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Development of pigment-cup eyes in the polychaete *Platynereis dumerilii* and evolutionary conservation of larval eyes in Bilateria

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SUMMARY

The role of *Pax6* in eye development in insects and vertebrates supports the view that their eyes evolved from simple pigment-cup ocelli present in their last common ancestors (Urbilateria). The cerebral eyes in errant polychaetes represent prototype invertebrate pigment-cup ocelli and thus resemble the presumed ancestral eyes. We have analysed expression of conserved eye specification genes in the early development of larval and adult pigment-cup eyes in *Platynereis dumerilii* (Polychaeta, Annelida, Lophotrochozoa). Both larval and adult eyes form in close vicinity of the optic anlagen on both sides of the developing brain ganglia. While *pax6* is expressed in the larval, but not in the developing, adult eyes, expression of *six1/2* from trochophora stages onwards specifically outlines the optic anlagen and thus covers both the developing larval and adult eyes. Using *Platynereis rhabdomeric opsin* as differentiation marker, we show that the first pair of adult eye photoreceptor cells is detected within bilateral clusters that transitorily express *ath*, the *Platynereis atonal* orthologue, thus resembling proneural sensory clusters. Our data indicate that – similar to insects, but different from the vertebrates – polychaete *six1/2* expression outlines the entire visual system from early developmental stages onwards and *ath*-positive clusters generate the first photoreceptor cells to appear. We propose that *pax6*-, *six1/2*- and *ath-positive* larval eyes, as found in today's trochophora, were present already in Urbilateria.

Key words: *Platynereis*, Eye, Evolution, Larval eyes, Adult eyes, *six*, *pax6*, Lochotrophozoa, Annelids

INTRODUCTION

The finding that *eyeless/pax6* and *sine oculis/six* transcription factors play important roles in eye development in insects and vertebrates (Cheyette et al., 1994; Chow et al., 1999; Halder et al., 1995a; Hill et al., 1991; Loosli et al., 1999; Quiring et al., 1994; Zuber et al., 1999) has initiated a heated debate about homology of eyes in Bilateria (Arendt and Wittbrodt, 2001; Gehring and Ikeo, 1999; Pichaud et al., 2001). All bilaterian eyes should trace back to a common, *pax6*-dependent precursor resembling the 'prototype eye' of Charles Darwin (Gehring and Ikeo, 1999). Still, the notion of eye homology across the Protostomia and Deuterostomia split is disputed from the morphological viewpoint (Nilsson, 1996; Salvini-Plawen and Mayr, 1977). To advance on the controversial issue of eye homology, molecular studies on diverse eye types have been initiated in Bilateria other than insects and vertebrates. In Protostomia, these involve the lens eyes in cephalopods (Tomarev et al., 1997), and the pigment-cup-eyes in nemertines (Loosli et al., 1996) and in planarians (Callaerts et al., 1999). In Deuterostomia, the simple pigment-cup eyes in amphioxus (Glardon et al., 1998) and in ascidians (Glardon et al., 1997) have been studied. A caveat for comparison, however, is that all eyes investigated so far in Protostomia are either highly derived (squid lens eyes and insect compound eyes) or developmental stages are not easily accessible (nemertines and planarians). We have therefore investigated eye development in the marine polychaete *Platynereis dumerilii*, which has been chosen for its ancestrality and central position in Lophotrochozoa, and is easy to keep in breeding culture (Arendt et al., 2001; Dorresteijn et al., 1993). This species forms one pair of larval, and two pairs of adult pigment-cup eyes, and all stages of embryonic and larval development can be easily studied.

The paired larval eyespots of the *Platynereis* trochophora larva are composed of only one pigment cell and one photoreceptor cell (Fig. 1A) (Rhode, 1992), and thus match the bilaterian prototype two-celled eye (Gehring and Ikeo, 1999). They are referred to as inverse, because the photoreceptor, the rhabdome, is oriented towards the concavity of the pigment cell. Similar larval eyes are found in the primary ciliary larvae of sipunculan worms, flatworms, molluscs and acon worms. Although structurally divergent to some extent, all larval eyes form at comparable positions left and right of the apical organ, and their widespread distribution in Bilateria makes them good candidates for interphyletic homology (Arendt and Wittbrodt,

2001). The two pairs of *Platynereis* adult pigment-cup eyes show a very characteristic structure with photoreceptoral cell processes traversing the pigment cell layer (Fig. 1B,C), shared with adult eyes in other carnivorous polychaetes, various molluscs, sipunculans, and onychophorans (Eakin and Westfall, 1965; Hermans and Eakin, 1974; Salvini-Plawen and Mayr, 1977). Deviating from the larval eyes, they are referred to as everse, because the rhabdomeric photoreceptors are oriented away from the concavity of pigment. *Platynereis* adult eyes represent a second separate type of eye that is distinct from the larval eyes that might equally be phylogenetically conserved, at least among Protostomia (Arendt and Wittbrodt, 2001).

We have studied four eye specification genes conserved in evolution with respect to the development of *Platynereis* larval and adult eyes. *pax6* orthologues are essential for eye formation in vertebrates (Chow et al., 1999; Hill et al., 1991; Walther and Gruss, 1991) and *Drosophila* (Halder et al., 1995a; Quiring et al., 1994). This has led to the hypothesis that *pax6* has an evolutionary conserved function in eye development (master control gene hypothesis) (Gehring and Ikeo, 1999; Halder et al., 1995a; Quiring et al., 1994). *pax6* orthologues are expressed in the developing lens eye of the squid (Tomarev et al., 1997), in the ocelli of regenerating nemertines (Loosli et al., 1996; Tarpin et al., 1999), in the frontal organ of amphioxus (Glardon et al., 1998), and in the ascidian ocellus (Glardon et al., 1997). Genes belonging to the *six1/six2* family of transcription factors are equally involved in eye development across phyletic boundaries. In *Drosophila*, *sine oculis* is expressed in, and required for, the formation of the entire visual system, comprising optic lobes, larval eyes (Bolwig organ), and developing lateral compound eyes and ocelli (Cheyette et al., 1994; Seimiya and Gehring, 2000). In planarians, *six1/2/sine oculis* is essential for the regenerating eyes (Pineda et al., 2000). The vertebrate *sine oculis* orthologues *six1* and *six2* are detected in differentiated ganglion cells of the retina in mouse (Kawakami et al., 1996; Oliver et al., 1995), and in *Xenopus* (Ghanbari et al., 2001). Orthologues of the bHLH transcription factor *atonal* are involved in cell type specification in the differentiating insect and vertebrate eyes. In the *Drosophila* eye disc, selection of R8 photoreceptor cells requires transient expression of *atonal* in a cluster of competent, 'proneural' cells (Jarman et al., 1994). Remarkably, *atonal* is not expressed in the R1-R7 photoreceptor precursors. It is required for their formation, however, because R1-R7 are induced by R8 (Jarman et al., 1994). *Atonal* is also indispensable for the formation of the larval Bolwig organ, where it is again active only in the first, but not in the secondary photoreceptor precursors (Daniel et al., 1999). Formation of the *Drosophila* ocelli also requires *atonal* function (Jarman et al., 1994). In mouse, frog, and fish, transitory expression of *ath5* precedes, and is required for, the determination of ganglion cells (Brown et al., 1998; Kanekar et al., 1997; Kay et al., 2001; Liu et al., 2001; Wang et al., 2001). Finally, the expression of *opsin* related genes is initiated in the differentiating photoreceptor cells. The opsin promoter is a direct downstream target of *pax6* in *Drosophila* (Papatsenko et al., 2001; Sheng et al., 1997). In Bilateria two distinct opsins can be distinguished: *rhabdomeric opsin (r-opsin)* that is expressed in invertebrate rhabdomeric receptor cells, and *ciliary opsin (c-opsin)* that is expressed in the vertebrate ciliary photoreceptor cells (Arendt and Wittbrodt, 2001).

We have isolated *pax6*, *six1/2*, *ath* and *r-opsin* orthologues from *Platynereis dumerilii* and investigated their expression in the developing eyes. Our study reveals distinct molecular identities for larval and adult eyes. While the developing larval eyes co-express *six1/2* and *pax6*, the developing adult eyes express *six1/2* only. Both the larval photoreceptors and the first differentiating photoreceptors of adult eyes emerge from cell clusters positive for *ath*. Our data reveal that the early, and common, expression of *pax6*, *six1/2* and *ath* transcription factors, as found in the *Platynereis* larval eye anlage is a shared feature across Protostomia, and this corroborates the notion that two-celled larval eyes, as found in the polychaete trochophora, were the evolutionary precursors for at least a subset of cerebral eyes in Bilateria (Arendt and Wittbrodt, 2001; Callaerts et al., 1997; Gehring and Ikeo, 1999; Halder et al., 1995b).

MATERIALS AND METHODS

Worm breeding culture

Embryonic, larval and developing adult stages were obtained from an established *Platynereis* breeding culture at the EMBL Heidelberg, following the protocol of (Dorresteijn et al., 1993).

Cloning of partial and full-length cDNAs

We isolated fragments of *Platynereis pax6*, *six1/2*, *ath* and *r-opsin* genes by nested PCR after reverse transcription of embryonic mRNA (48 hours). Degenerate primers for *pax6* (forward, GIGGIGTITTYGTIAAYGG; nested forward, TIGGIMGITAYTAY-GARACIGG; reverse, GCRAANACRTCNGGRTARTG; nested reverse, NGCYTCNGGNARRTCDATYT) were used for PCR: 5×(1 minute at 94°C, 2 minutes at 43°C, 4 minutes 72°C) then 35×(1 minute at 94°C, 2 minutes at 48°C, 4 minutes at 72°C) followed by 10 minutes at 72°C. Additional degenerate primers to detect potential paralogues (forward, GGICAYWSIGGNGTIAAYCA; nested forward, GGIGCIMGICCITGYGAYAT; reverse, ARNARNCKR-TCNCKDATYTCCCA; nested reverse, TCNCKDATYTCCC-ANGCRAA) were used under similar PCR conditions. Degenerate primers for *six1/2* (forward, CCNWSITTYGGNTTYACNCARGA; nested forward, ARGTNGCNTGYGTITGYGARGT; reverse KNGGNSWNGGRTANGGRTTRTG; nested reverse AARCAR-TAISWIGTYTCYTCICCRTC) were used for PCR: 5×(1 minute at 94°C, 2 minutes at 42°C, 4 minutes at 72°C) then 35×(1 minute at 94°C, 2 minutes at 47°C, 4 minutes at 72°C) followed by 10 minutes at 72ºC. Degenerate primers for *ath* (forward, ACNAA-YGTNGTNCARAARCA; nested forward, CARMGAMGNYTN-GCNGCNAAYGC; reverse, GCRTTDATRTANRTYTGNGCC-ATYTG; nested reverse, GCCATYTGGARNGTYTCRTAYTT) were used for PCR 5×(1 minute at 94°C, 2 minutes at 42°C, 4 minutes at 72°C) then 35×(1 minute at 94°C, 2 minutes at 47°C, 4 minutes at 72°C) followed by 10 minutes at 72°C. Degenerate primers for *r-opsin* (forward, CAYTGGACICARTTYCCICCIGT; nested forward, CARACGCCAGCIAAYATGTTYATHATHAA; nested nested forward, ATHCCICCITTYTTYGGITGG; reverse, ATNGCYTCIC-KRWAYTTIGGRTG; nested reverse, CTCTGCGTADATDATIGG-RTTRTGIAT) were used at 5×(1 minute at 94°C, 2 minutes at 43.3°C, 4 minutes at 72°C) then 35×(1 minute at 94°C, 2 minutes at 48.3°C, 4 minutes at 72°C) followed by 10 minutes 72°C.

Full-length clones were obtained by screening a 24 hour cDNA library (*pax6)*, or by 5′ and 3′ RACE on an embryonic 24 hour or 48 hour cDNA library using sequence-specific primers for *six1/2* (forward,

CACCTGCACAAGAACGAGTCGGTCCT; nested forward, GTCCTCAAAGCCAAGGCTGTCGTAG; reverse, CGTCTCCTCTCCGTCCCAGATAGTCC, nested reverse, CTCCTCACTCGGTATTTGCCCACAGC), *ath* (forward, ATGAACAGCCTGAACGGGG; nested forward, GTATGAAAGGGCAAAGACA; reverse, CCCCGTTCAGGCTGTTCAT; nested reverse, TGTTTTGCCCTTTCATAC), *r-opsin*: forward, TCCCTGAAGGATTCCAGACATCTTG; nested forward, AACTACACCTACGTCCTCGGCATGT; reverse, GCGAGCAGAGGGTGGATGAA; nested reverse, ACTCCAACGACGGCATAGGGTGTC) and plasmid specific primers (T7, T3) for 30 seconds at 94°C, 1 minute at 60°C and 4 minutes at 72°C. Identity of the clones was confirmed by sequencing [EMBL Nucleotide Sequence Database (Accession Numbers: *pax6*, AJ316541; *six1/2*, AJ316542; *ath*, AJ316543; *r-opsin*, AJ316544)].

Alignment and construction of phylogenetic trees

Protein sequences of a selected number of species were obtained from the database and aligned using CLUSTALX (Thompson et al., 1997). These alignments spanning the conserved domains such as HD (homeodomain), SD (Six domain), PD (paired domain) and bHLH (basic Helix-Loop-Helix) domain, were used to calculate a 1000-fold bootstrapped phylogenetic tree using the neighbour-joining method, excluding all positions with gaps in the alignment, and correcting for multiple substitutions, using the programme CLUSTALX (Thompson et al., 1997).

Whole-mount in situ hybridisation

Embryos were fixed in 4% paraformaldehyde/2× phosphate-buffered saline (PBS)-Tween (PFA/PTw) for 1 to 4 hours. An established in situ hybridisation protocol (Loosli et al., 1998) was followed with the modification of ProteinaseK treatment in 100 μg/ml for 4 minutes (24 hour larvae), or 10 minutes (72 hour young worm). After staining, embryos were refixed in (PFA/PTw), washed and cleared in 80% glycerol. Embryos were mounted in glycerol and pictures taken under Nomarski optics using a Zeiss Axiophot.

Immunostaining for acetylated tubulin

A commercially available MoAb to acetylated tubulin, clone no. 6-11B-1(SigmaT6793), was used that detects an interphyletically conserved epitope present in cilia and axons. Embryos and larvae were fixed as above, dehydrated in methanol, rehydrated in methanol/PTw, or taken from the postfixation solution after the in situ hybridisation procedure, and blocked for 2 hours in 1 ml 5% serum/PTW. Blocking solution was replaced by 150 μl monoclonal antibody (MoAb) to acetylated tubulin diluted 1:500 in serum/PTw, and incubated overnight at 4°C. Larvae were washed 6×10 minutes in PTW, and incubated for 2 hours at room temperature in sheep biotinylated Anti-Mouse IgG secondary Ab. After additional washes 6×10 minutes in PTw staining was performed using the Vectastain ABC Kit (Vector Laboratories). Post-staining treatment was done as described above.

RESULTS

Larval and adult eyes develop from the lateral optic anlagen

Larval eyes (Fig. 1A) are first visible at 22 hours of development as two faint spots of orange pigment in the larval episphere (Fig. 1D). Twenty-four hour trochophora larvae were immunostained with an anti-acetylated tubulin antibody (Fig. 1F) to visualise connections of larval photoreceptor cell axons to the larval central nervous system [for a general description

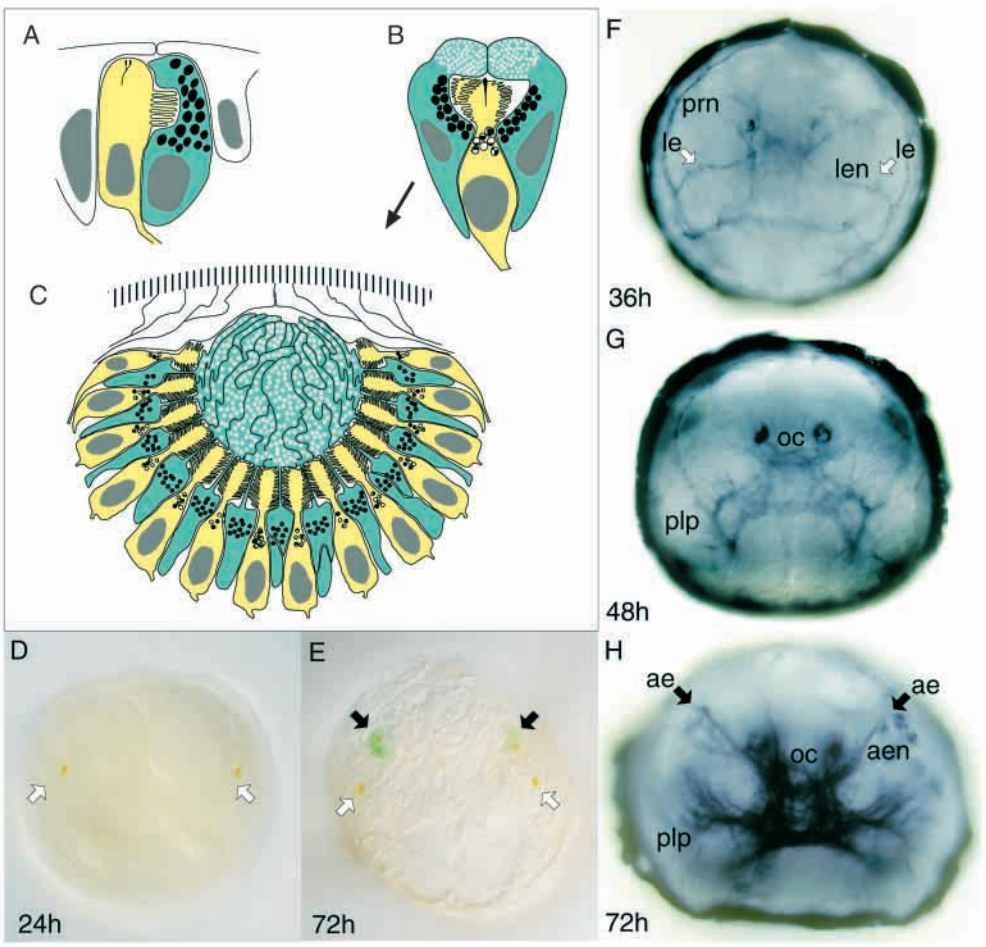

Fig. 1. Larval and adult eye development in *Platynereis dumerilii*. Ultrastructure of larval (A, 24 hours) and adult (B,C) eyes at 72 hours (B) and fully grown (C). Redrawn from EM micrographs (A,B; data not shown) and (C) after Fischer and Brökelmann (Fischer and Brökelmann, 1966). Yellow, rhabdomeric photoreceptor cells; green, pigment cells.
(D) Photomicrograph of 24 hour trochophora showing the yellow natural pigment of larval eyes (white arrows). (E) Larval (white arrows) and adult eyes at 72 hours. Green autofluorescence marks pigment cells of adult eye (black arrow).
(F-H) Staining of axonal scaffold with anti-acetylated tubulin antibody. Apical view showing larval axons and enervation of larval eyes at 36 hours of development (F). Axonal scaffold at 48 hours (G) and 72 hours (H), apical views. Black arrows, adult eyes. Abbreviations: aen, adult eye nerve; len, larval eye nerve; oc, optic commissure; plp, palpae anlagen; prn, prototroch ring nerve.

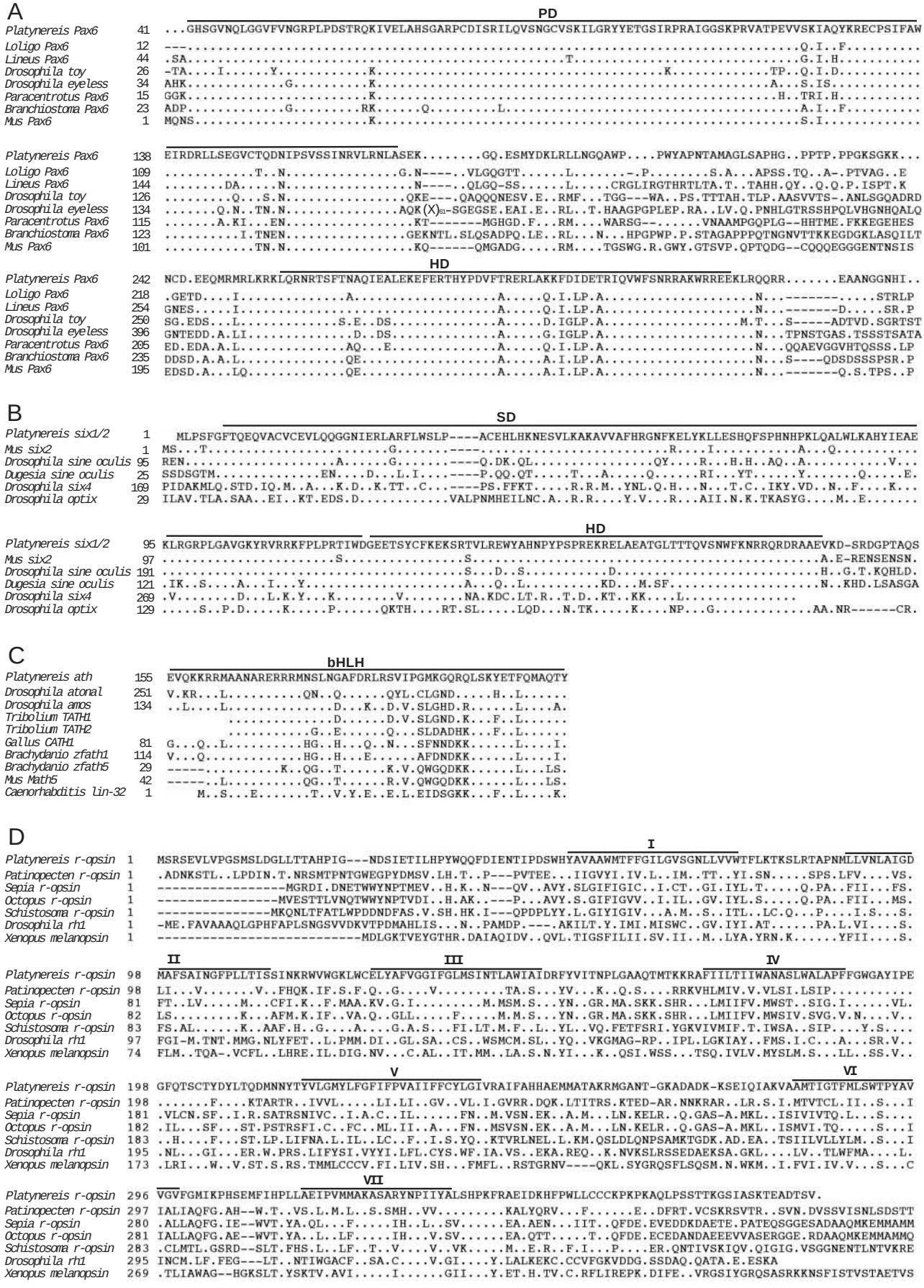
A
PD
Platynereis Pax6 41 ...GHSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQVSNGCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVSKIAQYKRECPSIFAW
Loligo Pax6 12 ---.....Q.I..F.....
Lineus Pax6 44 .SA.....T.....G.I.H.....
Drosophila toy 26 -TA....I.....Y.....K.....K.....TP..Q.I.D.....
Drosophila eyeless 34 AHK.....G.....K.....A...S.IS.....
Paracentrotus Pax6 15 GGK.....K.....H..TRI.H.....
Branchiostoma Pax6 23 ADP.....G.....RK.....Q.....L.....A.I..F.....
Mus Pax6 1 MQNS.....K.....S.I.....
Platynereis Pax6 138 EIRDRLLSEGVCTQDNIPSVSSINRVLRNLASEK.....GQ.ESMYDKLRLLNGQAWP....PWYAPNTAMAGLSAPHG..PPTP.PPGKSGKK...
Loligo Pax6 109 .....T..N.....G.N----..VLGQGTT.....L.....-.P.....S.A...APSS.TQ..A-.PTVAG..E
Lineus Pax6 144 .....DA.....N.....N----..QLGQ-SS.....L.....CRGLIRGTHRTLTA.T..TAHH.QY..Q.Q.P.ISPT.K
Drosophila toy 126 .....Q...S.N.....QKE---.QAQQQNESV.E..RMF...TGG---WA..PS.TTTAH.TLP.AASVVTS-.ANLSGQADRD
Drosophila eyeless 134 .....Q.N..TN.N.....AQK(X)61-SGEGSE.EAI.E..RL..T.HAAGPGPLEP.RA..LV.Q.PNHLGTRSSHPQLVHGNHQALQ
Paracentrotus Pax6 115 .....A.KI...EN.....KT-------MGHGD.F...RM....WARSG--.....VNAAMPGQPLG--HHTME.FKKEGEHES
Branchiostoma Pax6 123 .....I.TNEN.....GEKNTL.SLQSADPQ.LE..RL...N...HPGPWP.P.STAGAPPPQTNGNVTTKKEGDGKLASQILT
Mus Pax6 101 .....TN.N.....KQ------QMGADG.....RM....TGSWG.R.GWY.GTSVP.QPTQDG--CQQQEGGGENTNSIS
HD
Platynereis Pax6 242 NCD.EEQMRMRLKRKLQRNRTSFTNAQIEALEKEFERTHYPDVFTRERLAKKFDIDETRIQVWFSNRRAKWRREEKLRQQRR.....EAANGGNHI..
Loligo Pax6 218 .GETD....I.....A.....A.....Q.I.LP.A.....N...----------....STRLP
Lineus Pax6 254 GNES.....I.....A.....Q.I.LP.A.....N...-------D.....SR.P
Drosophila toy 250 SG.EDS...L.....S.E..DS.....A.....D.IGLP.A.....M.T...S-----ADTVD.SGRTST
Drosophila eyeless 396 GNTEDD.A.LI.....D..DS.....A.....G.IGLP.A.....N...TPNSTGAS.TSSSTSATA
Paracentrotus Pax6 265 ED.EDA.A.L.....AQ...E.....A.....Q.I.LP.A.....N...QQAEVGGVHTQSSS.LP
Branchiostoma Pax6 235 DDSD.A.A.L.....QE.....A.....A.I.LP.A.....N...S----QDSDSSSPSR.P
Mus Pax6 195 EDSD.A...LQ.....QE.....A.....A.I.LP.A.....N...-------Q.S.TPS..P
B
SD
Platynereis six1/2 1 MLPSFGFTQEQVACVCEVLQQGGNIERLARFLWSLP----ACEHLHKNESVLKAKAVVAFHRGNFKELYKLLESHQFSPHNHPKLQALWLKAHYIEAE
Mus six2 1 MS...T.....G.....----.....R....I.....A...Q.....
Drosophila sine oculis 95 REN.....A.....G.....----Q.DK.QL.....QY....R...H.H..AQ..A.....V...
Dugesia sine oculis 25 SSDSGTM.....EN....D...L.I....----P.QQ.QT.....T...A.....Q.....RI...YT.....Y.....Q.....E.
Drosophila six4 169 PIDAKMLQ.STD.IQ.M..A...K.D..K.TT..C...----PS.FFKT.....R.R.M..YNL.Q.H...N...T.C..IKY.VD..N..F....K...
Drosophila optix 29 ILAV.TLA.SAA..EI..KT.EDS.D.....VALPNMHEILNC.A..R.R....Y.V...R...AII.N.K.TKASYG....M..E.....
HD
Platynereis six1/2 95 KLRGRPLGAVGKYRVRRKFPLPRTIWDGEETSYCFKEKSRTVLREWYAHNPYPSPREKRELAEATGLTTTQVSNWFKNRRQRDRAAEVKD-SRDGPTAQS
Mus six2 97 .....S.....S.....A.E-RENSENSN.
Drosophila sine oculis 191 .....S...D..S.....D.....H..G.T.KQHLD.
Dugesia sine oculis 121 .IK..S....A...I...Y.....A...Q..L.....KD...M.SF.....N..KHD.LSASGA
Drosophila six4 269 .V.....D...L.K.Y...K.....V.....NA.KDC.LT.R..T.D..KT..KK....L.....
Drosophila optix 129 .....S..P.D.....K.....P.....QKTH....RT.SL.....LQD...N.TK.....K....NP...G.....AA.NR------CR.
C
bHLH
Platynereis ath 155 EVQKKRRMAANARERRRMNSLNGAFDRLRSVIPGMKGQRQLSKYETFQMAQTY
Drosophila atonal 251 V.KR...L.....QN..Q.....QYL.CLGND.....H..L.....
Drosophila amos 134 ..L....L.....D...K..D.V.SLGHD.R.....L....A.
Tribolium TATH1 .....D.....D.V.SLGND.K...F..L.....
Tribolium TATH2 .....G..E.....Q...SLDADHK...F..L.....
Gallus CATH1 81 G...Q..L.....HG..H...Q..N...SFNNDKK.....L....I.
Brachydanio zfath1 114 V...Q.....HG..H...E.....AFDNDKK.....L....I.
Brachydanio zfath5 29 -----.....K..QG..T.....K.V.QWGQDKK.....L...LS.
Mus Math5 42 -----..L.....QG..T.....R.V.QWGQDKK.....L...LS.
Caenorhabditis lin-32 1 M..S...E.....T..V.Y.E..E.L.EIDSGKK...F..L....K.
D
I
Platynereis r-opsin 1 MSRSEVLVPGSMSLDGLLTTAHPIG---NDSIETILHPYWQQFDIENTIPDSWHYAVAAWMTFFGILGVSGNLLVVWTFLKTKSLRTAPNMLLVNLAIGD
Patinopecten r-opsin 1 .ADNKSTL..LPDIN.T.NRSMTPNTGWEGPYDMSV.LH.T..P---PVTEE...IIGVYI.IV.L..IM..TT..YI.SN.....SPS.LFV....VS.
Sepia r-opsin 1 ----------MGRDI.DNETWWYNPTMEV..H.K..N---QV..AVY.SLGIFIGIC..I.CT..GI.IYL.T.....Q.PA..FII...FS.
Octopus r-opsin 1 ----------MVESTTLVNQTWWYNPTVDI..H.AK..---P...AVY.S.GIFIGVV..I.IL..GV.IYL.S.....Q.PA..FII...MS.
Schistosoma r-opsin 1 ----------MKQNLTFATLWPDDNDFAS.V.SH.HK.I---QPDPLYY.L.GIYIGIV...A.M..S..ITL..LC.Q...P....I.S...S.
Drosophila rh1 1 -ME.FAVAAAQLGPHFAPLSNGSVVDKVTPDMAHLIS...N..PAMDP.---.AKILT.Y.IMI.MISWC..GV.IYI.AT.....PA.L.VI....S.
Xenopus melanopsin 1 ----------MDLGKTVEYGTHR.DAIAQIDV..QVL.TIGSFILII.SV.II..M..LYA.YRN.K.....YFII....S.
II
III
IV
Platynereis r-opsin 98 MAFSAINGFPLLTISSINKRWVWGKLWCELYAFVGGIFGLMSINTLAWIAIDRFYVITNPLGAAQTMTKKRAFIILTIIWANASLWALAPFFGWGAYIPE
Patinopecten r-opsin 98 LI...V.....V..FHQK.IF.S.F.Q..G....V.....TA.S...YV...K..Q.S....RRKVHLMIV.V.VLSI.LSIP.....
Sepia r-opsin 81 FT..LV.....M...CFI.K..F.MAA.KV.G.I.....M.MSM.S...YN..GR.MA.SKK.SHR...LMIIFV.MWST..SIG.I.....VL.
Octopus r-opsin 82 LS.....K...AFM.K.IF..VA.Q..GLL.....F.....M.M.S...YN..GR.MA.SKK.SHR...LMIIFV.MWSIV.SVG.V.N....V..
Schistosoma r-opsin 83 FS.AL.....K..AAF.H..G....A....G.A.S...FI.LT.M.F..L..YL..VQ.FETFSRI.YGKVIVMIF.T.IWSA..SIP....Y.S....
Drosophila rh1 97 FGI-M.TNT.MMG.NLYFET..L.PMM.DI..GL.SA..CS..WSMCM.SL..YQ..VKGMAG-RP..IPL.LGKIAY..FMS.I.C...A...SR.V..
Xenopus melanopsin 74 FLM..TQA-.VCFL..LHRE.IL.DIG.NV...C.AL..IT.MM..LA.S.N.YI...K..QSI.WSS...TSQ.IVLV.MYSLM.S...LL..SS.V..
V
VI
Platynereis r-opsin 198 GFQTSCTYDYLTQDMNNYTYVLGMYLFGFIFPVAIIFFCYLGIVRAIFAHHAEMMATAKRMGANT-GKADADK-KSEIQIAKVAAMTIGTFMLSWTPYAV
Patinopecten r-opsin 198 .....F....KTARTR..IVVL.....LI.LI..GV..VL.I.GVRR.DQK.LTITRS.KTED-AR.NNKRAR..LR.S.I.MTVTCL.II..S...I
Sepia r-opsin 181 .VLCN.SF..I.R.SATRSNIVC..I.A.C..IL.....FN..M.VSN.EK..A.M...LN.KELR..Q.GAS-A.MKL..ISIVIVTQ.L...S....
Octopus r-opsin 182 .IL...SF...ST.PSTRSFI.C..FC..ML.II..A...FN..MSVSN.EK..A.M...LN.KELR..Q.GAS-A.MKL..ISMVI.TQ.....S...I
Schistosoma r-opsin 183 ..H....F...ST.LP.LIFNA.L.IL..LC..F..I.S.YQ..KTVRLNEL.L.KM.QSLDLQNPSAMKTGDK.AD.EA..TSIILVLLYLM..S...I
Drosophila rh1 195 .NL...GI...ER.W.PRS.LIFYSI.VYYI.LFL.CYS.WF.IA.VS..EKA.REQ..K.NVKSLRSSEDAEKSA.GKL....LV..TLWFMA....L.
Xenopus melanopsin 173 .LRI...W..V.ST.S.RS.TMMLCCCV.FI.LIV.SH...FMFL..RSTGRNV----QKL.SYGRQSFLSQSM.N.WKM..I.FVI.IV.V...S...C
VII
Platynereis r-opsin 296 VGVFGMIKPHSEMFIHPLLAEIPVMMAKASARYNPIIYALSHPKFRAEIDKHFPWLLCCCKPKPKAQLPSSTTKGSIASKTEADTSV.
Patinopecten r-opsin 297 IALIAQFG.AH--W.T..VS.L.M.L..S.SMH..VV.....KALYQRV...F.....E..DFRT.VCSKRSVTR..SVN.DVSSVISNLSDSTT
Sepia r-opsin 280 .ALLAQFG.IE--WVT.YA.QL...F.....IH..L..SV.....EA.AEN...IIT..QFDE.EVEDDKDAETE.PATEQSGGESADAAQMKEMMAMM
Octopus r-opsin 281 IALLAQFG.AE--WVT.YA..L..LF.....IH...V.SV.....EA.QTT.....T..QFDE.ECEDANDAEEEVVASERGGE.RDAAQMKEMMAMMQ
Schistosoma r-opsin 283 .CLMTL.GSRD--SLT.FHS.L..LF..T..V....V..VK.....M..E.R..F.I...P....ER.QNTIVSKIQV.QIGIG.VSGGNENTLNTVKRE
Drosophila rh1 295 INCM.LF.FEG---LT..NTIWGACF..SA.C....V.GI....Y.LALKEKC.CCVFGKVDDG.SSDAQ.QATA.E.ESKA
Xenopus melanopsin 269 .TLIAWAG--HGKSLT.YSKTV.AVI.....I.....GII...Y.ET.H.TV.C.RFLIREPK.DIFE..VRGSIYGRQSASRKKNSFISTVSTAETVS

Fig. 2. *Pd*-pax6, *Pd-six1/2*, *Pd-ath* and *Pd-opsin* alignments. Alignment of protostome and deuterostome *pax6* (A), six1/2 (B), atonal (C) and opsin (D) protein sequences. Conserved domains are indicated by a bar. Identical amino acids are indicated by a dot in the alignments, gaps are represented by '-'. Abbreviations: I to VII, conserved transmembrane domains in r-opsin; bHLH, basic helix loop helix; HD, homeodomain; PD, paired domain; SD, six domain.

of polychaete larval nervous systems see Heimler (Heimler, 1988)]. From the larval eyes (white arrows in Fig. 1F), traceable axons extend in opposite directions, medially towards the apical organ to contribute later to the optic commissure, and peripherally to connect to the prototroch ring nerve (Fig. 1F). Most likely these are collaterals of a single photoreceptor cell axon. Larval photoreceptor axons thus contribute to two bilateral nerves interconnecting prototroch ring nerve, larval eyes and apical organ (larval eye nerve, len, in Fig. 1F). Note that in the 2-day-old metatrochophora larva the episphere larval nervous system has been skewed medially, owing to the disproportionate growth of the ventral-peripheral anlagen of the palpae (plp in Fig. 1G).

The developing adult eyes (Fig. 1B) are morphologically visible at 53 hours of development, by the orange shading pigment in the first photoreceptor cell to form (not shown) Slightly later, at 60 hours, adult eyes consist of two

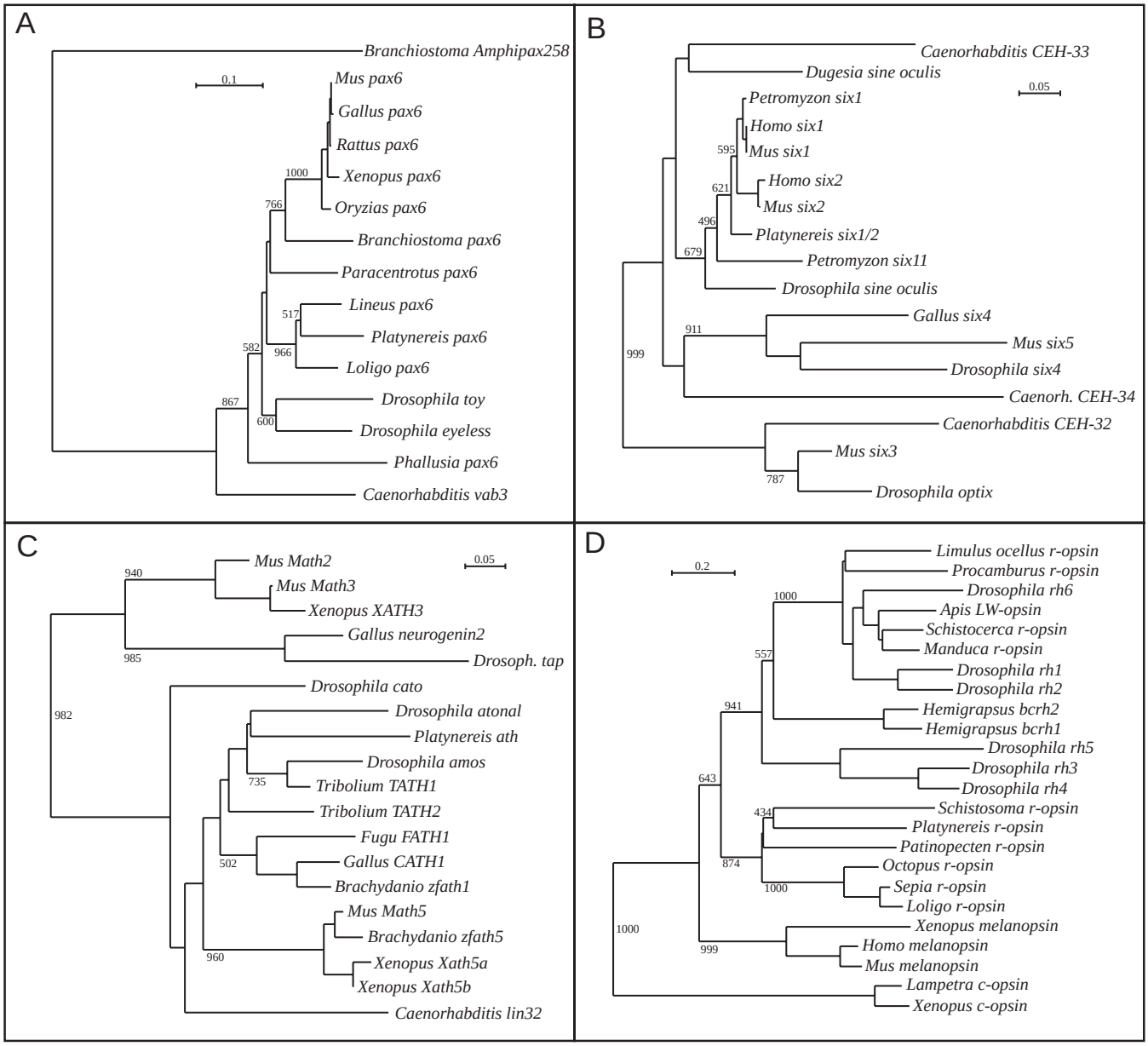

Fig. 3. Phylogenetic trees. Trees were calculated using amino acid sequences. Numbers at branching points are bootstrap values exceeding 500. (A) Phylogenetic tree of Pax6 proteins. Platynereis Pax6 is closest to the other lochotrophozoan Pax6 proteins described so far. (B) Phylogenetic tree of Six/Sine oculis proteins. Platynereis six1/2 clearly clusters within the Six1/Six2 subfamily. (C) Phylogenetic analysis of Atonal-related proteins shows close relationship of *Platynereis* ath with *Drosophila* atonal. (D) Clear distinction between rhabdomeric (r-) and ciliary (c-) opsis revealed by phylogenetic analysis. Platynereis opsin is a member of the r-opsins subgroup.

photoreceptor cells and two pigment cells (Fig. 1E) (Rhode, 1992). Notably, adult eye photoreceptor cells form only about three cell diameters dorsal from, and thus in close vicinity to, the larval eyes. This suggests that larval and adult eyes could trace back to common eye precursors (see below). Additional adult photoreceptor cells, pigment cells, and support cells are added continuously, and at 72 hours, the adult eye anlagen on each side have split into two (Rhode, 1992). The visible pigment in *Platynereis* has been isolated and characterised as a mixture of three pterin dimers with autofluorescent activity (Viscontini et al., 1970). Axons that emerge from adult eye photoreceptor cells connect to the axonal scaffold at the level of the dorsal brain commissure, the optic commissure (oc in Fig. 1G,H).

Episphere serial sections of the 72 hour episphere show that both larval and adult eyes form part of lateral cell masses that separate from the medial developing brain by layers of connective tissue (data not shown, and Fig. 6D), and that connect to the brain via the optic nerves (Fig. 1H). Given the continuous growth and the later large size of the adult eyes, it is likely that most cells of these masses will contribute to the developing adult eyes. We refer to the lateral masses as optic anlagen. They do not include the anlagen of the antennae or of the palpae.

Between 72 hours and 96 hours of development, the larval eyes and the adjacent ventral palpae move ventrally and medially, while the two pairs of developing adult eyes remain situated dorsolaterally (data not shown).

Cloning of *pax6*, *six1/2*, *ath* and *r-opsin* orthologues in *Platynereis*

A *Platynereis pax6* fragment of about 600 bp length spanning the paired box and the homeobox was isolated by low-stringency RT-PCR using degenerate primers derived from conserved regions in the paired domain and in the homeodomain. This was used to screen a *Platynereis dumerilii* 24 hour cDNA library (C. Heimann, unpublished), yielding seven *pax6* clones. Two library clones of 4.3 kb (pax6-17) and of 4.1 kb span the entire pax6 ORF. An extensive PCR search for possible additional *pax6* genes involved four pairs of degenerate upper and lower primers in all possible combinations. Eighty clones were picked after low-stringency colony hybridisation and 28 were sequenced, all of them representing the same, single *Platynereis pax6* gene. In line with this, Southern blot hybridisation of genomic DNA with a *Platynereis pax6* fragment yielded single bands under moderate stringency conditions (data not shown). An alignment with other bilaterian Pax6 proteins (Fig. 2A), as well as the construction of a phylogenetic tree involving bootstrap analysis (Fig. 3A) reveals that the *Platynereis pax6* gene obtained clearly clusters within the Pax6 family. It is most closely related to the *pax6* genes of nemertine and squid, two other representatives of the Lophotrochozoa.

Several overlapping *Platynereis six1/2* fragments in the range from 300 bp to 450 bp, encompassing parts of the Six-domain and of the homeodomain, were obtained by low-stringency PCR with degenerate primers. A full-length clone of 1.4 kb was then obtained by vector-anchored PCR from a 48 hour cDNA library (C. Heimann, unpublished). Southern blot hybridisation indicated the presence of a single *six1/2* gene in the *Platynereis* genome (data not shown). According to the alignment (Fig. 2B) and the phylogenetic tree (Fig. 3B), *Platynereis six1/2* is a firm member of the *six1/six2/sine oculis* subfamily of Six transcription factors. Notably, it is less diverged in sequence from *Drosophila sine oculis* and mouse *six1* and *six2* than *Dugesia sine oculis*, another Lophotrochozoan member of the group.

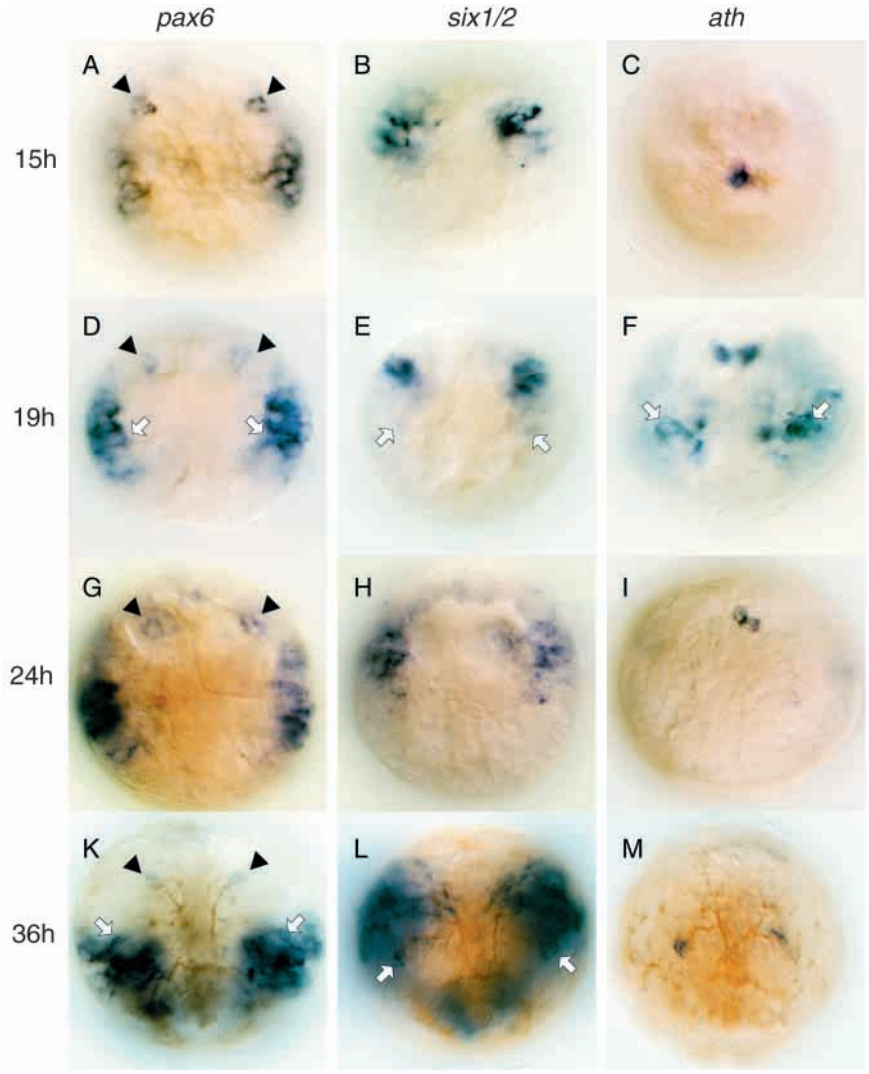

Fig. 4. *Platynereis pax6*, *Pd-six1/2* and *Pd-atonal* in larval eye development. Apical views of whole-mount in situ stained embryos showing the pattern *pax6* (A,D,G,K), *six1/2* (B,E,H,L) and *atonal* (*ath*) (C,F,I,M) expression at 15 hours (A-C), 19 hours (D-F), 24 hours (G-I) and 36 hours of development (K-M). In 36-hour-old larvae (K-M), the axonal scaffold was counterstained with anti-acetylated tubulin antibody. Developing or mature larval eyes are indicated by white arrows. Black arrowheads point at isolated dorsal cells constantly expressing *pax6*.

A *Platynereis ath* fragment of about 150 bp spanning the basic helix-loop-helix domain was isolated using low-stringency PCR with degenerated primers. Two longer fragments of 765 bp, including the entire N-terminus and basic helix-loop-helix domain were obtained by vector-anchored PCR from a 48 hour cDNA library (C. Heimann, unpublished). Both clones have identical protein sequences. Alignment (Fig. 2C) and bootstrap analysis (Fig. 3C) revealed that *Platynereis ath* is an atonal/ath1/5 orthologue.

Low-stringency PCR with degenerate primers yielded a 450 bp *r-opsin* fragment. A full-length clone of 1.4 kb was then obtained by vector-anchored PCR from a cDNA library. It contains an open reading frame of 1149 bp, starting from the first ATG and encoding 383 amino acids. In Fig. 2D, the deduced amino acid sequence is aligned with other invertebrate *r-opsins*. Bootstrap analysis reveals that the *Platynereis r-opsin* belongs to the subfamily of invertebrate *r-opsins* (Fig. 3D). Molecules belonging to this subfamily are active in rhabdomeric photoreceptors. The conserved series of amino acid residues between transmembrane segments V and VI which is required for binding and activation of G-protein (Fig. 2D) equals its counterparts in other Lophotrochozoan and Athropod opsins, but not in vertebrate c-opsins (Arendt and Wittbrodt, 2001). This indicates that *Platynereis* opsin interacts with the Gq-α subunit canonical for invertebrate r-opsins.

Larval eye precursors form at the intersect of the *pax6* and *six1/2* territories from *ath*-positive precursors

Expression of *pax6*, *six1/2* and *atonal* was analysed by whole-mount in situ hybridisation (Fig. 4). The *Platynereis pax6* and *six1/2* genes are expressed in the developing episphere, in bilateral patches of cells that laterally abut the prototroch. These patches are detected already at the late embryonic stage (15 hours, Fig. 4A,B), and persist in the early larval stage (19 hours, Fig. 4D,E), in the mature trochophora larva (24 hours, Fig. 4G,H), in the late trochophora (36 hours, Fig. 4K,L; 43 hours, Fig. 5A,B; 48 hours, Fig. 6A,B), well into the three-segmented young worm (data not shown). The *pax6* staining is located in the ventral half of the episphere, and the *six1/2* staining in the dorsal half of the episphere. In all stages examined, there is an overlap of expression in the lateral episphere. Two-colour co-staining of both *pax6* and *six1/2* transcripts with acetylated tubulin (which labels larval photoreceptor axons, see above) reveal that this overlap of expression covers the two-celled larval eyes at 24 hours (data not shown) and at 36 hours (arrows in Fig. 4K,L).

From embryonic (15 hours) to mature larval stages (24 hours), the patches of *pax6* expression extend more dorsally than the larval eyes (white arrows in Fig. 4D). These more dorsally located *pax6*-expressing cells, by position, might correspond to adult eye precursors. At 36 hours of development, *pax6* expression is lost in the more dorsal cells, so that the larval eyes now demarcate the dorsalmost extent of the *pax6* expression domain (Fig. 4K). However, *pax6* expression is maintained in a pair of isolated dorsal cells (arrowheads in Fig. 4A,D,G,K, and see below). Co-staining with acetylated tubulin revealed that these cells send out axons towards the optic commissure (Fig. 4K), identifying them as neurones. *pax6*-positive dorsal cells can also be detected at 43 hours (Fig. 5B), at 48 hours (Fig. 6A,D) and in the 72 hour developing young worm, where a group of *pax6*-positive cells is found at the very base of the differentiating adult eyes (data not shown). The *Platynereis pax6* gene is also expressed along the developing central nervous system of the body segments, similar to insect and vertebrate *pax6* genes (data not shown). Like *pax6*, also the *six1/2* gene is expressed at additional sites in the segmented body regions, starting in the mature larva (out of focus in Fig. 4L, and data not shown)

Expression of the *Platynereis ath* gene in the developing episphere is very dynamic. This is in line with the proneural function assigned to the insect and vertebrate orthologues. In the 15 hour embryonic episphere, a single cell is stained that, by position, represents a precursor cell of the apical organ (Fig. 4C). By 19 hours of development, two dorsomedial cells, as well as lines of three cells along the apical organ, are *ath*-positive (Fig. 4F). In addition, there was strong staining of *ath* matching the *pax6/six1/2* overlap at 19 hours of development (arrows in Fig. 4D-F). By the timepoint of their appearance and by position, these large cells transitorily positive for *ath* represent larval eye precursors. At 24 hours, when the larval eyes are fully differentiated, expression in the larval eye precursors, and in other cells, had disappeared (Fig. 4I). Starting at late larval stages (36 hours), another prominent site

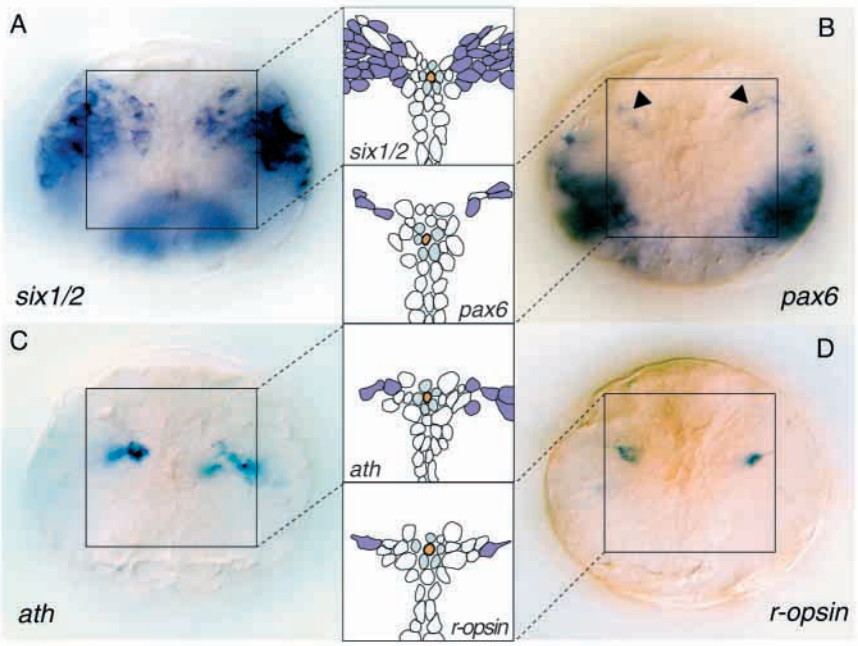

Fig. 5. *Pd-six1/2*, *Pd-atonal* and *Pd-opsin* at the onset of photoreceptor differentiation at 43 hours. Apical views at 43 hours of development. Expression of *six1/2* (A) *pax6* (B), *ath* (C) and *r-opsin* (D) in the differentiating adult eye. Black arrowheads point at isolated dorsal cells constantly expressing *pax6*. Cellular resolution of the whole-mount in situ analysis and the determinate development of Platynereis allow the direct comparison of the expression patterns using morphological landmarks (schematic drawings in the middle part of the figure).

of *ath* expression emerged in the episphere, medial to the larval eyes along the larval eye axons (Fig. 4M). This staining demarcated bilateral clusters of cells that slightly later give rise to the first adult eye photoreceptor cells.

Expression of the *r-opsin* gene was undetectable at embryonic and early larval stages. It was present in the larval eye photoreceptors in the mature larvae, though at low levels (data not shown).

A cluster of *atonal*-positive cells generates the first photoreceptor cell in the adult eye anlage

The next focus was on the molecular characterisation of adult eye development, to find out whether the same combination of *pax6*, *six1/2* and *ath* expression would also define the adult eye anlagen. As adult eye photoreceptor cells are morphologically visible at 53 hours only, we used *Platynereis r-opsin* expression as a molecular marker to identify differentiating adult eye photoreceptors. The first, *r-opsin*-positive adult eye photoreceptor cells were detected at 43 hours of development. These two cells form three cell diameters right and left of the apical organ (Fig. 5D). This position matched the bilateral clusters of *atonal*-expressing cells that were detected at the same time point in larvae from the same batch (Fig. 5C), and that were already present along the larval eye axons at 36 hours of development (Fig. 4M and see above). This match in position was revealed by a comparison of cellular patterns in the medial episphere (compare Fig. 5C with 5D). For this, we took advantage of the fact that cellular outlines are visible under Nomarski optics, and that the 2-day-old brain anlage in the *Platynereis* episphere represents a clear, transparent epithelium with clear morphological landmarks such as the apical organ. A plausible explanation for the overlap in expression, which takes into account the functional data from other systems (Brown et al., 1998; Jarman et al., 1994; Kanekar et al., 1997; Kay et al., 2001; Liu et al., 2001; Wang et al., 2001), is that the bilateral clusters of *ath* expression represent proneural clusters that generate the first photoreceptor cells of the adult eye. Remarkably, these clusters of *ath*-positive cells had already disappeared slightly later on, at 48 hours (data not shown), indicating that *ath* expression in photoreceptor precursors is very transient and precedes differentiation. As the cell lineage of adult eye photoreceptors is not yet known, we cannot exclude the possibility that additional photoreceptors added later to the developing adult eyes also trace back to initially *ath*-positive precursors.

Developing adult eyes express *six1/2*, but not *pax6*

Interestingly, the *ath*-positive clusters were enclosed in the *six1/2*, but not in the *pax6* expression territory (compare Fig. 5A,B,C). In addition, the comparison of cellular patterns revealed that the *atonal*-positive clusters did not match the isolated groups of cells that constantly express *pax6* (arrowheads in Fig. 5B and Fig. 6A; detected as early as 15 hours); instead, these *pax6*-positive cells were located dorsally adjacent to them. Therefore, at 43 hours of development, adult eye photoreceptor precursor cells, and the first differentiating photoreceptors, express *six1/2*, but are devoid of *pax6*. However, it is possible that *pax6* is expressed in adult eye precursor cells at earlier stages, given the more dorsal extension of the patches of *pax6* expression at embryonic and larval stages (Fig. 4A,D,G, see above).

In the 2-day-old metatrochophora, four adult eye photoreceptor cells were detected using *r-opsin* as a marker (Fig. 6C). These cells are now located in a more peripheral, dorsolateral position, almost abutting the prototroch. They are enclosed within the *six1/2* territory (Fig. 6B), but still do not express *pax6* (Fig. 6A). This is also true for the latest stage examined, the 3-day-old developing young worm (data not shown).

Platynereis pax6 in larval eyes, chemosensory palpae and antennae

It is evident that the two-celled larval eyes represent only a small subset of cells within the ventrolateral patches of *pax6* expression. To determine the fate of the remainder of *pax6*-positive cells, we analysed *pax6* expression in 72 hour developing young worms. At that stage, differentiation is well under way and facilitates identification of structures. In a ventroanterior view, three *pax6*-positive sensory organs and organ precursors can be identified (Fig. 7A). First, the larval eyes are still present and express *pax6* (arrows in Fig. 7A; also visible in the optical cross section in Fig. 7B). Second, the majority of *pax6*-expressing cells ventral to the larval eyes and adjacent to the ventromedial gland field constitute the anlagen of the palpae: two fields of mechano- and chemosensory receptor cells located right and left of the mouth that control food uptake (Hauenschild and Fischer, 1969). Third, the two medial cells on both sides of the apical organ represent the tip of the developing antennae (ant in Fig. 7A). The *Platynereis* antennae likewise host mechano- and chemosensory receptor cells (Hauenschild and Fischer, 1969). Expression of *pax6* in head chemosensory organs has been described for nemertines (Loosli et al., 1996), for cephalopods (Tomarev et al., 1997) and for vertebrates (Grindley et al., 1995; Walther and Gruss, 1991), thus representing another recurrent theme in Bilateria.

DISCUSSION

Developing larval and adult polychaete eyes differ in the expression of *pax6*

In accordance with the well-established role of *pax6* in eye development in Bilateria, our expression study also implicates *pax6* in *Platynereis* eye development. In the two distinct types of eyes present in *Platynereis*, namely larval and adult eyes, *pax6* is expressed in larval eye precursors and in the differentiated larval eyes, and possibly also in early adult eye precursors, although this has not been determined with certainty. However, we found that larval and adult eyes differed with respect to *pax6* deployment at differentiation stages. While the gene was expressed in the larval eyes at all stages examined, it was not detected in the developing adult eyes from the onset of photoreceptor differentiation onwards, well into the three-segmented young worm stage. This is all the more remarkable, as the *Platynereis* adult eyes exhibit life-long growth (Hauenschild and Fischer, 1969), with hundreds of cells added to the initial few-celled primordia (Fischer and Brökelmann, 1966; Rhode, 1992). Our data preclude a direct role of *pax6* in this process. More precisely, *pax6* appears not to be involved in the transcriptional control of *Platynereis* adult eye differentiation, such as the activation of *r-opsin* or of any other photoreceptor or pigment cell-specific downstream

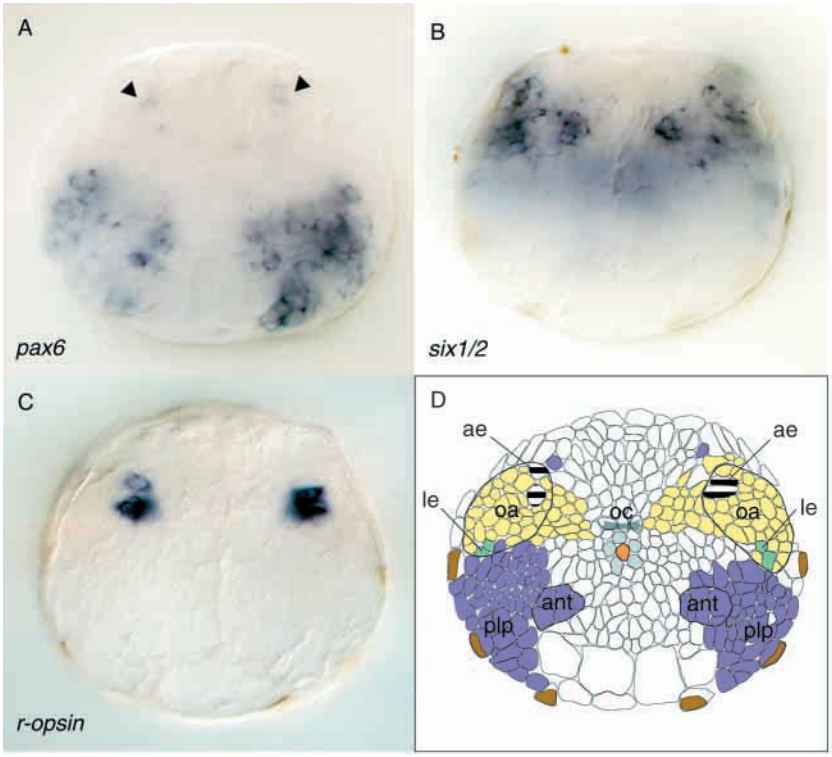

Fig. 6. Adult eye development in 2-day-old metatrochophora larvae. Apical views at 48 hours of development. Expression of *pax6* (A) *six1/2* (B) and *r-opsin* (C) in the growing adult eye. Black arrowheads indicate isolated dorsal cells constantly expressing *pax6*. (D) Schematic drawing of gene expression patterns at 48 hours of development. Natural pigment (brown spots) serves as morphological landmark. Abbreviations: ae, adult eyes; le, larval eyes; oa, optic anlagen; oc, optic commissure; ant, antennae; plp, palpae anlagen.

genes. We cannot completely rule out the existence of another *pax6* paralogue that might exert this function, but we consider this rather unlikely given our extensive searches. *pax6* is expressed, however, in a group of dorsal cells, located at the base of the developing adult eyes. By morphology and position, at least a subset of these cells might represent first order sensory interneurones of the visual system [where *pax6* is also expressed in mouse (Stoykova et al., 1996)]. It will be interesting to determine whether, at earlier developmental stages, these *pax6*-positive cells exert an influence on the formation of the adjacent adult eyes.

Adult cerebral eyes that differentiate in the absence of *pax6* also exist in Lophotrochozoans other than polychaetes. In the squid (Cephalopoda, Mollusca), *pax6* expression covers the early eye anlagen, but is not detected in the differentiating retina (Tomarev et al., 1997). This would suggest an evolutionary relationship between polychaete and cephalopod adult eyes that also exhibit a very similar ultrastructure. Homology of the distinct eye types found in molluscs and polychaetes, however, is an unresolved issue (Arendt and Wittbrodt, 2001; Bartolomaeus, 1992).

From a comparative point of view, the question arises of whether the co-existence of distinct eye types that differentiate with and without *pax6* also applies for other groups. Among Lophotrochozoans, separate types of cerebral eyes are present for example in Sipunculans (Salvini-Plawen and Mayr, 1977) that could well correspond to larval and adult eyes in polychaetes but have not yet been investigated at the molecular level. In Ecdysozoan insects, however, two distinct conserved eye types co-exist (Paulus, 1972; Paulus, 1979), the medial ocelli, and the lateral compound eyes [of which the Bolwig organs in Dipteran larvae are evolutionary derivatives (Daniel et al., 1999)]. Remarkably, neither of the *Drosophila pax6* orthologues (*eyeless* and *twin of eyeless*) are expressed in the differentiating lateral compound eye or Bolwig organ photoreceptors (Czerny et al., 1999; Quiring et al., 1994). However, evolutionary relationships of insect and polychaete eye types are obscure (Arendt and Wittbrodt, 2001; Salvini-Plawen and Mayr, 1977) and more molecular comparative data (also for *Drosophila* ocelli) will be needed to advance on this issue.

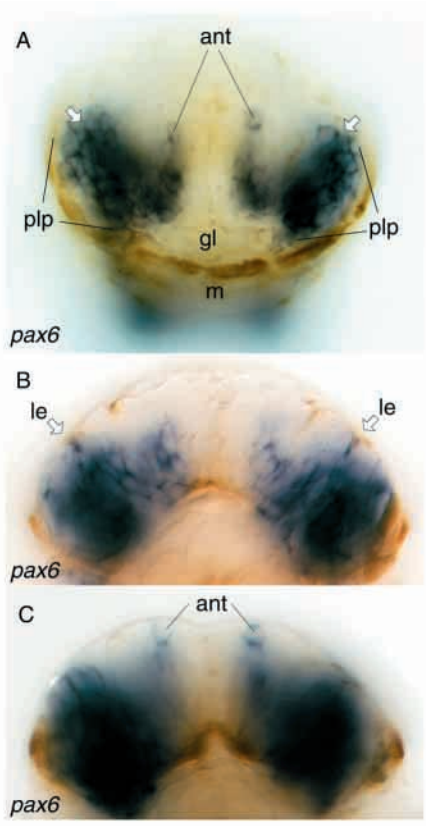

Fig. 7. *pax6* expression in the 72 hour episphere. Figure shows *pax6* expression in 72 hour young worms in a series of optical sections from a ventroanterior view. (A) Surface view showing *pax6* expression in larval eyes (white arrows), antennae (ant) and palpae (plp). (B) Deeper optical section with *pax6* expression in larval eyes (le, white arrows) and anlagen of the palpae. (C) More dorsal optical section showing *pax6* expression in antennae (ant), besides prominent expression in palpae.

It has been proposed that a direct role in photoreceptor cell differentiation should be ancestral for *pax6* genes (Gehring and Ikeo, 1999; Pichaud et al., 2001; Sheng et al., 1997). Clearly, this role does not apply for *Platynereis* adult eyes, but it does apply for the larval eyes that prominently express *pax6* at all stages examined. This supports ancestrality of polychaete larval eyes, and corroborates our notion that larval eyes are ancestral for Bilaterians. It will be important to analyse whether *pax6* is also active in the development of larval eyes in other ciliated larvae, such as the mollusc, echiurid and sipunculan trochophora-type larvae, or the enteropneust tornaria.

six1/2 expression defines the entire visual system – but only in Protostomia

The survey of *six1/2* expression in the developing *Platynereis* episphere from larval to adult stages (Figs 4-6) reveals a very specific and continuous expression that encompasses the developing larval and adult eyes, and that outlines the optic anlagen (Fig. 6D). Medially, *six1/2* expression also extends into the optic commissure. The polychaete optic commissure is considered an optic associative neuropil with many synaptic endings, rather than a simple fibre tract (Bullock and Horridge, 1965). Accordingly, *six1/2* expression defines the entire *Platynereis* visual system from early developmental stages onwards (Fig. 6D). In this respect *Platynereis* eye development resembles *Drosophila* eye development, where *sine oculis/six1/2* is required from early embryonic stages onwards for the formation of the entire visual system (Cheyette et al., 1994; Seimiya and Gehring, 2000). In planarians, *sine oculis/six1/2* is also expressed early on in the regenerating eye (Pineda et al., 2000). These findings indicate that the role of *six1/2* orthologues in early visual system specification could be evolutionarily ancient. The vertebrate *six1/2* expression data, however, are at variance with this notion. Deviating from the situation in insects and polychaetes, the vertebrate *six1* and *six2* genes are not involved in early eye development, but are detected only in the late differentiating retina (Ghanbari et al., 2001; Kawakami et al., 1996). Therefore, early development of visual systems differs across Bilateria, in that *six1/2* is active in Protostomia but not in the vertebrates. This is not the first case of overt non-conservation in bilaterian early eye development, given that the *rx* (*retina homeobox*) gene has proven crucial for eye formation in the vertebrates (Loosli et al., 2001; Mathers et al., 1997; Mathers and Jamrich, 2000; Zhang et al., 2000) but not in insects (Eggert et al., 1998) or in polychaetes (D. A. and J. W., unpublished). If it is not the early role of *six1/2* in eye specification that is conserved across Bilateria, what about the later (shared) expression of *six1/2* genes in differentiating cells of the developing eye?

The ganglion cells of the vertebrate retina: evolutionary counterparts to invertebrate rhabdomeric photoreceptors?

A common feature of *six1/2* involvement in vertebrate and in invertebrate eye development is the cell type-specific expression in the developing eye at differentiation stages. In *Drosophila* (Serikaku and O'Tousa, 1994), planarians (Pineda et al., 2000) and *Platynereis* (this study), the *six1/2*-positive cell types are rhabdomeric photoreceptor cells and pigment cells, while in the vertebrates, *six2* shows a conserved expression in pigment cells and ganglion cells in the late differentiating eyecup – not, however, in the ciliary photoreceptor cells (Ghanbari et al., 2001; Kawakami et al., 1996). Apart from the possible conservation of expression in pigment cells, this comparison indicates that invertebrate rhabdomeric receptor cells and vertebrate ganglion cells may be evolutionarily related. In line with this, there are additional characteristics that are specifically shared between the two cell types, and that might indicate common descent. First, at the morphological level, both send out their axons towards the optic centres of the brain. Second, invertebrate rhabdomeric photoreceptor cells emerge from *atonal/ath*-positive precursors, in insects (Daniel et al., 1999; Jarman et al., 1994) and in polychaetes (this study), and vertebrate ganglion cells emerge from *ath5*-positive precursors in mouse, frog and fish (Brown et al., 1998; Kanekar et al., 1997; Kay et al., 2001; Liu et al., 2001; Wang et al., 2001). Notably, vertebrate ciliary photoreceptors do not express *ath5* (Marquardt et al., 2001). A third similarity of rhabdomeric photoreceptor cells and ganglion cells is that they express orthologous *r-opsin* molecules (Arendt and Wittbrodt, 2001): All invertebrate photoreceptors so far examined (including those of *Platynereis*, this study), employ *r-opsin* molecules for photodetection. A vertebrate *r-opsin* orthologue has recently been identified, called *melanopsin*. It shows restricted expression in retinal ganglion cells but not in the ciliary photoreceptors (Provencio et al., 1998; Provencio et al., 2000).

Is there a plausible explanation for these resemblances in terms of eye evolution? We have recently hypothesised that primary ciliary larvae with 'rhabdomeric' eyespots (as found, for example, in today's trochophora and tornaria larvae) were present also in chordate ancestors (Arendt and Wittbrodt, 2001). Chordate descendants then lost the primary larvae, but might have inherited the larval eyes, in a way that today's ganglion cells are remnants of the ancestral rhabdomeric photoreceptors. They were then complemented by a population of ciliary photoreceptor cells.

Reconstructing the eyes in Urbilateria

Gehring and Ikeo (Gehring and Ikeo, 1999) have recently proposed that bilaterian eyes trace back to rather simple two-celled precursors. Based on a comparative survey of eye positions and morphologies, and of phototransductory cascades, we have further outlined that these precursors might have been a pair of larval eyes present in the ciliated larvae of Urbilateria, with rhabdomeric photoreceptors employing *r-opsin* (Arendt and Wittbrodt, 2001). This study implies that, besides *pax6*, *six1/2* and *ath* were also involved in the development of such larval eyes – as observed in the larval eyes of today's trochophora.

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