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Identification of *Bombyx Atonal* and functional comparison with the *Drosophila Atonal* proneural factor in the developing fly eye

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Abstract

The proneural genes are fundamental regulators of neuronal development in all metazoans. A critical role of the fly proneural factor Atonal (Ato^{Dm}) is to induce photoreceptor neuron formation in *Drosophila,* whereas its murine homolog, $Atonal7^{Mm}$ (aka Ath5) is essential for the development of the ganglion cells of the vertebrate eye. Here, we identify the *Bombyx mori ato* homolog (ato^{Bm}). In a pattern strikingly reminiscent of ato^{Dm}, the ato^{Bm} mRNA is expressed as a stripe in the silkworm eye disc. Its DNA-binding and protein-protein interaction domain is highly homologous to the Ato^{Dm} bHLH. Targeted expression of Ato^{Bm} in the endogenous ato^{Dm} pattern rescues the eyeless phenotype of the fly ato^{1} mutant and its ectopic expression induces similar gain-of-function phenotypes as Ato^{Dm}. Rescue experiments with chimeric proteins show that the non-bHLH portion of Ato^{Bm} (N-region) can effectively substitute for the corresponding region of the fly transcription factor, even though no apparent conservation can be found at the amino acid level. On the contrary, the highly similar bHLH domain of Ato^{Bm} cannot similarly substitute for the corresponding region of Ato^{Dm}. Thus, the $bHLH^{Bm}$ domain requires the Ato^{Bm} N-region to function effectively, whereas the $bHLH^{Dm}$ domain can operate well with either N-region. These findings suggest a role for the non-bHLH portion of Ato proteins in modulating the function of the bHLH domain in eye neurogenesis and implicate specific aa residues of the bHLH in this process.

Keywords

proneural factor; bHLH; neurogenesis

Introduction

Proneural genes play critical roles during neurogenesis in all metazoans. In *Drosophila*, nervous system development is largely controlled by two proneural gene families, the *achaete-scute* complex which includes *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*), and the *atonal* family which includes *atonal* (*ato*), *absent MD neurons and olfactory sensilla* (*amos*) and *cousin of atonal* (*cato*) (Bertrand *et al.*, 2002; Gonzalez *et al.*, 1989; Goulding *et al.*, 2000a; Goulding *et al.*, 2000b; Huang *et al.*, 2000; Jarman *et al.*, 1993; Villares and Cabrera, 1987).

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The *ato* family controls formation of several types of peripheral sensory organs mediating visual, olfactory and mechano-sensation. During development of these sensory organs, *ato* family proneural factors play a dual role. They confer neuronal potential as well as specify the neuronal progenitor type. First expressed in clusters of cells, each proneural factor soon becomes restricted to one cell (or a few) out of the proneural cluster. This cell develops into a founder neuron, which in turn recruits surrounding cells to also develop as neurons (Brand *et al.*, 1993; Campuzano and Modolell, 1992; Goulding *et al.*, 2000b; Jarman *et al.*, 1993).

The proneural gene *ato* is dedicated to the formation of photoreceptor neurons in all three visual systems of the fly (adult eye, ocelli and larval eye) as well as chordotonal organs (stretch receptors) and a few olfactory sensilla (Jarman *et al.*, 1993; Jarman *et al.*, 1994). As other proneural proteins, Ato contains a basic domain for DNA binding and a helix-loop-helix domain for protein-protein interactions (bHLH motif) (Massari and Murre, 2000). In the developing fly eye, Atonal is critical to the specification of the R8 founder photoreceptor-neuron. Thus, in the ato^{1} mutant (either homozygous or hemizygous over $Df(3R)p^{13}$, very few, if any, R8 photoreceptors develop and consequently none of the other R1-R7 neurons form as well. This results in a nearly complete lack of ommatidia or single eyes in the adult (Jarman *et al.*, 1994).

Bombyx mori is an important model organism, which belongs to the Lepidoptera order, the second largest order of Insecta with ~200,000 species (Nardi, 1995). Thanks to its accessible development, the availability of mutants and the completion of the Silkworm Genome Project (Duan *et al.*, 2010; Xia *et al.*, 2004), *Bombyx mori* has become a useful tool in insect genomics and genetic studies. The compound eyes of the adult are comprised of ~3,000 ommatidia and the developing eye epithelium can be easily dissected at the late larval stage. Based on the evolutionary relationship between Lepidoptera and Diptera as well as genetic studies of developmentally related genes (Nakao, 2010; Zhou *et al.*, 2009), we expect critical players in *Drosophila* neurogenesis to play a conserved role in the development of the *Bombyx* nervous system.

In this paper, we report the identification and cloning of the *ato* homologue from *Bombyx mori* (ato^{Bm}), and a comparative functional analysis of the Ato^{Bm} and Ato^{Dm} proteins using transgenic tools generated in *Drosophila*.

Results

Identification and conservation of the atonal gene in Bombyx mori

Genome sequencing projects greatly facilitate the identification and comparison of genes across diverse species. As our interest lay in the *ato* gene, we searched the *Bombyx mori* genome database by tBLASTn using the full length Ato^{Dm} protein sequence. Using this approach, we found a single genomic region (Genbank AADK01015472.1) that encoded a translation product with high similarity to Ato^{Dm} within the bHLH region and that in turn identified a single related cDNA (EST BP120230). A search of the Silkworm Genome Database (http://silkworm.genomics.org.cn/) with this EST led us to the annotated gene BGIBMGA013643. Through RT-PCR and 5′ RACE experiments on total larval head RNA (5^{th} instar – day 4), we were able to confirm the existence of a full-length open reading frame (ORF) of 480 bp identical to the genomic DNAand encoded by a putative transcription unit of ~2.6 kb, which we named ato^{Bm} (Fig. 1A; see Fig. S1 and legend for details).

The predicted transcription unit of Ato^{Bm} spans ~2.6 kb, including a 1967 bp 5′UTR, a 480 bp ORF, and a ~170 bp 3′UTR (Figs. 1A). The putative ato^{Bm} ORF encodes a 159 amino acids (aa) protein that is considerably shorter than Ato^{Dm} (312 aa) (Fig. 1A and B).

Similarly to Ato^{Dm}, the bHLH domain is located at the very end of the aa chain (C-terminus) and the transcription unit does not appear to have any introns. Comparative sequence analysis of the *Bombyx* and *Drosophila* proteins shows that within the bHLH region Ato^{Bm} shares 80% amino acid identity and 88% similarity with Ato^{Dm} (Fig. 1C), whereas outside the bHLH, there is no obvious conservation.

We also compared Ato^{Bm} with a number of other proneural proteins from the fly and vertebrates including the *Mus musculus* $Atonal7^{Mm}$(Tautz, 1989), *Xenopus laevis* $Atonal7^{Xl}$ (Sommer and Tautz, 1989), the *ato*-family members$Amos^{Dm}$, $Cato^{Dm}$, and $NeuroD^{Xl}$, as well as the more distantly related proneural factor Sc^{Dm} (Fig. 1C). The protein sequence alignment showed that none of the proteins are conserved outside the bHLH domain (not shown). As expected, conservation of the bHLH with Ato family members from vertebrate species, i.e. $Atonal7^{Mm}$ and $Atonal7^{Xl}$, is higher than conservation with the more distant fly proneural factor Sc^{Dm} (Fig. 1C).

Phylogenetic analysis and direct sequence comparison of the bHLH domains confirms that Ato^{Bm} is most closely related to Ato^{Dm} than other *ato* family members (Fig. 1D). In particular, Ato^{Bm} is more closely related to Ato^{Dm} than $Amos^{Dm}$ in aa sequence conservation. The Bombyx protein is also most similar to Ato^{Dm} in that its bHLH domain is located at the very end of the protein, whereas additional aa follow the second helix in other *ato* family factors, from just a few in Amos to extensive C-terminal region in other factors.

In conclusion, we believe to have identified the ortholog of Ato in *Bombyx mori*.

Expression pattern of ato^{Bm} in the eye disc of Bombyx

In the developing *Drosophila* eye, the *ato* gene is expressed soon after eye progenitor cells cease to divide. Thus, initial expression occurs in a stripe that bisects the disc into an eye progenitor region, anterior to the stripe, and a developing neuronal field, posterior to it (Fig. 2A) (Jarman *et al.*, 1994; Jarman *et al.*, 1995). Whereas initially transcribed in all eye precursor cells ready to undergo morphogenesis, *ato* expression soon resolves into evenly spaced clusters of 10–15 cells (intermediate groups) and thereafter down to a single expressing cell, that is thus selected as the founder R8 photoreceptor neuron (Fig. 2B) (Jarman *et al.*, 1994; Jarman *et al.*, 1995). The other R neurons emerge around R8 during the following hours in an Ato-independent fashion.

Similarly to *Drosophila*, the larger compound eye of *Bombyx* (3,000 ommatidia) also forms from a larval eye disc. Unlike the fruit fly, however, development of the eye disc follows a different pattern. Studies of eye disc formation in Lepidoptera are limited to *B. mori* and *Manduca sexta* (Koyama and Tanaka, 1954; Koyama, 1958; Allee *et al.*, 2006). In these species, adult eye primordium cells are initially part of the epidermis of the larval head. In fact, like other epidermal cells, they produce cuticle. By day 1-day 2 of L5, cuticle production in these cells is down-regulated, the cells detach from the cuticle, initiate rapid cell divisions and begin to invaginate to form the inverted eye imaginal disc. Morphogenesis of the eye would presumably begin soon after and may continue partly into pupation. Thus, *B. mori* eye discs were dissected on day 4 of L5 and *in situ* hybridization was carried out to detect ato^{Bm} expression.

Interestingly, in spite of these differences in disc development, the expression pattern of ato^{Bm} in the L5 eye disc was reminiscent of the pattern observed in the *Drosophila* eye epithelium. We detected the ato^{Bm} mRNA in a stripe, and within the stripe there were groups of more highly expressing cells, reminiscent of the intermediate groups seen in the fly (Fig. 2C, D). Noticeably, we did not observe rows of single expressing cells similar to the R8 cells of the fly eye disc; only few single cells were occasionally detected nearby

some clusters. It is unclear whether this discrepancy reflects a significant difference in the way ommatidial neuronal clusters assemble in the two species or, alternatively, if it results from the lower sensitivity of the *in situ* hybridization protocol in *Bombyx*. It may well be the latter case, because even in the fly, detection of *ato*^{Dm} expression in single cells is achieved only under optimal conditions.

Due to our very limited understanding of eye disc development and neurogenesis in *Bombyx*, the relationship between the *ato*^{Bm} mRNA stripe and developmental events in the eye disc is unclear. However, the striking similarities with *ato*^{Dm} expression in the fruit fly suggest that *ato*^{Bm} may play an analogous role in the formation of the ommatidial array during development of the moth eye.

The putative Ato^{Bm} can induce Ato-like gain-of-function effects in Drosophila

Depending on biological context, ectopic Ato^{Dm} expression has been shown to induce formation of supernumerary R8 neurons or chordotonal organs (Chien *et al.*, 1996; Jarman *et al.*, 1993; Sun *et al.*, 2000). Hence, we sought to establish whether the putative Ato^{Bm} homologue could also induce such effects.

In the developing wing disc, ectopic gene expression was induced using the *dpp-Gal4* driver along the anterior-posterior compartments boundary (Fig. 3A). As expected expression of Ato^{Dm} led to formation of ectopic chordotonal organs along the third wing vein that were clearly visible on the wing blade of the adult fly. Analogous expression of Ato^{Bm} also resulted in formation of a few ectopic chordotonal organs nearby vein 3 (Fig. 3A, arrows).

In the developing eye epithelium, broad over-expression of either Ato^{Bm} or the Ato^{Dm} within and posterior to the MF was induced using the *pGMR-Gal4* driver (Fig. 3B). The adult eyes of *pGMR-Gal4 UAS-ato*^{Dm} flies were “rough” that is the ordered packing of ommatidia was disrupted. In the developing eye discs, it was clear that Ato^{Dm} induces many extra R8 neurons, identified using the R8-specific marker Senseless (Sens) (Fig. 3B). In addition, staining with the pan-neural marker Elav showed that extra neurons in general, many not of the R8 type, were also present (Fig. 3B). Given the large number of R8 cells induced and the inductive role R8 plays in recruiting additional R-cells within the clusters (Carthew, 2007), this result was as expected for misexpression of Ato^{Dm}.

The adult eyes of *pGMR-Gal4 UAS-ato*^{Bm} flies were also “rough,” but mildly compared to *UAS-ato*^{Dm}-expressing eyes. In the developing eye discs, it was nonetheless clear that Ato^{Bm} could induce supernumerary R8 neurons, though not to such an extent to lead to a dramatic increase in total R cells (Fig. 3B).

These results strongly suggest that Ato^{Bm} functions in a similar way as Ato^{Dm} when ectopically expressed in the fruit fly, although its activity appears to be somewhat lower than the *Drosophila* factor.

Ato^{Bm} can rescue ato loss-of-function in Drosophila

To further explore the functional conservation between Ato^{Bm} and Ato^{Dm}, we developed a rescue assay in the fly based on the restoration of the adult eye in the *ato* loss-of-function. The *ato*^{1} allele is a semi-lethal recessive mutant allele that shows extensive loss of Ato-dependent sensory organs, including the eye, at larval and adult stages. Hemizygous *ato*^{1}/*Df(3R)p*^{13} escapers are nearly or completely eyeless, displaying only a few ommatidia when eye tissue is present (Fig. 4).

Previous rescue experiments in this mutant background made use of various Gal4 drivers with broad expression quite different from the very restricted and transient pattern of the

endogenous *ato*^{Dm} gene in the L3 eye disc (Jarman *et al.*, 1994; Maung and Jarman, 2007; Sun *et al.*, 2003). In addition, the UAS-cDNA transgenes used to express bHLH factors were generated at a time when site-directed transgenesis was not available (Bischof *et al.*, 2007). Thus, UAS transgenes displayed differences in expression levels due largely to their random sites of integration within the fly genome. In these cases, rescue results were variable and interpretable only when differences in the extent of rescue were very large. As a consequence, the findings cannot always be reconciled with aa conservation (Jarman *et al.*, 1994; Maung and Jarman, 2007; Sun *et al.*, 2003).

To set up an improved rescue assay, we generated new UAS transgenic lines in which each transgene was always inserted at the same site (51C on chromosome II; (Bischof *et al.*, 2007). This approach allowed us to avoid variability in expression levels due to insertion-site effects. Moreover, in order to reproduce the endogenous *ato*^{Dm} pattern as closely as possible, we generated a novel Gal4 driver (*ato*$^{5' EYE3'}$*-Gal4*) based on the eye enhancer elements of the fly gene (Supplementary Figure 2).

Using these tools, a robust rescue of the loss-of-eye phenotype in *ato*^{1}*/Df(3R)p*^{13} flies was obtained with *UAS-ato*^{Dm} (Figs. 4A). Expression of *UAS-ato*^{Bm} also resulted in a dramatic rescue of the adult and developing eye (Figs. 4 and 5C). However, in both cases, the ordered ommatidial packing was not restored suggesting that neuronal patterning in the L3 discs was somewhat disrupted. Staining of developing eye discs for Sens (R8) and Elav (all neurons) showed that supernumerary R8 cells were visible in the developing neuronal fields, though more so when Ato^{Dm} was expressed (Fig. 4B). This is likely due to higher than wt levels of Ato proteins' expression through the Gal4/UAS binary method. Interestingly, this effect was stronger with Ato^{Dm} than Ato^{Bm}, suggesting once again that the *D. melanogaster* protein is somewhat more 'effective' than the *B. mori* homologue.

Nonetheless, these findings confirm that the Ato^{Bm} factor behaves similarly to Ato^{Dm} in rescue experiments and thus closely mimics Ato^{Dm} function in the fruit fly eye.

Differential activity of the highly conserved Ato^{Bm} and Ato^{Dm} bHLH domains

As shown above (Fig. 1C), the conservation between Ato^{Bm} and Ato^{Dm} is very high within the bHLH domain. The few distinct aa within their respective bHLH domains do not map to any residues of known significance for protein folding, DNA-protein or protein-protein interactions (Ellenberger *et al.*, 1994; Ma et al., 1994). The similar rescue of *ato*^{1}*/Df(3R)p*^{13} eyes by Ato^{Dm} and Ato^{Bm} is consistent with a minimal effect of these aa changes on bHLH function. Thus, one might predict that the two bHLH domains would be nearly interchangeable such that a chimeric Ato^{DmBm} protein, encoding a fusion of the N-terminus from *ato*^{Dm} to the bHLH from *ato*^{Bm} (*N*^{Dm}*+bHLH*^{Bm} *= UAS-ato*^{DmBm}), would substitute quite well for the wt Ato^{Dm} factor. Once again, this transgene was inserted in the 51C site to prevent insertion site effects.

Surprisingly, using the same Gal4 driver (*ato*$^{5' EYE3'}$*-Gal4*), Ato^{DmBm}-expressing mutant flies consistently displayed eyes much smaller than those of either *ato*^{Dm} or *ato*^{Bm}-rescued animals (compare Figs. 5A to 4A; Figs. 5C and S2). The extent of rescue in the discs of L3 wandering-stage larvae (Fig. 5B) also correlated well with the adult phenotypes. Thus, in spite of the extensive conservation between the Ato^{Bm} and Ato^{Dm} bHLHs, these domains are not interchangeable.

Interchangeable activity of the highly divergent Ato^{Bm} and Ato^{Dm} N-fragments

On the contrary, no conservation can be detected between Ato^{Bm} and Ato^{Dm} outside the bHLH domains (N-regions). Thus, the N-regions may not be expected to effectively substitute for one another. The significantly less effective rescue by the Ato^{DmBm} chimera

than by the Ato^{Bm} protein is consistent with a necessary coupling of N-regions and bHLH domains.

To further test this hypothesis, we also compared the rescue of the *ato^{1}/Df(3R)p^{13}* phenotype by Ato^{Dm} to that by a chimeric Ato^{BmDm} protein, in which the Ato^{Bm} N-region was linked to the Ato^{Dm} bHLH domain (*N^{Dm}+bHLH^{Bm} = UAS-ato^{DmBm}*). Using the *ato$^{5'EYE3'}$-Gal4* driver, Ato^{BmDm}-expressing mutant flies consistently displayed eyes only a little smaller than Ato^{Dm} eyes and considerably larger than eyes expressing Ato^{DmBm} (compare Figs. 5A to 4; and Fig. 5C and S2). As before, the extent of rescue in the discs (Fig. 5B) correlated well with the adult phenotypes.

In conclusion, the N-region of Ato^{Bm} can robustly substitute for the corresponding region of the Ato^{Dm} transcription factor despite the lack of evolutionary conservation at the aa level.

Discussion

We report here the cloning of the *ato* gene of *Bombyx mori* and show that it is highly similar to fly Ato both in aa sequence of its bHLH domain and apparently also in its expression pattern (mRNA) in the developing eye disc of the silkworm. Based on these striking similarities, it is likely that Ato^{Bm} plays an analogous role to Ato^{Dm} in the formation of the ommatidial array during development of the *Bombyx* eye.

As mentioned above, the conservation between *ato^{Bm}* and *ato^{Dm}* is very high within the bHLH domain, whereas no obvious similarities can be found on the N-terminal side (Fig. 1C). This is also true for Ato homologs from other, non-*Drosophila* species. Interestingly, the 3 members that have been tested for *ato^{1}* rescue - Amos^{Dm}, Atonal7^{Mm} and Atonal7^{Xl} - all have robustly and similarly conserved bHLH domains (48/60, 47/60, 46/60) (Fig. 1C). Nonetheless, as previously shown, they each rescued *ato^{1}* to surprisingly different extent as compared to each other and Ato^{Dm} (Sun *et al.*, 2003). The reason for this may reside in the few distinct aa within their respective bHLH domains; or it may result from the activity of their very divergent N-terminal regions.

We sought to investigate these alternatives using the Ato^{Bm} protein. On one hand, based on the high conservation with Ato^{Dm} within the bHLH, we might have expected a nearly perfect rescue of the *ato^{1}* phenotype by Ato^{Bm}. On the other hand, considering the lack of conservation on the N-terminal side, a poor rescue could also have been expected. As shown here, we found that *UAS-ato^{Dm}* and *UAS-ato^{Bm}* can both rescue the loss of eye phenotype of the *ato^{1}* mutant to a similar extent, with the fly factor doing so a little better than the *Bombyx* one.

Given the high conservation between the bHLH domains of these proteins, such result may have simply reflected the proposed preeminence of the bHLH motif in defining the function of bHLH factors. In this scenario the contribution made by the N-termini to protein activity would be minor. The robust rescue induced by a chimeric protein composed of the *Bombyx* N-terminal region and the *Drosophila* bHLH domain, Ato^{BmDm}, initially appeared consistent with this view (Fig. 5). However, in this case, we would have also expected that the *Bombyx* bHLH domain induce a similar level of rescue when provided with the N-terminal region of Ato^{Dm}. Surprisingly, this was not the case. The Ato^{DmBm} fusion rescued poorly, at a much lower level that Ato^{Bm} itself or the Ato^{BmDm} chimera (Fig. 4 and 5). Thus, whereas the bHLH^{Bm} domain is nearly as effective as the bHLH^{Dm} when in the context of the full length Ato^{Bm} protein, it is not so when associated with the Ato^{Dm} N-region. In short, the bHLH^{Bm} domain requires the Ato^{Bm} N-region to function effectively, whereas the bHLH^{Dm} domain can operate sufficiently well in the context of a profoundly different N-sequence.

These findings strongly suggest that, in the eye, bHLH function is modulated by non-bHLH sequences, notwithstanding the proposed critical role of the bHLH motif in defining the function of bHLH factors (Chien et al., 1996; Nakada et al., 2003; Quan et al., 2004; Maung and Jarman, 2007). Given the high conservation in the bHLH, the small number of residues changed between the $bHLH^{Dm}$ and $bHLH^{Bm}$ appear to be significant in mediating interactions with the N-region. All 5 non-conserved changes (Fig. 1C) occur at positions that have not been associated with particular functions (folding, dimerization or DNA contact) (Ellenberger *et al.*, 1994; Ma et al., 1994). None occur within the DNA-binding basic region, two occur in helix 1, two in the loop, and one in helix 2. The helices, in particular, are known mediators of protein-protein binding suggesting a potential role for some of these residues in modulating interactions between the bHLH and the rest of the protein, either directly or indirectly.

Further analyses of modified Ato^{DmBm} proteins using the tools presented here and other approaches will permit a detailed investigation of how this effect is mediated at the molecular level.

Methods

Insect stock and transgenic lines

The silkworm strain *Dazao* was reared on an artificial diet at 25°C and 70%–80% relative humidity in the Zhang lab. Transgenic *Drosophila* lines were generated by site-specific transformation using the 51C or 68E insertion sites (Bischof *et al.*, 2007). *UAS-ato*^{Bm} and *UAS-ato*^{Dm} expression constructs were generated by cloning the full-length ORFs of *ato*^{Bm} and *ato*^{Dm} into the *pUAST-attB* transformation vector. The chimeric trangenes *UAS-ato*^{BmDm} and *UAS-ato*^{DmBm} were generated by fusing aa 1–85 of *ato*^{Bm} to aa 238–312 of *ato*^{Dm}, and aa 1–237 of *ato*^{Dm} to aa 86–159 of *ato*^{Bm}, respectively. The *ato*$^{5'EYE3'}$*-Gal4* transgene was constructed in the site-specific transformation vector *pattB* (Venken et al., 2006). 1.1 kb of the genomic DNA around the Ato^{Dm} promoter was first cloned in the pattB MCS; the 2.6 kb 5′ eye enhancer region was then inserted upstream of the 1.1 Kb; and lastly the 6.8 kb 3′ enhancer region was inserted downstream of the Gal4 ORF. The transgenic flies were generated by insertion at 68E. Other fly lines used were *dpp-Gal4* (Staehling-Hampton *et al.*, 1994), *pGMR-Gal4* (Freeman, 1996), *ato*$^{5'EYE3'}$*-Gal4* (Zhou and Pignoni, submitted), *ato*^{1} (Jarman *et al.*, 1994) and *Df(3R)p*^{13}.

Molecular biology and Bioinformatics

The bHLH domain of the *ato*^{Bm} gene was identified by tBLASTn search (NCBI) of the *Bombyx mori* genome and EST databases using the full length Ato^{Dm} protein sequence. Gene-specific primers for 5′ RACE and RT-PCR were designed based on the genomic sequence (Fig. S1), Total RNA was extracted from the heads of 5th instar larvae using a Trizol-based protocol (Chomczynski, 1993). One microgram of total RNA was DNAse (Promega, US) treated and then used as template for 1st strand cDNA synthesis using PrimeScript Reverse Transcriptase (Takara, Dallian, China). 5′-RACE was performed using the SMART™ RACE cDNA Amplification Kit (Clontech, USA) to extend ORF sequence into 5′UTR. Second strand PCR amplification was then performed in a total volume of 25 ul composed of 1× PCR buffer, 0.2 mM dNTP mix, 1.5 mM MgCl2, 1 U of *Taq* polymerase (Takara), 0.4 mM each primer, and 0.5 ml of the cDNA template. PCR products were analyzed by electrophoresis, gel-purified, cloned into pMD18-T (Takara), and sequenced commercially. The *ato*^{Bm} sequence has been deposited in GenBank (HQ888870). Protein sequences alignments and conservation analyses were done with ClustalX (Thompson *et al.*, 1997) and GeneDoc (Thompson *et al.*, 2002). Phylogenetic analysis was carried out by PAUP software (v.4.0b10) (Wilgenbusch and Swofford, 2003), with the maximum

parsimony method and bootstrapping sampled for 1,000 times (Wilgenbusch and Swofford, 2003). Figure 1D was generated with TreeView application (Page RD, 1996).

Whole-mount in situ hybridization

In situ hybridization was performed on the L5 day 4 larval eye discs of the silkworm and L3 eye discs of the fly with DIG-labeled RNA probes (Sullivan et al., 2000). Sense and antisense digoxigenin-labeled RNA probes were prepared using the cloned RT-PCR ato^{Bm} product; ato^{Dm} probe was prepared from a ORFs amplified from the genome and subcloned. Digoxigenin RNA labeling kit (Roche, USA). AP-conjugated anti-digoxigenin Fab fragments (Roche, USA) were used at 1:2000. Tissue was mounted in 80% glycerol.

Immunohistochemistry

Staining of *Drosophila* eye disc was carried out by standard protocols (Sullivan et al., 2000) with the following antibodies: rat mAb anti-Elav (DSHB), mouse mAb anti-Eya (DSHB), guinea pig Ab anti-Sens (Nolo *et al.*, 2000), rabbit anti-β-gal (1:1000) (Cappell) and secondary antibodies (anti-mouse, anti-rat, anti-rabbit, and anti-guinea pig) conjugated to Cy2, Cy3, and Cy5 (Jackson ImmunoResearch Laboratories). Tissue was mounted in 80% glycerol. All Ab stainings shown are images from confocal microscopy. Images were obtained with a Leica DM5500Q confocal system and processed with Adobe-Photoshop.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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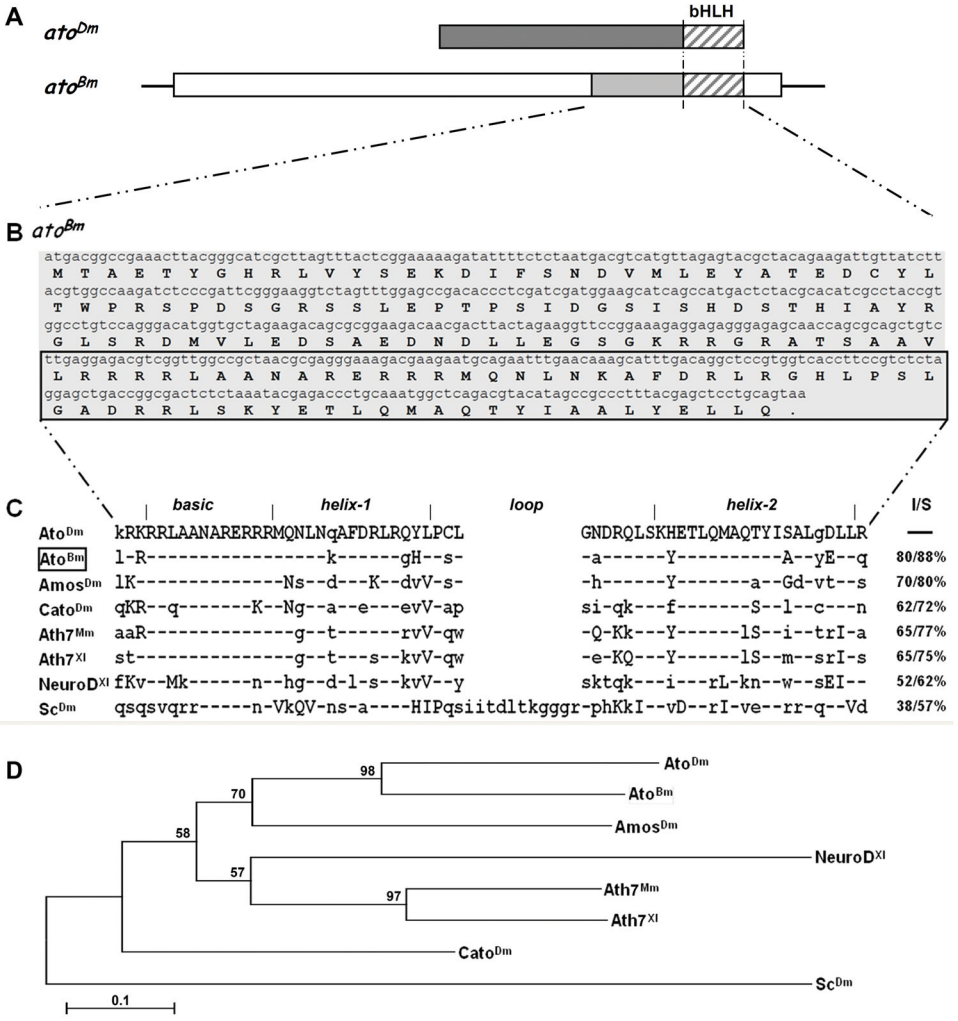

Figure 1. Identification of the *atonal* gene in *Bombyx mori*

A) Schematic drawing of the transcription unit for *ato*^{Bm} . Open boxes represent 5′ and 3′ UTR, shaded boxes represent ORF with the stripes marking the region encoding the bHLH. Structure of the *ato*^{Dm} transcript is shown above for comparison. The two proteins are 80% identical and 88% conserved in the bHLH region (striped boxes), but not conserved elsewhere. See Supplementary Figure 1 for more details. B) Full length ORF and translation of *ato*^{Bm}. The bHLH domain is marked by the box. C) Sequence alignment of the bHLH domains of selected proneural proteins (Mm=Mus musculus; Xl=Xenopus laevis). Conservation is given as I=Identities (%identical aa) and S=Similarity (% identical + similar aa) as compared to Ato^{Dm}. Residues identical to Ato^{Dm} are marked by a hyphen (−), conserved changes are in upper case, nonconserved changes are in lower case. GenBank accession numbers are as follows: Ato^{Dm} = NP731223; Ato^{Bm} = HQ888870; Amos^{Dm} = NP477446; Cato^{Dm} = NP477344; Ato7^{Mm} = NP058560; Ato7^{Xl} = NP001079289; NeuroD^{Xl} = 1096595; Sc^{Dm} = NP476803. D) Phylogenetic tree showing the relationship of *atonal* homologs from different species. The bootstrap 50% majority-rule consensus trees were made with the maximum parsimony method (PAUP, v.4.0b10) using multiple alignments of amino acid sequences. Statistical support (percentage) for each node was evaluated by bootstrap analysis with 1,000 replicates.

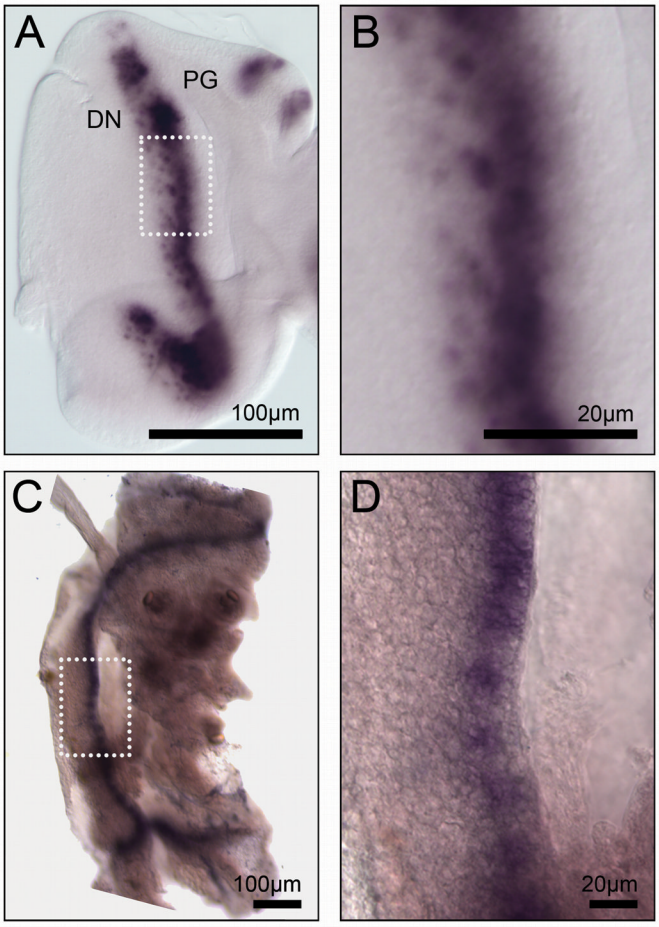

Figure 2. The *ato*^{Bm} mRNA is expressed as a stripe in the silkworm eye disc

In all figures, panels showing fly discs or adult eyes are oriented posterior to the left. A,B) *In situ* hybrization to *ato*^{Dm} mRNA in the wt L3 eye disc of *Drosophila*. *ato*^{Dm} is expressed in a stripe marking neuronal progenitor cells. C, D) *In situ* hybrization to *ato*^{Bm} mRNA in the wt eye disc (L5 day 4) of *Bombyx*. Strikingly, *ato*^{Bm} is also expressed in a stripe. Although eye morphogenesis in *B.mori* is not well understood, this pattern of *ato*^{Bm} expression suggests that neurogenesis may proceed in a similar fashion as in the fruit fly. Identification of the eye discs was based on position within the larval head region relative to the larval ocelli (which are unrelated to the fruit fly ocelli, being already fully differentiated and visible externally at the larva stage in this species).

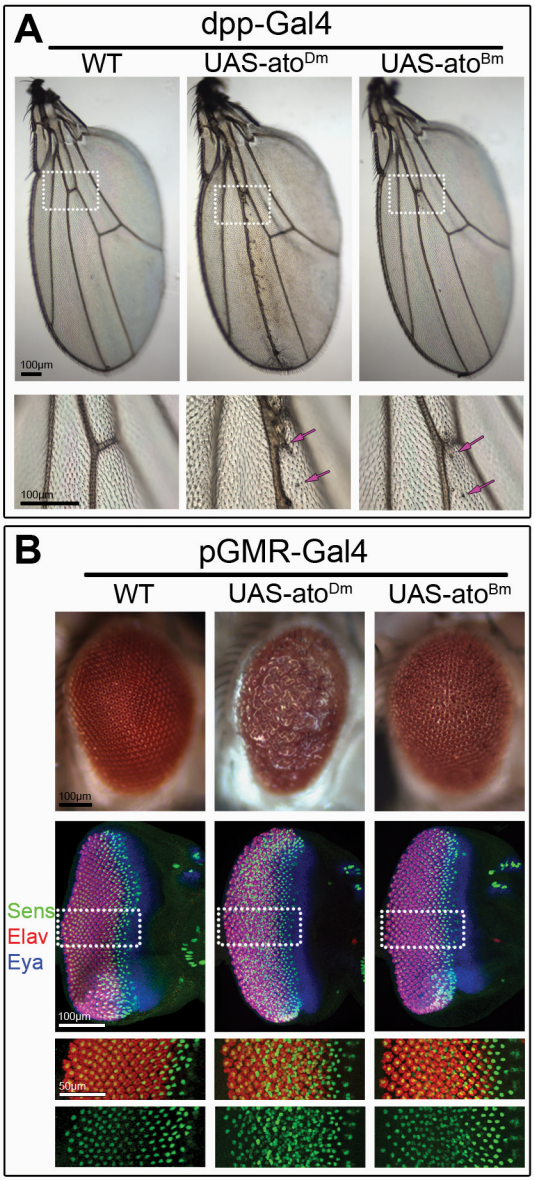

Figure 3. Mis-expression Ato^{Dm} and Ato^{Bm} induces related phenotypes in the wing and the eye

A) Mis-expression Ato^{Dm} or Ato^{Bm} in the developing wing disc under the control of *dpp-Gal4* induces formation of ectopic chordotonal organs (arrow) along the 3rd wing vein. Lower panel shows high magnification of region marked in upper panel. B) Mis-expression Ato^{Dm} or Ato^{Bm} under the control of *pGMR-Gal4* disrupts eye development leading to the formation of a 'rough' adult eye in which the spatial arrangement of the ommatidia is disrupted. Developing disc corresponding to the adult eyes shown above were stained for Sens to mark R8 neurons, the pan-neural marker Elav to highlight all photoreceptors and the retina-identity determinant Eya to mark the developing eye disc. Single channels for Sens and Elav are shown below. Misexpression of Ato^{Dm} or Ato^{Bm} results in the formation of supernumerary R8 neurons (Sens-positive). In both wing and eye, Ato^{Dm} has a stronger effect than Ato^{Bm} but the resulting phenotypes are consistent with a similar activity of the two proteins in these experiments.

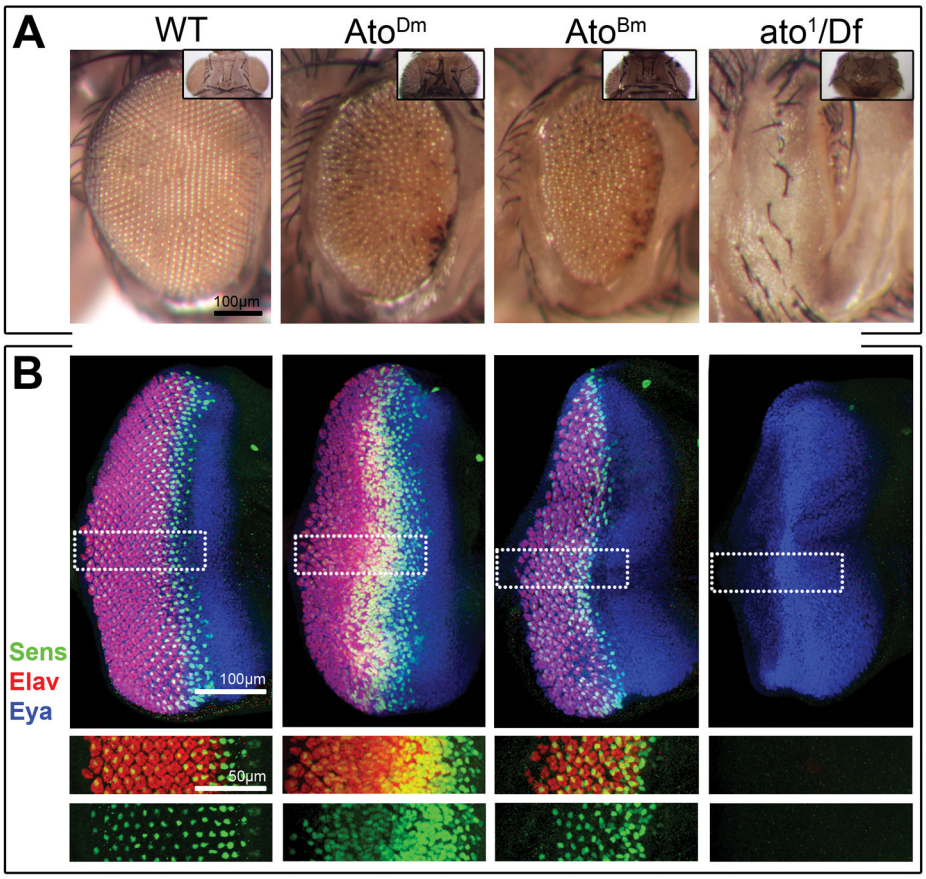

Figure 4. Ato^{Bm} can rescue the loss of eye of the *ato^{1}* mutant

A) Adult compound eyes of wt, Ato^{Dm}-rescued, Ato^{Bm}-rescued and ato^{1}-mutant flies (left to right). Inset shows dorsal views of corresponding fly genotypes. Quantitative data are shown in Fig. 5C. Control 'wt' genotype is *ato$^{5'EYE3'}$-Gal4 ato^{1}/+;* rescued flies genotypes are *UAS-ato^{X}/+ ; ato$^{5'EYE3'}$-Gal4 ato^{1}/Df(3R)p^{13}*; *ato* mutant genotype is *ato$^{5'EYE3'}$-Gal4 ato^{1}/Df(3R)p^{13}*. B) L3 eye discs of wt, Ato^{Dm}-rescued, Ato^{Bm}-rescued and ato^{1}-mutant flies (left to right). Discs are stained for Sens to mark R8 neurons, the pan-neural marker Elav to highlight all photoreceptors and the retina-identity determinant Eya to mark the developing eye disc. Single channels for Sens and Elav are shown below.

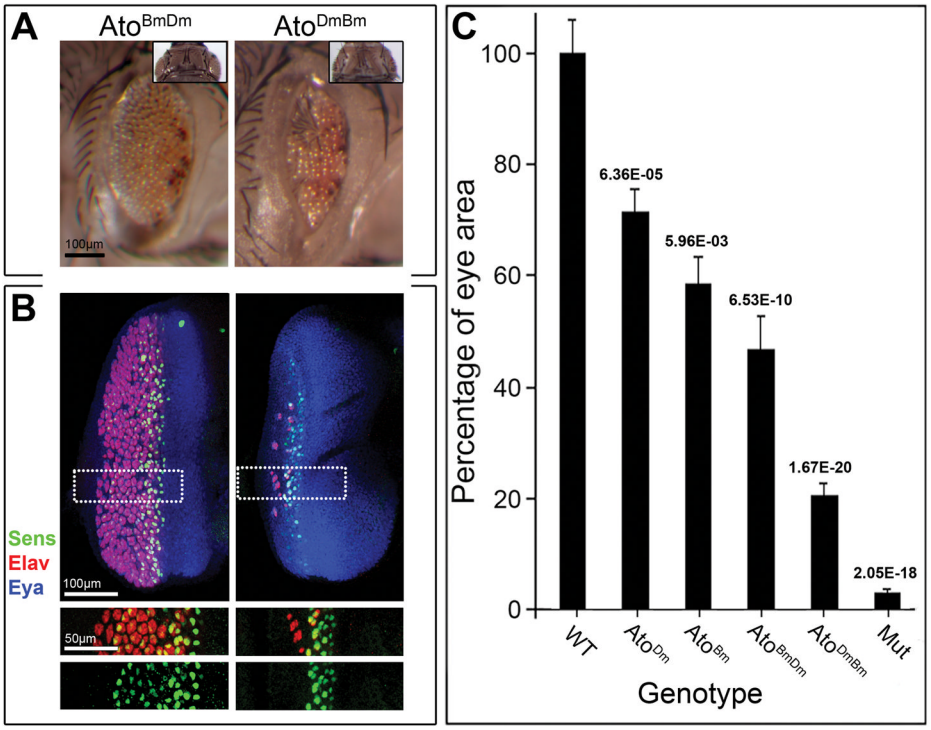

Figure 5. The N-regions but not the bHLH domains can effectively substitute for each other in the rescue assay

A) Adult compound eyes of Ato^{BmDm}-rescued, Ato^{DmBm}-rescued *ato^{1}*-mutant flies (left to right). Inset shows dorsal views of corresponding fly genotypes. Compare to wt, Ato^{Dm}-rescued, Ato^{Bm}-rescued and *ato^{1}*-mutant in Fig. 4 for extent of rescue. Quantitative data are shown in Fig. 5C. Rescued flies genotypes are *UAS-ato^{X}/+; ato$^{5' EYE3'}$-Gal4 ato^{1}/ Df(3R)p^{13}*. B) L3 eye discs of eyes of Ato^{BmDm}-rescued, Ato^{DmBm}-rescued *ato^{1}*-mutant flies (left to right). Discs are stained for Sens to mark R8 neurons, the pan-neural marker Elav to highlight all photoreceptors and the retina-identity determinant Eya to mark the developing eye disc. Single channels for Sens and Elav are shown below. C) Quantitative data comparing the eye size of wt, Ato^{Dm}-rescued, Ato^{Bm}-rescued, Ato^{BmDm}-rescued, Ato^{DmBm}-rescued and *ato^{1}*-mutant flies (N=10, 15, 40, 35, 25, 10 respectively). Columns show relative eye size compared to wt; bars show standard deviation; numbers on the top of each column indicate the *p* value from *t-test* between each sample and the one to the left. See Supplementary Figure 3 for details of data collection, analysis and males-females breakdown.