) Injected organisms. a, auricles; e, eye spots. Bars, 0.5 mm.



(Pineda *et al.*, 2000). Such eye inhibited organisms can re-induce the eye later on if we stop dsRNA injections for a period of three weeks, indicating that planarians are model organisms where the morphogenetic mechanisms are always activated and ready to induce new structures or morphogenetic changes.

#### Acknowledgements

This work was supported by grants from the DGICYT (to E.S.) (PB98-1261-CO2-01), and by FPI fellowship (to M.M.) from Ministerio de Ciencia y Tecnología; and (to D.P.) from Universitat de Barcelona.

#### References

- CALLAERTS, P., MUÑOZ-MARMOL, A.M., GLARDON, S., CASTILLO, E., SUN, H., LI, W.H., GEHRING, W.J. and SALÓ, E. (1999). Isolation and expression of a *Pax-6* gene in the regenerating and intact Planarian *Dugesia(G)tigrina*. *Proc. Natl. Acad. Sci. USA* 96: 558-563.
- CARRANZA, S., BAGUÑÀ, J. and RIUTORT, M. (1997). Is the Platyhelminthes a Monophyletic Primitive group? An assessment using 18S rDNA sequences. *Mol. Biol. Evol.* 14: 485-497.
- GEHRING, W.J., and IKEO, K. (1999). Pax 6 mastering eye morphogenesis and eye evolution. *Trends Genet.* 15: 371-377.
- KUMAR, J.P. and MOSES, K. (2001). EGF receptor and Notch signaling act upstream of *Eyeless/Pax6* to control eye specification. *Cell* 104: 687-697.
- PINEDA, D., GONZALEZ, J., CALLAERTS, P., IKEO, K., GEHRING, W.J. and SALÓ, E. (2000). Searching for the prototypic eye genetic network: *sine oculis* is essential for eye regeneration in planarians. *Proc. Natl. Acad. Sci. USA* 97: 4525-4529.
- PINEDA, D., GONZALEZ, J., MARSAL, M. and SALÓ, E. (2001). Evolutionary conservation of the initial eye genetic pathway in planarians. *Belg. J. Zool.* 131 (Supplement): 77-82.
- SAKAI, F., AGATA, K., ORII, H. and WATANABE, K. (2000). Organization and regeneration ability of spontaneous supernumerary eyes in planarians- eye regeneration field and pathway selection by optic nerves-. *Zool. Sci.* 17: 375-381.
- SEIMIYA, M. and GEHRING, W.J. (2000). The *Drosophila* homeobox gene *optix* is capable of inducing ectopic eyes by an *eyeless*-independent mechanism. *Development* 127: 1879-1886.
- WAWERSIK, S. and MAAS, L. R. (2000). Vertebrate eye development as modeled in *Drosophila*. *Human Molecular Genetics* 9: 917-925.