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Novel function of the class I bHLH protein Daughterless in the negative regulation of proneural gene expression in the *Drosophila* eye

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Two types of basic helix–loop–helix (bHLH) family transcription factor have functions in neurogenesis. Class II bHLH proteins are expressed in tissue-specific patterns, whereas class I proteins are broadly expressed as general cofactors for class II proteins. Here, we show that the *Drosophila* class I factor Daughterless (Da) is upregulated by Hedgehog (Hh) and Decapentaplegic (Dpp) signalling during retinal neurogenesis. Our data suggest that Da is accumulated in the cells surrounding the neuronal precursor cells to repress the proneural gene *atonal* (*ato*), thereby generating a single R8 neuron from each proneural cluster. Upregulation of Da depends on Notch signalling, and, in turn, induces the expression of the Enhancer-of-split proteins for the repression of *ato*. We propose that the dual functions of Da—as a proneural and as an anti-proneural factor—are crucial for initial neural patterning in the eye.

Keywords: Daughterless; atonal; Notch signalling; retinal neurogenesis; *Drosophila* eye

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INTRODUCTION

Basic helix–loop–helix (bHLH) proteins have important functions in the generation of various types of neurons during neurogenesis (Guillemot *et al*, 1993; Turner & Weintraub, 1994; Guillemot, 1995; Lee, 1997; Dambly-Chaudiere & Vervoort, 1998). In *Drosophila*, several bHLH genes, including *atonal* (*ato*), *Achaete-scute complex* (*ASC*) and *amos*, are expressed with spatially regulated patterns to specify various sensory neurons (Ghysen & Dambly-Chaudiere, 1988). In contrast to the presence of several tissue-specific class II proteins, Daughterless (Da) is the only known class I bHLH protein in *Drosophila* (Caudy *et al*, 1988b; Smith & Cronmiller, 2001). Similar to other class I proteins, Da is expressed in a broad range of tissues and is involved in diverse developmental processes depending on its class II bHLH-binding partners (Caudy *et al*, 1988a; Cline, 1989; Cronmiller & Cummings, 1993; Cummings & Cronmiller, 1994; King-Jones *et al*, 1999).

Interestingly, it has been reported that Da is ubiquitously expressed in the eye disc but upregulated in the morphogenetic furrow (hereafter referred to as furrow) where retinal neurogenesis occurs (Brown *et al*, 1996). Hence, expression of Da might be regulated in coordination with neurogenesis in the developing eye. During retinal neurogenesis, a group of proneural cells is selected from a population of uncommitted cells in the furrow. Subsequently, a single cell is further selected from each proneural group as the founder photoreceptor cell R8. The generation of proneural groups and selection of R8 require the class II bHLH proneural gene *ato* (Jarman *et al*, 1994). Similar to other class II bHLH proteins, Ato forms heterodimers with Da to initiate retinal development (Jarman *et al*, 1994; Brown *et al*, 1996; Chen & Chien, 1999). However, it is unknown how the upregulation of Da in the furrow is controlled and whether it has a specific function in patterning the neural retina.

Here, we revisited the expression pattern of Da in the eye disc to understand the basis of upregulation of Da and its role in retinal neurogenesis. We show that the expression of Da is dynamically regulated in the furrow by several mechanisms, including Hedgehog (Hh), Decapentaplegic (Dpp) and Notch signalling pathways. Furthermore, we provide evidence that Da has both proneural and anti-proneural functions, and that both Da and Notch signalling cooperatively repress the expression of Ato for R8 cell selection.

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RESULTS AND DISCUSSION

Distinct patterns of upregulation of Da in the furrow

Da is upregulated in the furrow region (Fig 1A–C), which is consistent with a previous observation (Brown *et al*, 1996). Surprisingly, however, we found that there are two distinct patterns of Da upregulation (Fig 1D–F). The first pattern is a broad, low-level upregulation in the furrow (hereafter referred to as basal level; Fig 1F, green arrow). The second pattern is a stronger expression of Da (hereafter referred to as high level) selectively in the non-neural cells surrounding the Ato-positive R8 cells between proneural clusters (Fig 1F, red arrow). We tested whether this previously unrecognized pattern of expression of Da is specific by examining eye discs containing *da* loss-of-function (LOF) clones. Both the basal and high-level expressions of Da in the furrow were lost in the LOF clones of da^3, a null allele (Fig 1H–J), showing the specificity of the pattern of Da expression.

Hh and Dpp signalling pathways regulate Da expression

The basal level of Da upregulation overlaps with the domain of Ato expression near the furrow (Fig 1F,G), where they function together to regulate neurogenesis. As the furrow progression and expression of Ato are controlled by Hh and Dpp signalling (Wiersdorff *et al*, 1996; Strutt & Mlodzik, 1997; Borod & Heberlein, 1998; Greenwood & Struhl, 1999; Curtiss & Mlodzik, 2000; Fu & Baker, 2003), we reasoned that regulation of Da expression in the furrow might be linked to these signalling pathways.

To test whether Hh signalling is required for the expression of Da, we examined Da expression in hh^1 mutant eye discs in which the production of Hh ceases after the mid-third instar stage, resulting in reduced expression of Ato and arrest of furrow progression (Heberlein *et al*, 1993; Lim & Choi, 2004). The expression of Da was downregulated in hh^1 mutant eye discs (data not shown). We also generated LOF clones of *smoothened* (*smo*), a crucial component for Hh signal transduction (Strutt & Mlodzik, 1997). Da expression was significantly reduced in *smo* mutant clones spanning the furrow (Fig 2A–C), suggesting that Hh signalling is required for the expression of Da. However, the expression of Da was not completely eliminated in hh^1 mutant eye discs (data not shown) or in *smo* LOF clones (Fig 2C). As Dpp signalling is partly required for the expression of Ato, we tested whether Dpp signalling is also necessary for the expression of Da by analysing LOF clones of *mad* (*mothers against dpp*), an essential factor for Dpp signalling transduction (Wiersdorff *et al*, 1996). Da expression showed little reduction in *mad* mutant clones (Fig 2F), indicating that Dpp signalling by itself is not essential for Da expression. By contrast, the expression of Da was almost completely abolished in LOF clones of *smo* and *mad* double-mutant cells in the furrow region (Fig 2G–I). Thus, the Hh and Dpp signalling pathways are crucial but partly redundant for the expression of Da. We also found that loss of function of Ato reduced the level of Da expression in the furrow (supplementary Fig S1 online). Therefore, several factors, including Ato, coordinate the accumulation of Da in the furrow.

High-level expression of Da has an anti-proneural function

To test whether the upregulation of Da in the furrow has a function in neurogenesis, we generated da^3 LOF clones and examined the effects of *da* mutation on the expression of Ato and neuronal differentiation (Fig 3A–F). Consistent with the previous

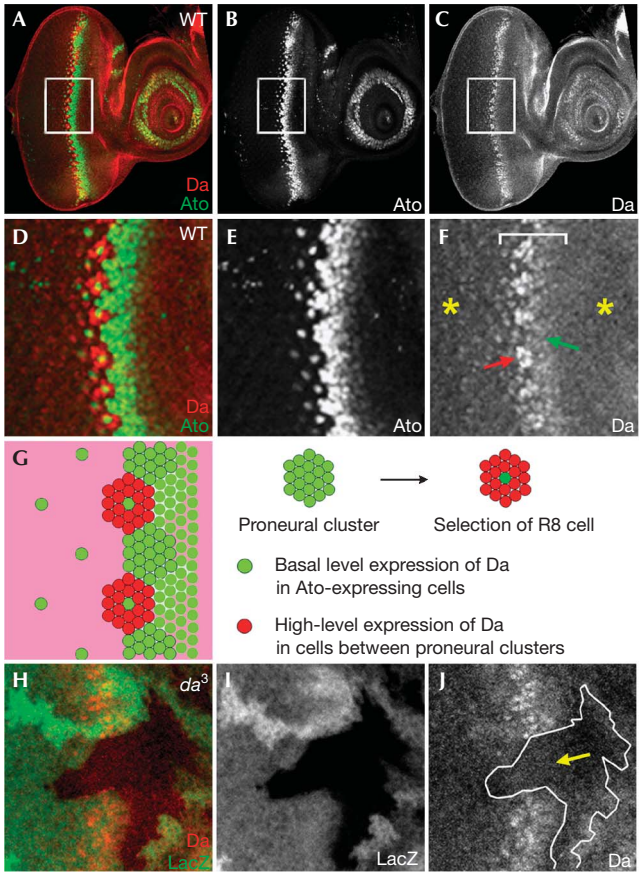

Fig 1 | Expression pattern of Daughterless and Atonal in the developing eye disc. (**A–G**) Third instar eye disc stained for Da and Ato. Areas around the furrow (marked with rectangles) in (**A–C**) were magnified in (**D–F**), respectively. (**G**) Schematic diagram of (**D–F**). In the furrow region (**F**, bracket), Da is expressed at a relatively low level in all Ato-expressing cells (**F**, green arrow), but it is highly expressed in the cells surrounding the singled-out Ato-positive R8 cells just behind the furrow (**F**, red arrow). Outside the furrow, Da is expressed broadly at a low level (**F**, asterisks). (**H–J**) An eye disc containing a da^3 LOF clone stained for β-Gal (clone marker; green) and Da (red). The expression of Da was lost in the da^3 mutant cells (**J**, arrow), confirming the specificity of the antibody. The white line marks the da^3 mutant clone boundary. In this and all subsequent images, antibodies used for staining are indicated in each panel. Confocal section images are combined to show protein expressions in a single image. Posterior is to the left and dorsal is to the top in all discs, unless otherwise indicated. Ato, atonal; Da, Daughterless; LOF, loss of function.

observations (Brown *et al*, 1996; Chen & Chien, 1999), loss of *da* resulted in ectopic expansion of Ato expression in the mutant clone (Fig 3C, arrow), suggesting that Da is crucial for repressing the expression of Ato.

Despite ectopic expression of Ato, most of the cells in *da* LOF mutant clones could not differentiate into photoreceptor cells, as indicated by the lack of neuronal markers such as Senseless (R8 marker) and Elav (pan-neural marker; Fig 3D–F). Hence, the expression of ectopic Ato is insufficient to induce retinal differentiation in the absence of Da. However, local

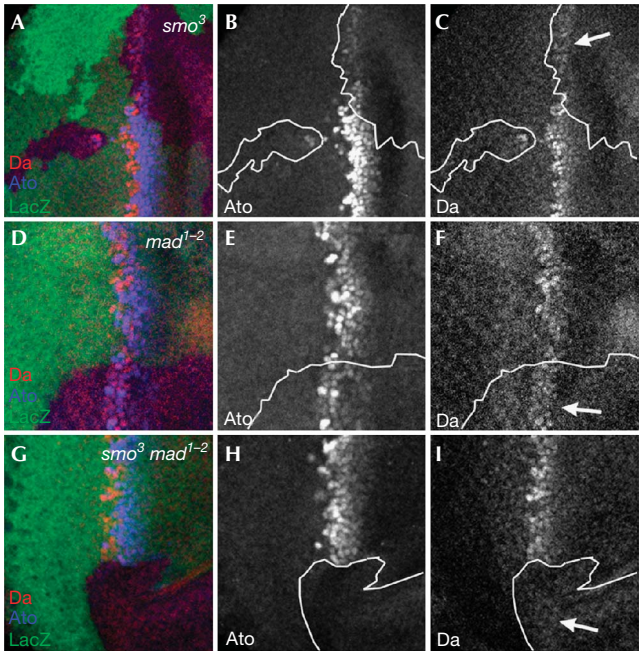

Fig 2 | Hedgehog and Decapentaplegic signalling pathways are required for the expression of Da in the furrow. Eye discs containing LOF clones of *smo*^{3} (**A–C**), *mad*$^{1-2}$ (**D–F**), and *smo*^{3}*mad*$^{1-2}$ (**G–I**) were stained for β-Gal (clone marker; green), Da (red) and Ato (blue). The expression of Da was downregulated but not completely lost in *smo*^{3} mutant clones (**C**, arrow). The expression of Da was reduced weakly in *mad*$^{1-2}$ LOF clones (**F**, arrow). In double LOF clones, *smo*^{3}*mad*$^{1-2}$, the expression of Da was eliminated (**I**, arrow). Expression of Ato was also downregulated in LOF clones of *smo*^{3} or *mad*$^{1-2}$, or both (**B**,**E**,**H**). White lines mark LOF clones. Da, Daughterless; Dpp, decapentaplegic; Hh, Hedgehog; LOF, loss of function; *mad*, *mothers against dpp*; *smo*, *smoothened*.

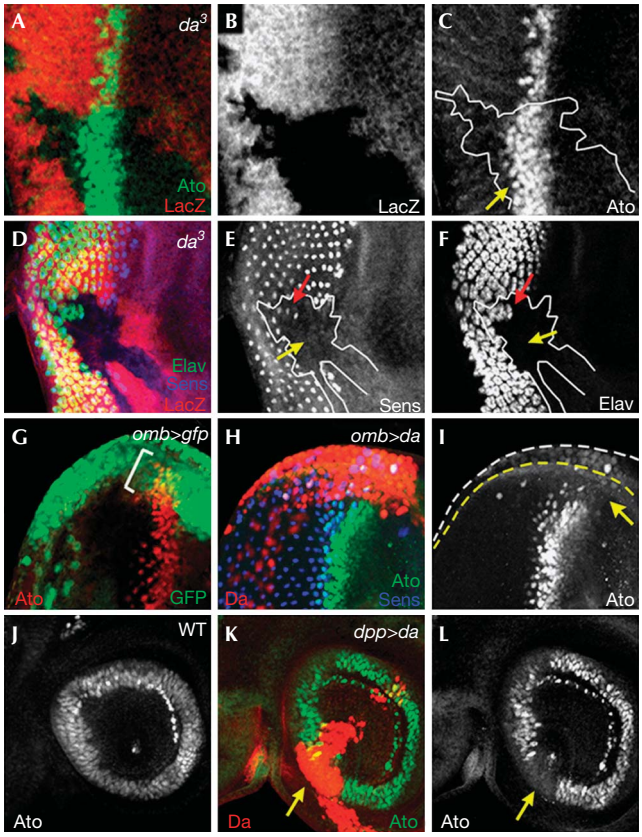

Fig 3 | High-level expression of Daughterless represses Atonal expression. (**A–F**) Loss of *da* caused expanded expression of Ato in the clone boundary (**C**, arrow) and loss of photoreceptor differentiation (**D–F**, yellow arrows in **E**,**F**). Red arrows indicate occasional local differentiation in the posterior region of the clone. *da* mutant cells are marked by the absence of β-Gal staining. (**G**) The expression of GFP (green) by the *omb-Gal4* driver in the dorsal margin of the eye disc proper overlaps with the endogenous expression of Ato (red, bracket). (**H**,**I**) Overexpression of *da* by *omb-Gal4* repressed Ato expression in the furrow near the dorsal margin of the eye disc (**I**, arrow). (**J**) Expression of Ato in the wild-type antenna disc. (**K**,**L**) Overexpression of Da by *dpp-Gal4* downregulated the expression of Ato in the *dpp* domain of antenna (arrows). Ato, atonal; Da, Daughterless; Dpp, decapentaplegic; GFP, green fluorescent protein; *omb*, *optomotor blind*.

differentiation was occasionally detected near the posterior end of some clones (Fig 3D–F). This might be due to the perdurance of Da in LOF clones, although we cannot exclude other possibilities such as partial non-autonomy or partial independence of photoreceptor differentiation from Da in the posterior region of the eye disc.

To support the idea that a high level of Da expression is required for the repression of Ato, we examined a temperature-sensitive allele of *da* (*da*^{ts}) that causes conditional partial loss of function of Da at the restrictive temperature. In *da*^{ts} mutant eye discs, Ato was expressed in several cells rather than a single R8 cell per proneural cluster (supplementary Fig S2 online). In addition, we tested the effects of conditional expression of Da by temperature shifts of *heat-shock* (*hs*)-*da* flies. Ato was repressed by the overexpression of Da after a longer heat shock but not after a shorter heat shock (supplementary Fig S3 online). These observations support the idea that enriched Da expression in the cells surrounding each R8 cell is required for generating a single R8 cell by the inhibition of Ato expression.

The expanded expression of Ato in *da* mutant clones might, in part, be due to the failure of *da* mutant cells to induce lateral inhibition of Ato expression (Chen & Chien, 1999; Frankfort & Mardon, 2002). It is also possible that Da might be involved in the cell-autonomous repression of Ato expression. To test this possibility, we overexpressed Da in the dorsoventral margin of the eye disc using the *optomotor blind* (*omb*)-*Gal4* driver (Fig 3G). The overexpression of Da downregulated Ato expression in the expression domain of *omb* (Fig 3H,I). Furthermore, the overexpression of Da in the antenna disc using the *dpp-Gal4* driver resulted in Ato repression in the expression domain of *dpp* (Fig 3J–L). Taken together, our data from LOF and overexpression analyses suggest that the high-level expression of Da is necessary and sufficient for the cell-autonomous repression of Ato during the selection of R8.

Da regulates Enhancer-of-split expression in the furrow

Both Da and Notch (N) are essential for the selection of R8 by repressing Ato expression in non-R8 precursors within proneural clusters. Hence, Da might be involved in N-dependent lateral

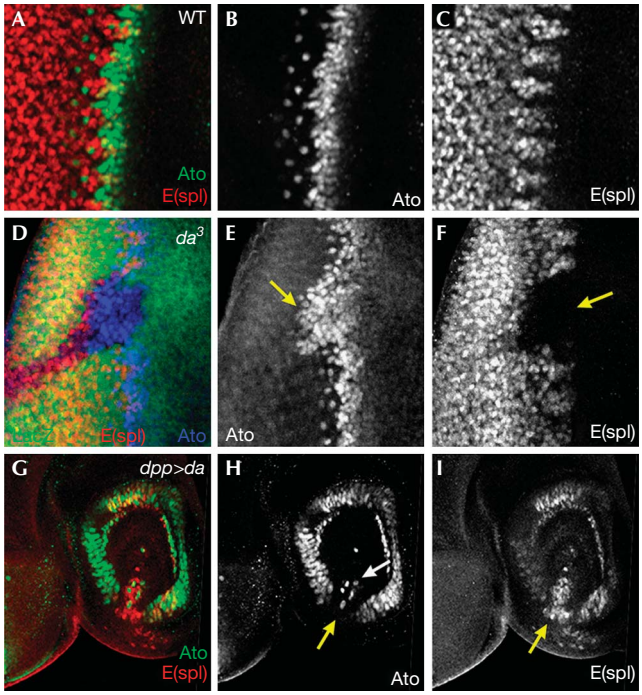

Fig 4 | Daughterless activates the expression of Enhancer-of-split. (**A–C**) Complementary expression patterns of Ato and E(spl) along the furrow. (**D–F**) Expression of E(spl) was absent or downregulated in a da^3 LOF clone (**F**, arrow). The expression of Ato was expanded in the same clone (**E**, arrow). (**G–I**) Overexpression of *da* by *dpp-Gal4* activated the expression of E(spl) (**I**, arrow) while suppressing the expression of Ato (**H**, yellow arrow). A few Ato-positive cells were occasionally found in the overexpression domain of Da (**H**, white arrow). Ato, atonal; Da, Daughterless; Dpp, decapentaplegic; E(spl), Enhancer-of-split; LOF, loss of function.

inhibition. Furthermore, the overexpression of ASC proneural factors, together with Da, can synergize with Suppressor of hairless and N to activate the expression of Enhancer-of-split (E(spl)) in cultured cells (Cooper *et al*, 2000). As E(spl) is expressed complementary to the expression of Ato (Baker *et al*, 1996; Dokucu *et al*, 1996) in the same cells expressing a high level of Da (Figs 1F,4A–C), we tested whether Da alone could regulate the expression of E(spl) *in vivo* (Fig 4D–F). The expression of E(spl) proteins was reduced in da^3 mutant cells (Fig 4F), showing that Da is required for the expression of E(spl) *in vivo*. Furthermore, the overexpression of Da with *dpp-Gal4* could induce the expression of ectopic E(spl) in the *dpp* domain of the antenna disc (Fig 4I). These results indicate that a high level of Da expression is necessary and sufficient for the activation of E(spl) expression.

As E(spl) is the main mediator of N signalling, Ato repression by a high level of Da might be dependent on the expression of E(spl). To test this possibility, we used the Mosaic Analysis with a Repressible Cell Marker (MARCM) method (Lee *et al*, 2000) to generate *E(spl)* LOF clones in which the expression of Da is induced by *tubulin (tub)-Gal4*. Da overexpression in *E(spl)* LOF clones did not show a significant repression of Ato (data not shown). Similarly, overexpression of E(spl)mδ in *da* LOF clones did not show noticeable repression of Ato (supplementary Fig S4 online). These data suggest that both Da and E(spl) are required for positive feedback regulation and for repression of Ato during lateral inhibition. However, it is also possible that other bHLH family genes of the *E(spl)* complex loci might be required, or that the overexpression of E(spl) or Da by *tub-Gal4* in MARCM assays might not be strong enough to repress the expression of *ato*. By contrast, Da expression by *dpp-Gal4* induces the expression of E(spl), even in the proximal sector of the antenna disc where Ato is not expressed (Fig 4G–I). *amos*, the proneural gene for olfactory sensilla, is not expressed in the antenna disc at this time (zur Lage *et al*, 2003). Thus, a high level of Da can induce E(spl) in the absence of Ato, although Da might act with other class II proteins to promote the expression of E(spl).

Notch signalling is essential for high-level expression of Da

As N signalling is activated in the same cells surrounding R8 founder neurons, we examined whether Da expression is affected by removing the function of *N* using a temperature-sensitive allele, N^{ts} (Fig 5A–D). Consistent with previous observations (Baker *et al*, 1996), the loss of function of *N* at the restrictive temperature resulted in several Ato-positive cells per proneural cluster (Fig 5B). Furthermore, the transient loss of *N* activity abolished the high-level of Da expression between the proneural clusters but did not eliminate the basal level of Da expression in the same cells (Fig 5D). This suggests that N signalling is essential for the high-level upregulation of Da expression. As shown earlier (Fig 2), the expression of *da* is regulated by Hh and Dpp signalling, as well as Ato (supplementary Fig S1 online). Thus, it is possible that the regulation of Da by Hh and Dpp might be mediated by Ato-dependent N signalling in the non-R8 precursor cells.

To investigate further the role of N signalling in the expression of Da, we tested whether E(spl) proteins mediate the function of N in inducing a high level of Da expression (Fig 5E–H). As expected from earlier studies (Ligoxygakis *et al*, 1998), loss of *E(spl)* caused ectopic expression of Ato in *E(spl)* mutant clones because of the lack of N-mediated lateral inhibition (Fig 5G). Interestingly, the high level of Da expression was suppressed, but the basal level of Da expression was still detected in *E(spl)* mutant clones (Fig 5H), as seen in N^{ts} mutant eye discs (Fig 5D). Thus, E(spl) is required for the high level but not for the basal level of Da expression. In contrast to da^3 LOF mutant cells that fail to differentiate in spite of ectopic Ato expression (Fig 3D–F), *E(spl)* LOF mutant cells not only expressed ectopic Ato but also differentiated into ectopic photoreceptors (Fig 5I–L). Thus, the basal level of Da expression remaining in *E(spl)* LOF clones (Fig 5H, yellow arrow) is sufficient for the formation of a functional complex with Ato to induce neural differentiation.

On the basis of the above observations, we propose a model in which Da has dual functions as a proneural and as an anti-proneural factor depending on the expression level during early retinal neurogenesis (Fig 5M). The anti-proneural function of Da proposed in our model provides an explanation for the abnormal upregulation of Ato in *da* mutant cells in the furrow, although the LOF experiments are also consistent with the pre-existing view that Da promotes the function of Ato (Chen & Chien, 1999). In Ato-positive neural precursors, low levels of Da expression are sufficient to form heterodimers with Ato to function as a proneural factor. In neighbouring cells, the N–E(spl) pathway further upregulates the expression of Da, which, in turn, induces more

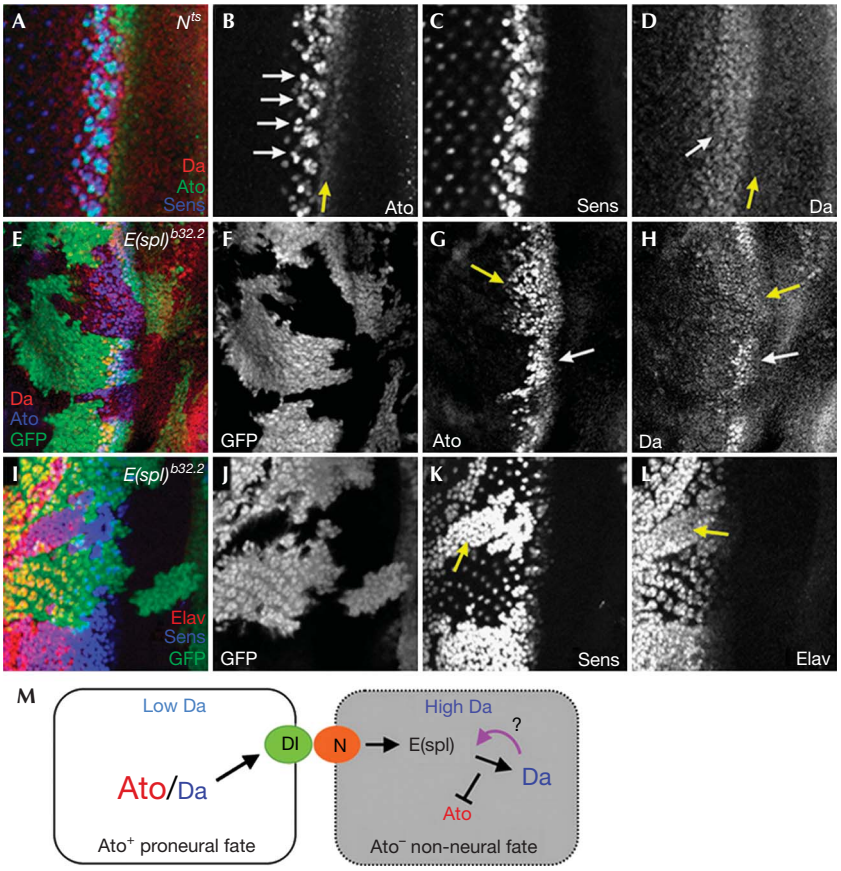

Fig 5 | The Notch signalling pathway is required for the high level of Daughterless expression. (**A–D**) Loss of *N* abolished high-level expression of Da, but had little effect on the low-level expression of Da in the furrow (**D**, white arrow). (**E–H**) Loss of *E(spl)* inhibited the high-level expression of Da, but a low level of Da still remained (**H**, compare the mutant (yellow arrow) and wild-type (white arrow) regions). (**I–L**) Low levels of Da expression remaining in *E(spl)* LOF clones were sufficient for retinal differentiation as shown by the expression of Senseless (Sens (R8 marker) and Elav (pan-neural marker) arrows). (**M**) A model for function of Da during retinal neurogenesis (see text). Da, Daughterless; E(spl), Enhancer-of-split; LOF, loss of function; N, Notch.

expression of E(spl). This putative feedback regulation might provide a mechanism for more effective lateral inhibition of Ato expression for the selection of R8. Interestingly, Da can form a homodimer and bind to DNA *in vitro* (Murre *et al*, 1989; Jafar-Nejad *et al*, 2003). Thus, in Ato-negative cells surrounding the R8 precursors, a high level of Da expression might enforce the formation of Da homodimers and/or heterodimers with other unknown bHLH proteins to repress the expression of *ato*. It would be interesting to see whether mammalian type I bHLH proteins such as E proteins might also be specifically regulated to have distinct developmental functions as seen in the case of Da.

METHODS

Generation of LOF mosaic clones and overexpression studies. LOF clones were generated by the FLP/FRT system (Xu & Rubin, 1993). Gal4-UAS system was used for overexpression studies. See the supplementary information online for more details, including mutant and transgenic flies used in this study.

Immunocytochemistry. Third instar eye imaginal discs were dissected in phosphate-buffered saline on ice, fixed in 2% paraformaldehyde-lysine-periodate fixative and stained. See the supplementary information online for more details.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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