

Conserved and divergent functions of *Drosophila atonal*, amphibian, and mammalian *Ath5* genes

Yan Sun,^a Shami L. Kanekar,^{b,1} Monica L. Vetter,^b Sharon Gorski,^{c,2} Yuh-Nung Jan,^d Tom Glaser,^e and Nadean L. Brown^{f,*,3}

^aInstitute of Molecular Pathology, 1030 Vienna, Austria

^bDepartment of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT 84132, USA

^cDepartment of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, USA

^dHHMI and Departments of Physiology and Biochemistry, University of California, San Francisco, San Francisco, CA, USA

^eDepartments of Internal Medicine and Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109, USA

^fDepartment of Pediatrics at Children's Memorial Institute for Education and Research, Northwestern University Medical School, Chicago, IL 60614, USA

*Author for correspondence (e-mail: nadean.brown@cchmc.org)

¹Present address: Myriad Genetics, 320 Wakara Way, Salt Lake City, UT 84109, USA.

²Present address: Genome Sciences Centre, 600 West 10th Avenue, Vancouver, BC, Canada V5Z456.

³Present address: Divisions of Developmental Biology and Ophthalmology, Children's Hospital Research Foundation, Cincinnati, OH 45229, USA.

SUMMARY Insect and vertebrate eyes differ in their formation, cellular composition, neural connectivity, and visual function. Despite this diversity, *Drosophila atonal* and its vertebrate Ortholog in the eye, *Ath5*, each regulate determination of the first retinal neuron class—R8 photoreceptors and retinal ganglion cells (RGCs)—in their respective organisms. We have performed a cross-species functional comparison of these genes. In *ato*¹ mutant *Drosophila*, ectopic *Xenopus Ath5* (*Xath5*) rescues photoreceptor cell development comparably with *atonal*. In contrast, mouse *Ath5*

(*Math5*) induces formation of very few ommatidia, and most of these lack R8 cells. In the developing frog eye, ectopic *atonal*, like *Xath5*, promotes the differentiation RGCs. Despite strong conservation of *atonal*, *Xath5*, and *Math5* structure and shared function, other factors must contribute to the species specificity of retinal neuron determination. These observations suggest that the *atonal* family may occupy a position in a gene hierarchy where differences in gene regulation or function can be correlated with evolutionary diversity of eye development.

INTRODUCTION

Theories of eye evolution have been debated for over a century and center around the hypothesis that the vast diversity of visual organs arose through convergent evolution. However, molecular and genetic studies (Halder et al. 1995; Loosli et al. 1998; Neumann and Nusslein-Volhard 2000; Oliver et al. 1995; Xu et al. 1997) have challenged this idea. It has been suggested that a common Urbilaterian ancestor may have possessed an ancestral eye selector gene whose expression and function was associated with a primitive light-sensing organ (Arendt and Wittbrodt 2001; Callaerts et al. 1997; Carroll et al. 2001). In the past decade such an eye selector gene, *Pax6*, was identified and intensely studied (Gehring and Ikeo 1999; Kumar and Moses 2001). The ability of *Pax6* to promote eye formation is strongly conserved across many metazoan phyla (Halder et al. 1995; Loosli et al. 1996; Tomarev et al. 1997; Ghardon et al. 1998; Callaerts et al. 1999;

Chow et al. 1999). Indeed, insect and mammalian *Pax6* genes retain strongly conserved protein function (Halder et al. 1995) and *cis*-regulation (Kammandel et al. 1999; Xu et al. 1999). Although *Pax6* specifies an eye fate, it is unlikely to directly impose morphologic, structural, or functional differences among eye types. Diversification might be explained through the downstream genes that *Pax6* regulates. For example, the number and type of *Pax6* “target genes” may vary in different animal species or target gene function may have diverged.

One vertebrate gene that acts as a *Pax6* target gene is the basic helix-loop-helix (bHLH) transcription factor, *Ath5* (Brown et al. 1998). *Pax6* binding sites have recently been reported in the promoter region of mouse *Ath5* (Marquardt et al. 2001), although they have not been tested functionally. In zebrafish, frogs, chickens, and mice, *Ath5* regulates the formation of the first retinal neuron, retinal ganglion cells (RGCs) (Vetter and Brown 2001). Mutations in zebrafish or mouse *Ath5* cause a complete loss of RGCs (Brown et al.

2001; Wang et al. 2001; Kay et al. 2001), and ectopic *Ath5* expression promotes RGC fate in the chick or frog eye (Kanekar et al. 1997; Liu et al. 2001). *Ath5* is the Ortholog of *Drosophila atonal*, which is the proneural gene for photoreceptor and chordotonal neurons (Jarman et al. 1993, 1994). *atonal* mutants lack the first photoreceptor neuron, the R8 cell, and ectopic expression of *atonal* during eye development induces extra R8 cells (Dokucu et al. 1996; Sun et al. 2000). Although *Ath5* mutations cause the selective agenesis of RGCs in zebrafish and mice, loss of *atonal* function leads to near eyelessness in flies (Jarman et al. 1994, 1995; Brown et al. 2001; Wang et al. 2001; Kay et al. 2001). This is due to fundamental differences in the mechanisms of fly and vertebrate eye development (reiterated cell–cell induction versus renewable progenitor cells).

Previously we compared the activity of ectopic *Xenopus* and *Mus Ath5* during frog eye development and found they do not promote the same cell types (Brown et al. 1998). Thus, the frog and mouse *Ath5* genes specify RGC fate inherently but cannot substitute for one another. This further implies that amphibians and mammals have divergent aspects of retinal development. Indeed, zebrafish, frog, chick, and mammalian retinogenesis differ in their spatial patterning and temporal progression (Easter 2000; Jarman 2000). To test this idea further, we directly compared the activity of *atonal*, *Xenopus Ath5* (*Xath5*), and mouse *Ath5* (*Math5*) in the context of frog and fly eye formation. We found that *atonal* induces frog RGCs identically to *Xath5* and *Xath5* reciprocally rescues fly eye formation in an *atonal* mutant background nearly as well as *atonal* itself. In contrast, *Math5* rescues *atonal* mutant eyes poorly and in a manner that resembles the bHLH gene, *scute*. We propose that *Xath5* and *atonal* are functionally interchangeable during fly or frog retinal neuron formation but *Math5* requires a mammalian-specific environment to initiate retinal neuron determination properly.

MATERIALS AND METHODS

Fly stocks and transgenic lines

The *gal4-7* enhancer trap, UAS-*ato*, and UAS-*sc* constructs were previously described (Sun et al. 2000). Full-length complementary DNAs for *Xenopus Ath5* (*Xath5*, accession number U93170), *Xenopus NeuroD* (accession number U28067), and mouse *Ath5* (*Math5*, accession number AF071223) were inserted into the pUAST vector by conventional cloning and transgenic fly lines were created using standard microinjection techniques. UAS-*ato*, UAS-*sc*, UAS-*Xath5*, UAS-*NeuroD*, UAS-*Math5*, or *gal4-7* transgenic lines were genetically recombined with the *ato*¹ loss of function mutation (Jarman et al. 1995; Sun et al. 2000). At least three independent transgenic lines for UAS-*Xath5*, UAS-*NeuroD*, and UAS-*Math5* were tested in this way. The coding regions within each construct were confirmed by DNA sequencing, and

comparable activities were observed among independent lines for each construct. In all fly experiments retinal expression was induced as follows, using UAS-*ato* as an example: *w-; gal4-7, ato*¹/*TM6Tb* X *w-; UAS-ato/UAS-ato; ato*¹/*TM6Tb*. Tb+ larvae or adults were analyzed from each cross.

Scanning electron microscopy

Drosophila heads were fixed in 4% buffered glutaraldehyde for 2 h and dehydrated through a graded alcohol series. Samples were washed twice in hexamethyldisilazane (Polysciences, Warrington, PA), desiccated overnight, mounted, and sputter coated with colloidal gold. Lateral views of eyes were recorded and analyzed using an Amray 1000B (SEM Tech Solutions, North Billerica, MA) scanning electron microscope. Polaroid photomicrographs were digitally scanned and processed using Photoshop 4.0 software (Adobe Systems Inc., San Jose, CA).

Histology

Sample preparation and sectioning are described in Brown et al. (1991). Sections (1 μm) were collected serially and stained with 1% toluidine blue/1% sodium borate (Sigma, St. Louis, MO). These were photographed through a Zeiss microscope (Carl Zeiss International, USA) using a 35-mm camera and Ektachrome 160T slide film (Eastman Kodak, USA). Images were digitized using a Coolscan slide scanner (Nikon, Melville, NY) and Adobe Photoshop 5.5 software.

Immunohistochemistry

Mouse anti-Scabrous (1:200) (Lee et al. 1996), rat anti-Elav (1:500) (O'Neill et al. 1994), and mouse anti-Rough (1:100) (Kimmel et al. 1990) hybridoma culture supernatants were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Mouse anti-Boss ascites (1:1000) was a gift from Helmut Kramer. Boss/Elav double or Scabrous single labels followed Cagan et al. (1992), except that imaginal discs were directly dissected in PLP fixative for Scabrous staining experiments. Rough/Elav double labels were performed as described by Kimmel et al. (1990). Directly conjugated secondary antibodies or biotinylated secondary antibodies and streptavidin-conjugated fluorochromes (Jackson ImmunoResearch Laboratory West Grove, PA) were used for antibody detection. Labeled whole-mount imaginal discs were imaged using either Biorad MRC600 or Olympus Fluoview confocal microscopes and Adobe Photoshop 5.5 or Image J software (<http://rsb.info.nih.gov/ij/download/>).

Xenopus in vivo lipofection

Xath5, *scute*, or *atonal* complementary DNA expression plasmids were constructed in pCS2 (Turner and Weintraub 1994). Plasmid DNA was transfected into the eye primordia of stage 17–18 frog embryos as previously described (Holt et al. 1990). The embryos were grown until stage 41 and then analyzed for effects on retinal cell fate as previously described (Kanekar et al. 1997). pEGFP-C1 plasmid DNA (BD Biosciences Clontech, Palo Alto, CA) was included to mark the transfected cells. Images of labeled sections were digitally captured by a Xillix Microimager PMI CCD camera using Openlab software (Improvision Lexington, MA).

RESULTS

***Xath5* and *Math5* do not rescue the *Drosophila atonal* mutation identically**

Drosophila Atonal shares significant homology in its bHLH domain with *Xath5* and *Math5* and with *Math1* and *Xath1* that are not expressed in the mammalian eye but act as an Atonal Orthologs in other regions of the vertebrate nervous system (Kim et al. 1997; Ben-Arie et al. 2000). Therefore, vertebrate *Ath1* and *Ath5* genes have been termed semi-Orthologs of Atonal (Brown et al. 2001, 2002; Kay et al. 2001). The amino acid homologies of these genes (Fig. 1) suggest conservation of DNA binding (basic domain) and protein interaction (HLH domain) functions. In particular, the basic domains of Atonal, *Math1*, *Xath5*, and *Math5* are 100% identical (Fig. 1). We wanted to compare further the functions of the three genes that function in the eye. Atonal is normally expressed in developing eye imaginal discs within the morphogenetic furrow (MF) (Jarman et al. 1994). Sun et al. (2000) demonstrated that *atonal* expression within and posterior to the MF restores a sizable portion of the *ato¹* mutant eye. We therefore tested *Xath5* and *Math5* for rescue of the *ato¹* eye phenotype, using the same binary Gal4-UAS system (Brand and Perrimon 1993) and *gal4-7* driver (Sun et al. 2000) to activate the transcription of each UAS construct during eye development. We also compared the activity of another more distantly related vertebrate bHLH gene, *Xenopus NeuroD* (Fig. 1), as a control. Each vertebrate gene was inserted downstream of UAS binding sites, and multiple independent transgenic fly lines were generated and tested. We compared the extent of eye rescue by scanning electron microscopy of adult flies ectopically expressing *atonal*, *scute*, *Xath5*, *NeuroD*, and *Math5* (Fig. 2, A–C and G–I). These eyes were also sectioned histologically to evaluate the full complement of ommatidial photoreceptor cells (Fig. 2, D–F and J–L).

In these experiments *Xath5* restored fly eye development nearly as well as *atonal* (compare Fig. 2, C with B and F with E), including cells that morphologically resemble R8

photoreceptors in the proximal retina (arrow in Fig. 2F). However, *Math5* rescued only a small number of ommatidia (<25 per eye) (Fig. 2, G and J), and none of these contained photoreceptor cells with clear R8 cell morphology. All *Math5*-rescued ommatidia examined contained fewer than the normal complement of eight photoreceptor cells. Among the three UAS-*Math5* fly lines tested two displayed no rescue, whereas a third induced small numbers of ommatidia (Fig. 2, G and J). This phenotype is similar to that of *gal4-7* driven *scute* expression during fly eye development (Fig. 2, H and K) (Sun et al. 2000). Although *scute* is not normally expressed during photoreceptor neuron formation and is predicted to have different DNA-binding properties (Chien et al. 1996), it can rescue a small number of ommatidia when expressed by eye disc cells within and posterior to the MF (Sun et al. 2000). Consistent with its known role in interommatidial bristle formation (Brown et al. 1991), *scute*-rescued eyes contain numerous bristles (Fig. 2H). However, *Math5* was unable to induce interommatidial bristles (Fig. 2G). *Xenopus NeuroD*, a relatively divergent member of the Atonal family (Fig. 1) (Brown et al. 1998; Hassan and Bellen 2000), rescued the *ato¹* eye phenotype equivalently to *atonal* or *Xath5* (Fig. 2, I and L).

***Xath5* and *NeuroD* induce early stages of ommatidial formation but *Math5* does not**

In the *Drosophila* eye, R8 is the founding photoreceptor neuron in each ommatidium and *atonal* is a key regulator of R8 determination (Jarman et al. 1994). To test whether *Xath5* or *Math5* can induce R8 cells, we examined eye imaginal discs for Bride-of-sevenless (Boss) protein expression. Boss is an early marker for terminally differentiated R8 cells (green punctate staining in Fig. 3). We colabeled eye discs with the pan-differentiation marker, *Elav*, to delineate photoreceptor neurons within forming ommatidia (cells in red in Fig. 3). UAS-*atonal* was previously shown to induce Boss expression in *ato¹* eye discs, whereas UAS-*scute* similarly expressed within and posterior to the MF rarely does (Sun et al. 2000).

| | Basic | Helix 1 | Loop | Helix 2 | % Identity |
|-----------|--|---------|-------------------------|---------|------------|
| Dm Atonal | RRLAANARERRRMQNLNQAFDRLRQYLPCLGND | ----- | RQLSKHETLQMAQTYISALGDLL | | 100 |
| Xl Ath5 |G..T...S..KVV.QW.E | ----- | K...Y.....LS..M..SRI | | 70 |
| Mm Ath5 |G..T...RVV.QW.Q | ----- | KK...Y.....LS..I..TRI | | 70 |
| Mm Ath1 |HG..H...Q..NVI.SFN | ----- | KK...Y.....I..N..SE.. | | 70 |
| Xl Ath1 |HG..H...Q..NVI.SFN | ----- | KK...Y.....I..N..S.. | | 71 |
| Xl NeuroD | ..MK.....N..HG..D.L.S..KVV..YSKT | ----- | QK...I...RL.KN..W..SEI | | 54 |
| Dm Scute |N.VKQV.NS.A...HI.QSIITDLTKGGGRPHKKI | ----- | ..VD..RI.VE..RR.QD.V | | 38 |
| Consensus | RR NARER R N LR P | | LSK TL A YI L | | |

Fig. 1. Alignment of bHLH domains for proteins analyzed in this cross-species functional comparison. ClustalW alignment of 56 (Atonal, *Xath5*, *Math5*, *Math1*, *Xath1* *NeuroD*) or 64 (Scute) amino acids that comprise the basic, helix 1, loop, and helix 2 conserved domain. Consensus amino acids are indicated along the bottom, with the amino acid letter denoting perfect conservation. Dashes represent spaces introduced for optimal alignment of the basic and loop subdomains between scute and the other proteins. A “.” represents amino acids identical to Atonal, and bold type indicates those residues that are identical or similar in three or more proteins.

We compared the ability of UAS-*Xath5*, UAS-*Math5*, and UAS-*NeuroD* to induce Boss expression. Both *Xath5*-rescued (Fig. 3C) and *NeuroD*-rescued eyes (Fig. 3F) expressed Boss immediately posterior to the MF (Fig. 3B). However, *Math5* induced only sparse Boss expression, usually in 1–3 ommatidia per eye disc (vertical arrows in Fig. 3D). This phenotype is identical to that of UAS-*scute* (Fig. 3E), although UAS-*scute* discs contained more ommatidia.

It is possible that *Math5* may activate very early steps of fly R8 or ommatidial development that cannot be maintained into later stages of retinal formation. To test this hypothesis, we examined eye discs for expression of Rough. This

homeodomain transcription factor is normally expressed by MF cells that abut but do not overlap those expressing Atonal (Dokucu et al. 1996). At different times during R8 determination and differentiation, Rough represses Atonal expression (Kimmel et al. 1990; Dokucu et al. 1996). Rough-positive cells in the MF are at the “precluster” stage of development (green nuclear staining in Fig. 4A), which precedes R8 cell selection and high levels of Atonal expression. Precluster expression of Rough also precedes Elav within terminally differentiated photoreceptor cells (red cells in Fig. 4). Posterior to the MF, Rough is coexpressed with Elav in R2, R3, R4, and R5 cells (Kimmel et al. 1990) (Fig. 4A). In *atonal*-rescued eye discs, both precluster (green nuclei) and R cell expression (red cells with green nuclei) of Rough can be discerned (Fig. 4B). In UAS-*Xath5* (Fig. 4C) and UAS-*NeuroD* (Fig. 4F) eye discs, Rough is mainly expressed within precluster cells, but in older larvae we observed Rough coexpression with Elav posterior to the MF (not shown). In UAS-*Math5* (Fig. 4D) and UAS-*scute* (Fig. 4E) discs, however, precluster Rough expression was not observed, although Rough is expressed by more mature R cells that coexpress Elav. Thus, *atonal*, *Xath5*, and *NeuroD* rescue the early precluster cell development that precedes R8 selection, whereas *Math5* and *Scute* do not. In contrast, all bHLH genes tested facilitate late Rough expression in differentiating R cells.

Expression of Scabrous is absent in *Math5*-rescued eye discs

In the anterior MF ubiquitous expression of Atonal protein is rapidly refined to groups of cells from which R8s emerge (Jarman et al. 1994, 1995; Dokucu et al. 1996). Simultaneously, restricted groups of Atonal-expressing cells and

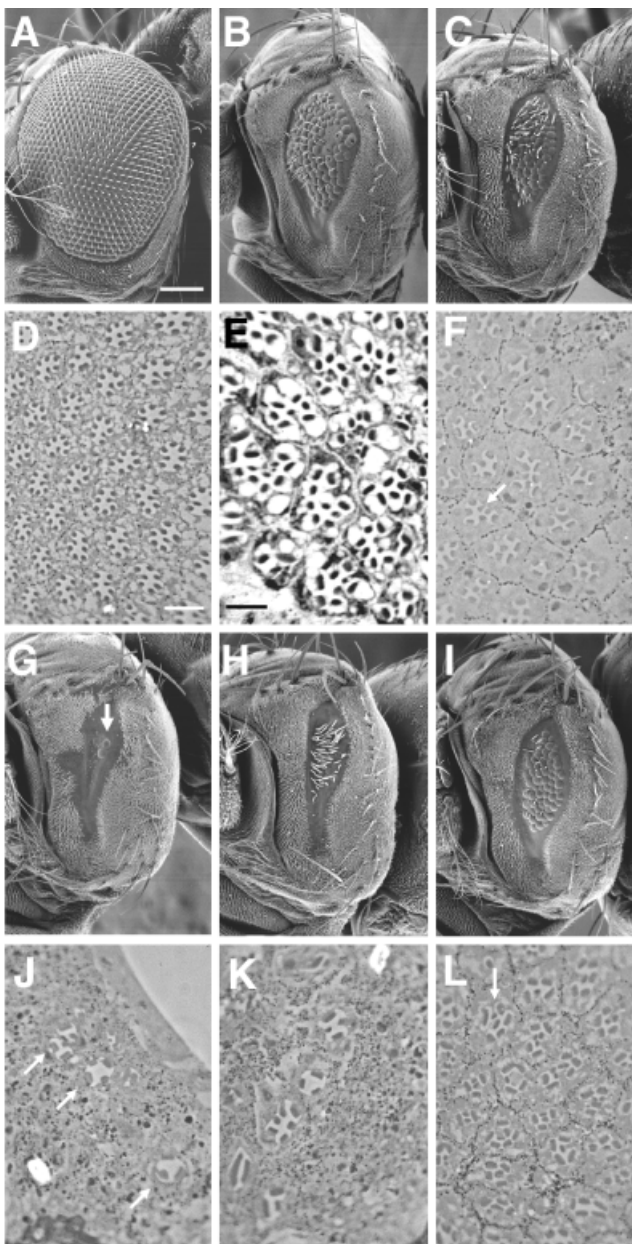


Fig. 2. *Xath5* and *Math5* rescue fly photoreceptor development differently. Scanning electron micrographs (A–C, G–I) and histologic sections (D–F, J–L) of adult *Drosophila* compound eyes. All histologic sections are at the proximal level of the retina where R8 photoreceptor cells are found. (A and D) *w-; gal4-7, ato1/TM6Tb* eyes are normal in size (A) and photoreceptor cell composition (D). (B and E) *atonal*-rescued eye with extra R8 cells in many ommatidia (E and Sun et al. 2000). (C and F) *Xath5*-rescued eye is slightly smaller size than *atonal* rescue. Cells that morphologically resemble R8s are present in these ommatidia (arrow in F). (G and J) *Math5*-rescued eye with two ommatidia (arrow in G). The total number of ommatidia observed varied from 2 to 20 ($n \geq 20$ eyes). Histologic sections of these rare ommatidia contain fewer than eight photoreceptor cells per ommatidium (arrows in J) with no clearly identifiable R8 cells. (H and K) *Scute*-rescued eye. *Scute* induces extra interommatidial bristles (H) and small numbers of ommatidia, mostly lacking R8 cells (K and Sun et al. 2000) (I and L) *NeuroD*-rescued eye is the same size as *Xath5*-rescued eye (compare I with C) and has R8-containing ommatidia (arrow in L). Scale bars: A, 100 μm ; D, 10 μm ; E, 3 μm .

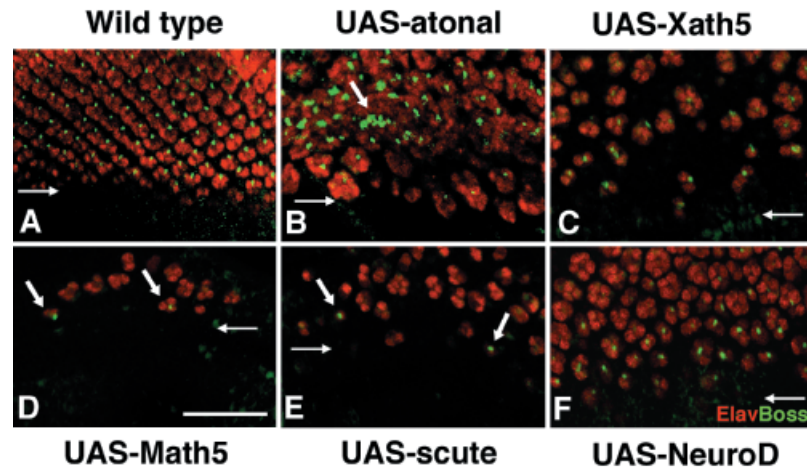


Fig. 3. *Xath5* induces fly R8 formation but *Math5* does so rarely. *Drosophila* imaginal discs double labeled with anti-Boss (green) to mark R8 photoreceptor cells and anti-Elav (red) to mark differentiated photoreceptor neurons. In each panel, a horizontal arrow marks the position of morphogenetic furrow (MF) and anterior is down. (A) *w⁻; gal4-7, ato¹/TM6Tb* control. Elav expression highlights normal ommatidial patterning. One R8 cell expressing Boss (green punctate staining) is present within each ommatidium posterior to the MF. (B) As reported by Sun et al. (2000), *atonal* induces extra R8 cells (vertical arrows) and ommatidial patterning is slightly disorganized. (C) *Xath5* induces R8 cell formation. Ommatidial patterning is similar to *atonal* rescues (compare B and C). (D) *Math5* induces very few ommatidia, and these are always located within a small posterior patch of the imaginal disc. Vertical arrows point to two ommatidia with Boss expression (in green). (E) Infrequent induction of R8-containing ommatidia (vertical arrows) by *UAS-scute*. (F) *UAS-NeuroD* rescued eye discs with R8 cells, as in *UAS-Xath5* (compare F and C). Scale bar, 25 μ m.

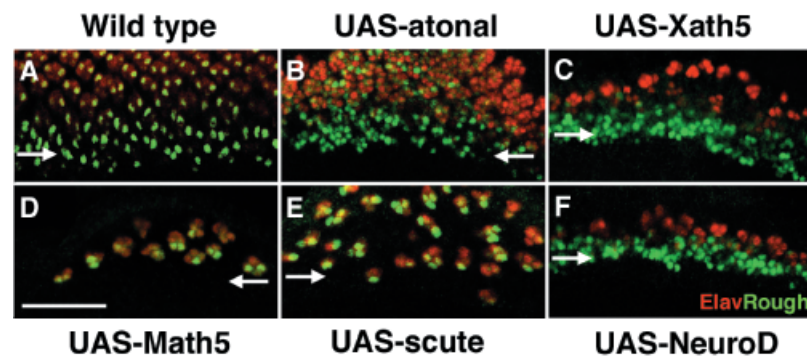


Fig. 4. *Math5* does not induce ommatidial precluster formation. Confocal micrographs of Rough (green) and Elav (red) expression in developing *Drosophila* eye discs. In all panels, horizontal arrows mark the position of the morphogenetic furrow (MF) and anterior is down. (A) *w⁻; gal4-7, ato¹/TM6Tb* control. In the MF, Rough is expressed within R8, R2, and R5 cells of ommatidial preclusters (green nuclei). After differentiation these cells coexpress Rough and Elav (cells with green nuclei and red cytoplasm). (B) *atonal*-rescued eyes initiate precluster formation within the MF. Some ommatidia have extra Rough-positive nuclei. (C) *Xath5*-rescued discs initiate precluster formation, marked by Rough expression (green nuclei alone) in the MF. (D) In *UAS-Math5* eye discs, cells expressing only Rough were never observed. However, many differentiated photoreceptors (labeled in red) also express Rough. (E) *Scute*-rescued eye disc also fail to initiate Rough precluster expression. Similar to *Math5*, many differentiated photoreceptors coexpress Rough and Elav. (F) Rough MF precluster expression in *NeuroD*-rescued eyes. Scale bar, 25 μ m.

nascent R8 cells secrete Scabrous, which acts to inhibit excess R8 specification (Frankfort and Mardon 2002; Hsiung and Moses 2002). Scabrous precedes Boss and is the earliest known marker of forming R8 cells (Baker et al. 1990). We tested the ability of *atonal*, *Xath5*, *Math5*, *NeuroD*, and *scute* to induce Scabrous expression in *ato¹* rescued eye discs (Fig. 5). Figure 5A shows the normal pattern of Scabrous

expression within secretory vesicles of MF cells. Initially, this expression is found in more precluster cells than will become R8 cells. However, once R8 is selected, Scabrous is rapidly restricted to the forming R8 neuron. In *UAS-atonal* rescued eyes, Scabrous is expressed by cells immediately posterior to the MF (Fig. 5B). This delay is due to the *gal4-7* driver, which activates UAS constructs several rows posterior to the normal

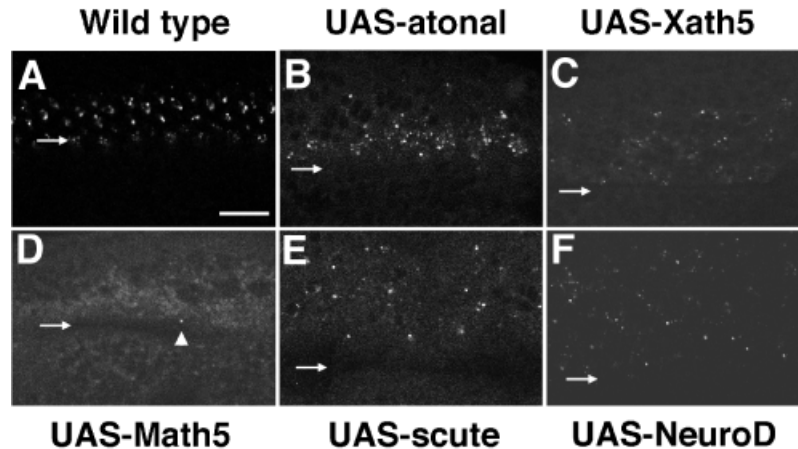


Fig. 5. Differing expression of Scabrous in *ato*¹ imaginal discs when *atonal*, *Xath5*, *NeuroD*, *Math5*, or *scute* are ectopically expressed. Confocal micrographs of Scabrous expression in the MF. (A) *w⁻;gal4-7, ato¹/TM6Tb* control. Normal expression highlights ommatidial precluster formation within the morphogenetic furrow (MF). (B) In *atonal*-rescued eyes, Scabrous expression initiates immediately posterior to the MF in a broad band of cells. (C) *Xath5*-rescued eyes also initiate Scabrous expression immediately posterior to the MF similarly to *atonal*. (D) *Math5* fails to activate Scabrous expression. The arrowhead points to the lone Scabrous-positive cell observed throughout these experiments. (E) *scute* induces randomly spaced Scabrous-expressing cells posterior to the MF. (F) The expression of Scabrous in *NeuroD*-rescued eye discs was nearly identical to that of *Xath5*-rescues. Scale bar, 25 μ m.

up-regulation of Atonal or Scabrous protein in the anterior MF (Sun et al. 2000). Both UAS-*Xath5* and UAS-*NeuroD* induce Scabrous expression similarly to UAS-*atonal* (compare Fig. 5, B, C, and F). In UAS-*Math5* discs, Scabrous expression was virtually absent. Only one Scabrous-expressing cell (arrowhead in Fig. 5D) was found in 50 eye discs tested. More cells expressing Scabrous were observed in *scute*-rescued eye discs (Fig. 5E), but these are randomly spaced throughout the posterior disc. Thus, *Math5* and *scute* differ in their ability to activate Scabrous in *ato* mutant eyes.

***atonal* induces vertebrate RGCs in the developing frog eye**

To determine whether *atonal* can function equivalently to *Xath5* or *Math5* in the context of a vertebrate eye, we overexpressed it in *Xenopus* retinal progenitors by in vivo lipofection (Holt et al. 1990). In these experiments, lipofection of *Xath5* DNA caused an increase in the representation of RGCs (Fig. 6, B and D) compared with green fluorescent protein (GFP) control lipofections (Fig. 6, A and D) as was previously shown (Kanekar et al. 1997). In the same context, however, lipofection of *Math5* DNA paradoxically promotes a bipolar cell fate rather than an RGC fate (Brown et al. 1998).

Drosophila atonal behaved similarly to *Xath5* upon DNA lipofection, causing a dramatic increase in the representation of labeled cells in the ganglion cell layer (Fig. 6, C and D). Overexpression of *Drosophila scute*, a nonretinal bHLH gene chosen for comparison, did not have this effect (Fig. 6D). Instead, *scute* caused an increase in bipolar cell differentiation

(data not shown; Moore et al. 2002). Both of these *Drosophila* bHLH genes can promote neural development in cleavage-stage frog embryos and ectopic neurogenesis in neural plate stage embryos (data not shown) similar to *Xath5* or *Math5* (Kanekar et al. 1997; Brown et al. 1998).

Our findings together with previous functional comparisons of *Xath5* and *Math5* (Brown et al. 1998) suggest that *atonal* and *Xath5* act analogously in both the fly or vertebrate frog retina. However, *Math5* does not function like *atonal* or *Xath5* when tested outside the context of the mammalian retina. In the fly and frog eye, *Math5* instead behaves like *scute*, another bHLH gene that does not normally function during fly retinal development.

DISCUSSION

Current debates regarding eye evolution focus on the emergence of a growing number of conserved developmental genes and pathways. Genes from different species are termed Orthologs when they share coding sequence homology, expression patterns, and endogenous function (Abouheif 1997). Beyond these criteria, however, few Orthologs have been reciprocally tested for conserved function in both invertebrate and vertebrate eyes. Such assessments are needed to identify divergent functions that correlate with different eye types. Because early (*Pax6*) and late (photoreceptor *opsins* and lens *crystallins*) acting proteins appear to have invariant roles in highly polymorphous bilaterian eyes (Arendt and Wittbrodt 2001 and references therein), it is plausible that gene networks required for early eye specification and visual

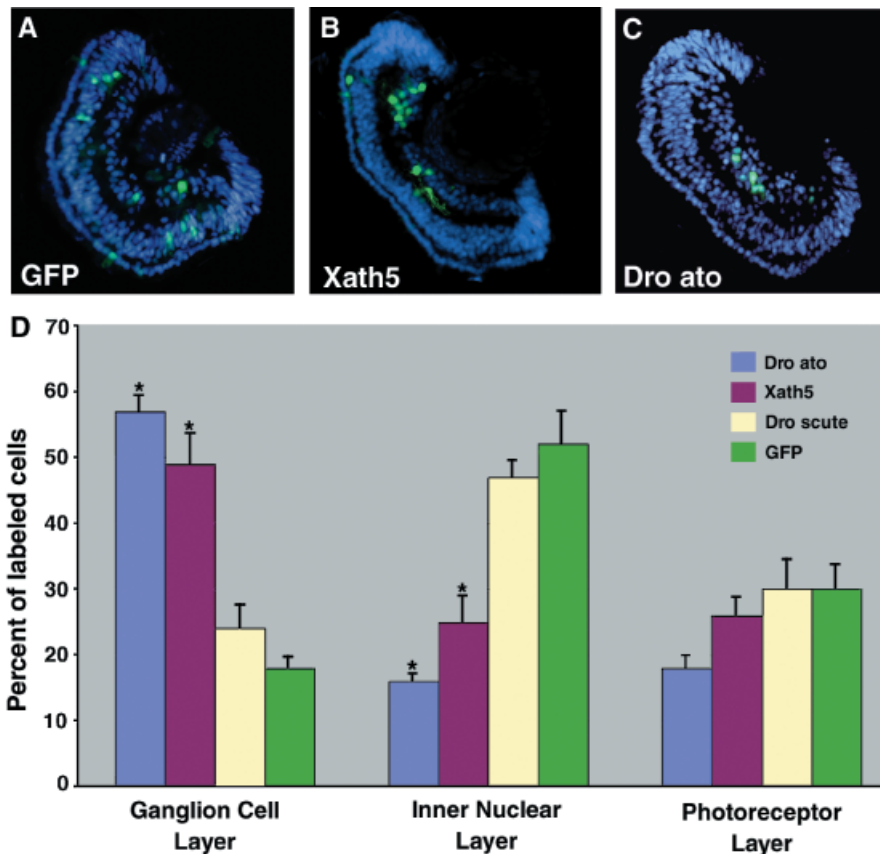


Fig. 6. *Drosophila atonal* mimics *Xath5* over expression by promoting vertebrate retinal ganglion cell fate. (A–C) Cryostat sections through the retina of stage 41 embryos lipofected at stage 17 with green fluorescent protein DNA alone or in combination with either *Xath5* or *Drosophila atonal* (*Dro ato*) DNA as indicated. Nuclei were stained with Hoechst dye (blue) to visualize the retinal cell layers. Overexpression of *Xath5* (B) or *Dro ato* (C) caused an increase in the representation of GFP-labeled cells in the ganglion cell layer. (D) Overexpression of either *Xath5* or *Dro ato* promoted an increase in retinal ganglion cell differentiation compared with control GFP lipofections, whereas overexpression of *Drosophila scute* did not. $n = 472$ cells from five embryos for GFP, 939 cells from six embryos for *Xath5*, 634 cells from five embryos for *scute*, and 950 cells from seven embryos for *Dro ato*. Error represents SEM; the asterisks represent significant difference as compared with GFP controls, $P < 0.01$ by Student's t -test.

function have significantly diverged. Here we compared the cross-species functions of *Drosophila atonal*, *Xenopus Xath5*, and *Mus Math5* during insect and amphibian eye development.

***Drosophila* proneural genes induce vertebrate retinal neurons**

Ectopic expression of *atonal* in the developing *Xenopus* eye biases retinal progenitors toward an RGC fate. *atonal* thus functions as well as *Xath5* in this vertebrate eye assay. By comparison, *Drosophila scute* promotes bipolar cell fate in DNA lipofection and 16-cell RNA injection studies. This result is consistent with the behavior of its vertebrate Orthologs, *Xenopus Xash1* and mouse *Mash1* (Kanekar et al. 1997; Brown et al. 1998; Moore et al. 2002) and the differing roles of *atonal* and *scute* in *Drosophila*. During fly neurogenesis, *atonal* and *scute* exhibit mutually exclusive

expression patterns and promote chordotonal and photoreceptor versus external sensory neuron fates, respectively. Thus, specification of neuronal subtype might be controlled through the spatiotemporal regulation of these genes. However, we expressed *atonal* and *scute* equivalently in time and space within the frog retina. This is similar to vertebrate neurogenesis where multiple bHLH genes function simultaneously, sometimes within the same cell. How do *atonal* and *scute* promote different vertebrate retinal fates? As in *Drosophila*, they may have different intrinsic properties and so activate different target genes (Chien et al. 1996; Sun et al. 2000) or may respond differently to regulatory mechanisms.

Recently, Moore et al. (2002) demonstrated that phosphorylation of some *Xenopus* bHLH proteins modulates the timing of their function and, subsequently, the neuronal fates they promote. In the frog retina, GSK3 β kinase phosphorylates NeuroD and Xash1 and so delays their action,

but it does not phosphorylate Xath5. *Drosophila* Scute can be similarly phosphorylated in *Xenopus* by GSK3 β kinase, and this modulates its activity to induce bipolar neurons upon its ectopic expression (Moore et al. 2002). However, when coexpressed with a dominant negative (dn) form of GSK3 β , Scute instead induces RGCs. Thus, posttranslational regulation of Scute by GSK3 β controls the *timing* of Scute function in the frog eye just as it does for its Ortholog, Xash1 (Moore et al. 2002). In principle, posttranslational modification of *Drosophila* bHLHs may also help regulate the timing of fly neurogenesis. However, not all differences between bHLH factors can be accounted for by protein phosphorylation. During *Xenopus* retinal development, *Math5* overexpression promotes bipolar differentiation (Brown et al. 1998), but this activity is not altered by coexpression with dnGSK3 β (Moore et al. 2002). The different effects of ectopic *Math5* and *Xath5* in the *Xenopus* retina thus cannot be attributed to GSK3 β phosphorylation. This suggests that frog and mouse retinal environments are not equivalent.

Is *Math5* a functional Ortholog of *atonal* and *Xath5*?

Our further functional comparison of mammalian *Math5* within the developing *Drosophila* eye demonstrates that it cannot functionally substitute for *atonal* or *Xath5*. Nevertheless, within the mouse eye *Math5* is the proneural gene for RGCs (Brown et al. 2001; Wang et al. 2001), demonstrating that it functions equivalently to other known vertebrate *Ath5* genes (Kanekar et al. 1997; Liu et al. 2001; Kay et al. 2001). For example, zebrafish *Ath5* mutants exhibit a loss of RGCs (Kay et al. 2001), whereas ectopic expression *Xenopus* or chick *Ath5* promotes excess RGC formation (Kanekar et al. 1997; Liu et al. 2001). Instead, within both the frog and fly retina, mouse *Math5* acts like *Drosophila scute* (this study) or *Mash1* (Brown et al. 1998; Moore et al. 2002). Several mechanisms can explain this conundrum. First, the tempo of eye development differs widely among mice, frogs, and fruit flies. *Xenopus* retinogenesis is completed in 1 day and *Drosophila* retinogenesis in 3 days. This contrasts with the rodent eye where retinal neurogenesis occurs during a 3-week period. Neither *atonal* nor *Xath5* have been tested in the rodent eye, so is it unclear whether they can induce RGCs during a protracted period of retinal development. Interestingly, ectopic *Math5* can induce RGCs in the chick retina (Liu et al. 2001), which develops on a time scale similar to rodents. *Cath5* activity has not yet been tested in the *Xenopus* or *Drosophila* assays.

Second, *Math5* may be unable to form functional heterodimers with *daughterless*, the *Drosophila* bHLH partner for *atonal*. Perhaps amino acid differences between Xath5 and Math5 or a putative transactivation domain in the C-terminus of Xath5, but not Math5, allow Xath5 to function with

Daughterless in the fly eye. We do not favor this explanation because Scute and Daughterless make functional heterodimers elsewhere in the fly nervous system and the phenotypes of *Math5* and Scute are nearly identical in both the fly and frog retina. Instead, *Math5* may interact differently with other components of the transcriptional complex. This suggests that *Math5* protein has low activity outside of a mammalian cell. *Math5* may require a mammalian-specific modification or cofactor. Alternatively, a conserved fly/frog component may prevent *Math5* from inducing either fly R8 or frog RGC neurons.

Finally, *atonal*, *Xath5*, and *Math5* may activate different target genes within their respective eye types. *Atonal* transcriptionally regulates *scabrous* by binding to its promoter in the fly eye (data not shown), whereas other bHLH proneural genes regulate *scabrous* outside the eye (Mlodzik et al. 1990). Here we show that *Xath5*, *NeuroD*, and *scute* (but not *Math5*) activate *scabrous* in the developing fly eye. *Scabrous* expression is more widely spaced in discs rescued by *Xath5* and *NeuroD* compared with *atonal*. However, *Scabrous* expression is extremely dispersed in UAS-*scute* eyes and essentially absent in UAS-*Math5* eyes. Because *Math5* and *Atonal* share 100% amino acid homology in their DNA-binding domains, our results cannot be simply explained by divergent basic domains binding DNA differently. Because no vertebrate eye homologue of *scabrous* has been described, it may represent a downstream pathway component that is not shared by all three eye types. Downstream transcriptional targets have been identified for each of the bHLH genes tested here, but none has been shown to function in fly, amphibian, and mouse retinal development.

Although *Math5* does not act analogous to *atonal* or *Xath5* in the fly and frog retina, it is a semi-Ortholog of *atonal* (Sharman 1999). This designation is based on the intrinsic function of vertebrate *Ath5* genes in zebrafish, frog, chick, and mouse retinal development; the partitioning of *atonal* function between vertebrate *Ath5* and *Ath1* genes; and the failure of loss or gain of function *Ath1* experiments to provoke eye phenotypes. The expression patterns and functions of *Math1* and *Math5* in mice are minimally overlapping but together encompass those of *atonal* in fruit flies. This implies that *atonal* was duplicated during evolution and tissue enhancers partitioned between the two *Ath* genes. After divergence, *Math1* acquired a new function within intestinal secretory cells that is not paralleled in *Drosophila* (Yang et al. 2001). Recently, Wang and colleagues (2002) performed a comprehensive functional comparison of *atonal* and *Math1*. In fruit flies, ectopic *Math1* rescued all *atonal* mutant phenotypes, including R8 photoreceptor formation (Wang et al. 2002). In mice, homologous recombination of *atonal* into the *Math1* locus fully compensated for loss of *Math1*, even within developing intestinal secretory cells (Wang et al. 2002).

The ability of *Math1* activity to rescue *Drosophila* photoreceptor development allows us to speculate that *Math5* retinal function was modified relatively recently. The properties of *Math5* are thus relatively derived in comparison with *Math1*, which appears to retain more basal characteristics. Phylogenetic and amino acid analyses support this idea (Brown et al. 2002). In particular, 10 bHLH amino acid residues that resolve Ath5 or Ath1 from Atonal show pattern differences between these clades (Brown et al. 2002). Interestingly, all affected residues are predicted to affect protein–protein interactions and not DNA binding. These studies point to the importance of testing the functional equivalence of *Math1* and *Math5* in mice by reciprocal substitution. If mutant phenotypes fail to rescue, this would suggest that differences in (unconserved) protein structure and/or tissue-specific factors are responsible for the divergence of *Math1* and *Math5* function.

We propose that the *atonal* gene family represents one position, within a network of early eye development genes, where gene regulation and/or protein interaction have diverged during eye evolution. To understand whether functional divergence within the *atonal* gene family is correlated with eye types, it will be necessary to identify and study these genes in more animal taxa.

Acknowledgments

We thank Carol Sattler of the McArdle Cancer Laboratory at the University of Wisconsin for histology, Bruce Donohoe of the University of Michigan Microscopy & Image Analysis Laboratory for scanning electron microscopy, Bob Holmgren for the use of his microscope, Helmut Kramer for anti-Boss antibody, and the Developmental Studies Hybridoma Bank, under the auspices of the NICHD and maintained by The University of Iowa. We are indebted to Bill Goossens for technical advice, Jim Lauderdale for valuable discussions, and David Blackburn and Ross Cagan for critical reading of the manuscript. This work was supported by NIH grants EY12274 (to M. L. V.), EY14259 (to T. G.), and EY13612 (to N. L. B.); the Pew Scholars Program in the Biomedical Scholars Program, sponsored by the Pew Charitable Trust (to M. L. V.); and the Howard Hughes Medical Institute (to Y.-N. J.).

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