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Formation of the Asymmetric Pineal Complex in Zebrafish Requires Two Independently Acting Transcription Factors

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Abstract

The pineal complex of zebrafish consists of a pineal organ and a left-sided parapineal organ. Mutation of the *floating head* (*flh*) gene, which encodes a homeodomain protein, causes premature termination of pineal cell division without affecting specification or asymmetric placement of the parapineal. The *from beyond* (*fby*) mutation, a premature stop codon in the T-domain-containing protein Tbx2b, disrupts formation of the parapineal while leaving the pineal largely intact. However, *flh* is reported as being required for *tbx2b* transcription. To resolve the paradox that *flh*and *tbx2b* mutants have opposite phenotypes but have been placed in the same genetic pathway, we have examined transcriptional cross-regulation in single *flh* or *fby* mutants and genetic epistasis in double mutants. Careful analysis shows that *flh* is not required for *tbx2b* transcription and double mutants exhibit an additive phenotype. We conclude that Flh and Tbx2b regulate separate programs of pineal and parapineal development.

Keywords

homeodomain; t-box; epiphysis; epithalamus; dorsal diencephalon

Introduction

The epithalamus, or dorsal diencephalon, of the vertebrate brain includes the left and right habenular nuclei (habenulae) and the melatonin-secreting pineal complex. The habenulae receive afferent input from forebrain regions including the septum, globus pallidus, and lateral hypothalamus, and send efferent axons to the midbrain (Sutherland, 1982). The pineal complex includes the pineal organ, which is placed just to the left of the midline (Liang et al., 2000), and an accessory organ called the parapineal in lampreys and fish (Concha and Wilson, 2001). The parapineal organ is also asymmetrically placed in the brain, a phenomenon first described by Hill (1892). In zebrafish, the parapineal lies adjacent to the left habenula and dictates left-right (L-R) differences in size, neuropil and gene expression between the left and right habenulae (Concha et al., 2003; Gamse et al., 2003).

The zebrafish pineal complex anlage gives rise to both the pineal and parapineal organs. Lineage labeling at 22–24 hours post-fertilization (h) reveals that the anterior portion of the pineal complex anlage gives rise to both pineal and parapineal cells, while more posterior regions produce pineal cells only (Concha et al., 2003). Two transcription factors have been identified that regulate pineal or parapineal development: the homeodomain protein Floating head (Flh) and the T-box domain protein T-box 2b (Tbx2b, previously known as Tbx-c). Flh

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The Supplementary Information referred to in this article can be viewed online.

activity promotes the generation of pineal cells; in *flh*^{n1} mutants, neurogenesis initiates in the pineal complex but ends prematurely at 18 somites (s) stage (Masai et al., 1997). By contrast, Tbx2b is required for the specification and migration of parapineal cells; in *fby*^{c144} mutants (a mutation in *tbx2b*), few or no parapineal cells develop and those that do fail to migrate to the left side of the brain (Snelson et al., 2008).

The regulatory relationship between *flh* and *tbx2b* is unclear. In the pineal complex anlage, *flh* expression begins at 80% epiboly (Masai et al., 1997), about 4 hr prior to *tbx2b* expression (Snelson et al., 2008). Expression of *tbx2b* and *flh* overlaps extensively within the pineal complex anlage at 24 h (Snelson et al., 2008). A previous publication (Cau and Wilson, 2003) suggested that Flh is required for expression of *tbx2b*. However, the phenotypes of *flh* and *fby* mutants are very different, arguing against the two genes acting in the same pathway. Asymmetrically-placed parapineal cells form *in flh* mutant larvae (Gamse et al., 2002, 2003), but not *fby* mutant larvae (Snelson et al., 2008). This observation prompted a re-evaluation of *tbx2b* and *flh* cross-regulation.

Examination of *flh* and *tbx2b* mRNA in *fby* and *flh* mutants reveals that they do not regulate one another's expression, and *flh;fby* double mutants exhibit an additive phenotype. Additionally, in *flh* mutants, specification and asymmetric migration of parapineal cells are indistinguishable from wild-type (WT) embryos. These results indicate that *flh* and *tbx2b* act independently of one another during pineal versus parapineal development.

Results

Parapineal Cell Division Ends Prior to Pineal Cell Division

To identify the period of development when pineal and parapineal cells are dividing, we performed BrdU pulse labeling at stages between 10 s and 30 hr and examined the fate of labeled cells at 4 days post-fertilization (d). To identify pineal and parapineal cells, embryos carrying the transgene *Tg-(foxd3:gfp)*^{fkg3} were used (Gilmour et al., 2002). Peak labeling of parapineal cells occurred when embryos received a half-hour pulse of BrdU at the 15 s stage (Fig. 1A,E,F and Table 1); BrdU pulses before (10 s) or after (18 s or later) labeled few or no parapineal cells (Fig. 1E,F and Table 1). Pineal cells also have maximal incorporation of BrdU at 15 s; interestingly, a second smaller peak of labeling occurs at 24 h (Fig. 1C,E,G and Table 1). In *flh* mutant larvae, pineal and parapineal BrdU labeling at the 10 s stage is similar to WT and increases at 15 s; however, almost no BrdU labeling of the pineal or parapineal occurs at or after 18 s (Fig. 1B,D,F,G and Table 1). The number of *foxd3:gfp*-positive cells in the pineal organ is reduced (Table 1), consistent with a premature end to pineal neurogenesis in *flh* mutants (Masai et al., 1997).

The Parapineal Organ in *flh* Mutants Develops Similarly to WT

In *flh* mutants, a single asymmetrically placed parapineal organ is always observed (Gamse et al., 2002) (although its placement is reversed in half of mutant larvae due to disrupted midline formation; Halpern et al., 1995). To confirm that the timing and migration of parapineal cells in *flh* mutants is similar to WT, time-lapse imaging was used in embryos carrying the transgene *Tg(foxd3:gfp)*^{fkg3} (Fig. 2). Similar to WT (Snelson et al., 2008; see Supplemental Movie 1, which can be viewed online), in *flh* mutants *foxd3:gfp*-positive parapineal cells are first visible at 31 h (Fig. 2A); cells continue to migrate unilaterally in a cluster between 32 and 46 h (Fig. 2B–P and Supplemental Movie 2).

To test whether the correct number of parapineal cells are specified in *flh* mutant larvae, we examined the expression of the gene *growth factor independent-1* (*gfi-1*), which is expressed in parapineal cells from 48 h onward; no *gfi-1* expression is seen in pineal cells (Dufourcq et

al., 2004). In WT larvae, an average of 10 *gfi-1* expressing cells are found on the left side of the brain in a tight cluster at 4 d (Fig. 3A and Table 2). Similarly, *flh* mutants form 10 *gfi-1*-expressing cells on average (Fig. 3B and Table 2).

To confirm that parapineal cells in *flh* mutant larvae do not express a pineal-specific marker, expression of the Fret43 antigen was examined in the context of the transgene *Tg (foxd3:gfp)*^{fkg3} at 4 days. In WT larvae, Fret43 (labeled by the Zpr-1 antibody; Larison and Bremiller, 1990), is expressed on red-green double cone cells found throughout the pineal organ (Masai et al., 1997) but excluded from the parapineal organ (Fig. 3C and Table 2). As in WT, none of the parapineal cells in *flh* mutants express Zpr-1 (Fig. 3D and Table 2).

To assay the ability of the parapineal in *flh* mutants to dictate L-R differences in the habenulae, expression of the asymmetrically expressed gene *leftover* (*lov*) (Gamse et al., 2003) was examined at 4 days. In both WT and *flh* mutant larvae, expression of *lov* was found in more cells of the habenula adjacent to the parapineal than in the contralateral habenula (Fig. 3E,F).

In sum, all of the characteristics of the parapineal, including number of cells specified, asymmetric migration, and ability to direct habenular asymmetry, are comparable between *flh* mutants and WT.

Flh and Tbx2b Do Not Regulate One Another's Expression

To evaluate cross-regulation of *flh* and *tbx2b*, we examined expression of each gene in the pineal complex of *tbx2b* and *flh* mutants. In WT embryos, expression of *tbx2b* begins at the 6 s stage in bilateral groups of cells in the pineal complex anlage, which soon merge to form one medial domain. At 24 h, expression is robust (Dheen et al., 1999; Ruvinsky et al., 2000; Snelson et al., 2008; Fig. 4A,B). *flh* expression begins at 80% epiboly in the pineal complex anlage (Masai et al., 1997), and is found in a single medial domain at 6 s and 24 h stages (Fig. 4C,D). Double labeling reveals that expression of *tbx2b* overlaps with the *flh* expression domain at 6 s and 24 hr; *flh* expression extends further medially than *tbx2b* at 6 s (Fig. 4E,F). In *flh* mutants, *tbx2b* expression is still detected at both 6 s and 24 hr (Fig. 4G,H). The total number of *tbx2b*-expressing cells in the pineal complex anlage of *flh* mutants is reduced relative to wild type, consistent with the reduced size of the pineal organ that will develop. In *fby* mutants, the number of *flh*-expressing cells detected at 6 s and 24 h is similar to WT (Fig. 4I,J). The data demonstrate that *tbx2b* and *flh* expression is maintained in *flh* and *fby* mutants, respectively.

flh;fby Double Mutants Have an Additive Phenotype

A sensitive test for genetic interaction is examining the phenotype of homozygous *flh;fby* double mutant embryos. In *fby* single mutants at 4 days, the pineal organ is similar in size to WT, but the *gfi-1*-expressing parapineal cells are reduced in number, remain near the midline, and fail to upregulate *lov* in the left habenula (Snelson et al., 2008; Fig. 5A,C,E).

Similar to *fby* single mutants, in *flh;fby* double mutants the *gfi-1*-expressing parapineal cells are fewer in number than WT and do not migrate leftward (Fig. 5B). Additionally, *lov* expression in double mutants is not upregulated in the left habenulae, similar to *fby* single mutants (Fig. 5F). The number of GFP-expressing pineal cells in double mutants is reduced to a level similar to the number of pineal cells in *flh* single mutants. In addition, the number of Zpr-1-labeled cells in double mutants is ~5-fold reduced compared to WT and indistinguishable from *flh* single mutants (Fig. 5D and Table 2).

These data indicate that *flh;fby* double mutants have an additive phenotype, exhibiting phenotypes in the pineal and parapineal organs characteristic of each single mutant.

Discussion

Previous work showed that Flh is a key regulator of pineal neurogenesis (Masai et al., 1997, Cau and Wilson, 2003) and suggested that Flh activates *tbx2b* transcription in pineal complex precursors (Cau and Wilson, 2003). The latter result implies that *flh* mutants should show defects in parapineal formation, which requires *tbx2b* function (Snelson et al., 2008). However, *flh* mutants develop a parapineal that is indistinguishable from WT larvae with regard to asymmetric migration and number of cells specified. Moreover, disruption of *flh* function does not eliminate the initiation or maintenance of *tbx2b* transcription in the pineal complex. Examination of homozygous double mutants reveals an additive phenotype and supports parallel rather than serial action of *flh* and *tbx2b*. These data argue strongly against *tbx2b* and *flh* acting in the same genetic pathway during the development of the pineal complex (Fig. 5G).

On the basis of mutational analyses and BrdU labeling, we propose the following model. Expression of *flh* and *tbx2b* before the 15 s stage defines a field of precursor cells that can divide to produce pineal or parapineal cells. An initial burst of cell division at the 15 s stage generates many new pineal complex cells; *tbx2b* activity assigns parapineal fate to some of these cells while the remainder develop with pineal fate. Subsequently, division of parapineal precursor cells drops off sharply, suggesting that *tbx2b* is largely dispensable for parapineal development after the 15 s stage. A method to conditionally inactivate *tbx2b* in the pineal complex will be necessary to test this hypothesis.

Beginning at 18 s, *flh* activity is required for continued production of cells in the pineal anlage (Masai et al., 1997, and this study). Almost all of the cells that divide after 18 s contribute to the pineal lineage. It is not clear why division of cells that give rise to the parapineal organ slows after the 15 s stage while division of pineal progenitors continues. One possibility is that separate groups of pineal and parapineal progenitor cells exist, and the latter group largely stops dividing after 15 s. At 6 s, the *tbx2b*-positive and *tbx2b*-negative cells within the *flh*-expressing domain may represent parapineal and pineal progenitors, respectively. A second explanation is that the same progenitor cells can give rise to either pineal or parapineal cells initially, but primarily produce pineal cells after 15 s. Careful lineage labeling of single cells in the pineal complex anlage will distinguish between these two scenarios.

A small number of pineal and parapineal cells still develop in *flh;fby* double mutant embryos. Presumably, there are other genes that contribute to proliferation and/or control specification of pineal and parapineal cells; however, these genes have yet to be isolated. Forward genetic screening in a sensitized background such as a *tbx2b* hypomorph should identify these genes.

The independent roles that *tbx2b* and *flh* play in the development of the pineal complex contrasts with the regulation of *tbx2b* expression by *flh* during notochord development. In *flh* mutants, *tbx2b* expression is absent in notochord precursor cells found in the chordoneural hinge (Dheen et al., 1999). In the notochord, *flh* appears to maintain *tbx2b* expression by blocking the action of another T-box transcription factor, *spadetail/tbx16 (spt)* (Dheen et al., 1999). By contrast, *spt* is not expressed in the pineal anlage (Ruvinsky et al., 1998), which may explain why *flh* and *tbx2b* activity are uncoupled in this tissue. Identification of other genes that are expressed in both the pineal complex anlage and notochord precursors, similar to *flh* and *tbx2b*, will illuminate the genetic networks involved in the patterning of these two tissues, and allow a comparison of evolutionary conservation and divergence between these networks.

Experimental Procedures

Zebrafish

Zebrafish were raised at 28.5°C on a 14/10 hr light/dark cycle and staged according to hours (h) or days (d) post-fertilization. The wild-type AB strain (Walker, 1999); the transgenic lines Tg(*foxd3:*GFP)^{fkg17} and Tg(*foxd3:*GF-P)^{fkg3} (Gilmour et al., 2002), and mutants carrying the spontaneous allele *flh*^{n1} (Halpern et al., 1995) and the ethylnitrosourea-induced null allele *fby*^{c144} (Snelson et al., 2008) were used.

RNA In Situ Hybridization

Whole-mount RNA in situ hybridization was performed as described previously (Gamse et al., 2003), using reagents from Roche Applied Bioscience. RNA probes were labeled using fluorescein-UTP or digoxygenin UTP. To synthesize antisense RNA probes, *znot* (*flh*) (Talbot et al., 1995) and pBK-CMV-*leftover* (Gamse et al., 2003) were linearized with *Eco*RI and transcribed with T7 RNA polymerase; pCRII-*tbx2b* (Snelson et al., 2008) with *Bam*HI and T7 RNA polymerase; and pBS-*gfi1* (Dufourcq et al., 2004) with *Sac*II and T3 RNA polymerase. Embryos were incubated at 70°C with probe and hybridization solution containing 50% formamide (or 65% for *cyc*). Hybridized probes were detected using alkaline phosphatase-conjugated antibodies and visualized by 4-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) staining. All in situ data was collected on a Leica DM6000B microscope with a 20× or 40× objective.

BrdU Labeling

For pulse labeling with bromodeoxyuridine (BrdU, Roche), embryos were incubated with ice-cold 10 mM BrdU dissolved in embryo medium plus DMSO at 2% for 10–15 s embryos, 5% for 18-s-stage embryos, or 10% for 24-30-h embryos, over a period of 30 min. Embryos were then washed extensively with embryo medium and raised to 4 d, when they were fixed in 4% paraformaldehyde for 2 hours at room temperature.

Time-Lapse Imaging

For time-lapse imaging, WT or *flh* mutant embryos carrying the transgene (*foxd3*:GFP)^{fkg3} were mounted in a 0.8% solution of low melt agarose containing 0.003% PTU and anesthetized using 0.04% Tricaine. Images were collected on a Zeiss/Perkin Elmer spinning disk confocal microscope with a 40× oil-immersion objective every 15 min from 31 to 46 hr, and analyzed using Volocity software (Improvision).

Immunofluorescence

For whole-mount immunohistochemistry, 4 d larvae were fixed in 4% paraformaldehyde overnight (for anti-Zpr-1/anti-GFP) or 2 hr (for anti-BrdU/anti-GFP). Samples were permeabilized by treatment with 10 μg/ml Proteinase K (Roche Applied Bioscience) and refixed in 4% paraformaldehyde. For BrdU labeling, samples were additionally washed in deionized water following refixation, and incubated in 2 N HCl for 1 hr. All samples were blocked in PBS with 0.1% Triton X-100, 2% sheep serum, 1% DMSO, and 10% BSA (PB-STrS). For antibody labeling, rabbit anti-GFP (1:1,000, Torrey Pines Biolabs), mouse anti-Zpr-1 (1:250, Zebrafish International Resource Center, Eugene, OR), and mouse anti-BrdU antibody (1: 200, developed by Stephen Kaufman, obtained from the Developmental Studies Hybridoma Bank, University of Iowa) were used. Larvae were incubated overnight in primary antibody diluted in PBSTrS. Primary antibody was detected using goat-anti-rabbit or goat-anti-mouse secondary antibodies conjugated to the Alexa 568 or Alexa 488 fluorophores (1:350, Molecular Probes). Immunofluorescence data were collected on a Zeiss/Perkin Elmer spinning

disk confocal microscope or a Zeiss LSM 510 Meta with a 40× oil-immersion objective and analyzed with Volocity software (Improvision).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Cau E, Wilson SW. Ash1a and Neurogenin1 function downstream of Floating head to regulate epiphysial neurogenesis. Development 2003;130:2455–2466. [PubMed: 12702659]

Concha ML, Wilson SW. Asymmetry in the epithalamus of vertebrates. J Anat 2001;199:63–84. [PubMed: 11523830]

Concha ML, Russell C, Regan JC, Tawk M, Sidi S, Gilmour DT, Kapsimali M, Sumoy L, Goldstone K, Amaya E, Kimelman D, Nicolson T, Grunder S, Gomperts M, Clarke JD, Wilson SW. Local tissue interactions across the dorsal midline of the forebrain establish CNS laterality. Neuron 2003;39:423–438. [PubMed: 12895418]

Dheen T, Sleptsova-Friedrich I, Xu Y, Clark M, Lehrach H, Gong Z, Korzh V. Zebrafish tbx-c functions during formation of midline structures. Development 1999;126:2703–2713. [PubMed: 10331981]

Dufourcq P, Rastegar S, Strahle U, Blader P. Parapineal specific expression of gfi1 in the zebrafish epithalamus. Gene Expr Patterns 2004;4:53–57. [PubMed: 14678828]

Gamse JT, Shen YC, Thisse C, Thisse B, Raymond PA, Halpern ME, Liang JO. Otx5 regulates genes that show circadian expression in the zebrafish pineal complex. Nat Genet 2002;30:117–121. [PubMed: 11753388]

Gamse JT, Thisse C, Thisse B, Halpern ME. The parapineal mediates left-right asymmetry in the zebrafish diencephalon. Development 2003;130:1059–1068. [PubMed: 12571098]

Gilmour DT, Maischein HM, Nusslein-Volhard C. Migration and function of a glial subtype in the vertebrate peripheral nervous system. Neuron 2002;34:577–588. [PubMed: 12062041]

Halpern ME, Thisse C, Ho RK, Thisse B, Riggleman B, Trevarrow B, Weinberg ES, Postlethwait JH, Kimmel CB. Cell-autonomous shift from axial to paraxial mesodermal development in zebrafish floating head mutants. Development 1995;121:4257–4264. [PubMed: 8575325]

Hill C. Development of the epiphysis in Coregonus Albus. J Morphol 1892;5:503–510.

Larison KD, Bremiller R. Early onset of phenotype and cell patterning in the embryonic zebrafish retina. Development 1990;109:567–576. [PubMed: 2401210]

Liang JO, Etheridge A, Hantsoo L, Rubinstein AL, Nowak SJ, Izpisua Belmonte JC, Halpern ME. Asymmetric nodal signaling in the zebrafish diencephalon positions the pineal organ. Development 2000;127:5101–5112. [PubMed: 11060236]

Masai I, Heisenberg CP, Barth KA, Macdonald R, Adamek S, Wilson SW. floating head and masterblind regulate neuronal patterning in the roof of the forebrain. Neuron 1997;18:43–57. [PubMed: 9010204]

Ruvinsky I, Silver LM, Ho RK. Characterization of the zebrafish tbx16 gene and evolution of the vertebrate T-box family. Dev Genes Evol 1998;208:94–99. [PubMed: 9569350]

Ruvinsky I, Oates AC, Silver LM, Ho RK. The evolution of paired appendages in vertebrates: T-box genes in the zebrafish. Dev Genes Evol 2000;210:82–91. [PubMed: 10664151]

Snelson CD, Santhakumar K, Halpern ME, Gamse JT. Tbx2b is required for the development of the parapineal organ. Development 2008;135:1693–1702. [PubMed: 18385257]

Sutherland RJ. The dorsal diencephalic conduction system: a review of the anatomy and functions of the habenular complex. Neurosci Biobehav Rev 1982;6:1–13. [PubMed: 7041014]

Talbot WS, Trevarrow B, Halpern ME, Melby AE, Farr G, Postlethwait JH, Jowett T, Kimmel CB, Kimelman D. A homeobox gene essential for zebrafish notochord development. Nature 1995;378:150–157. [PubMed: 7477317]

Walker, C. Methods in Cell Biology. London: Elsevier; 1999. Haploid screens and gamma-ray mutagenesis; p. 43-70.

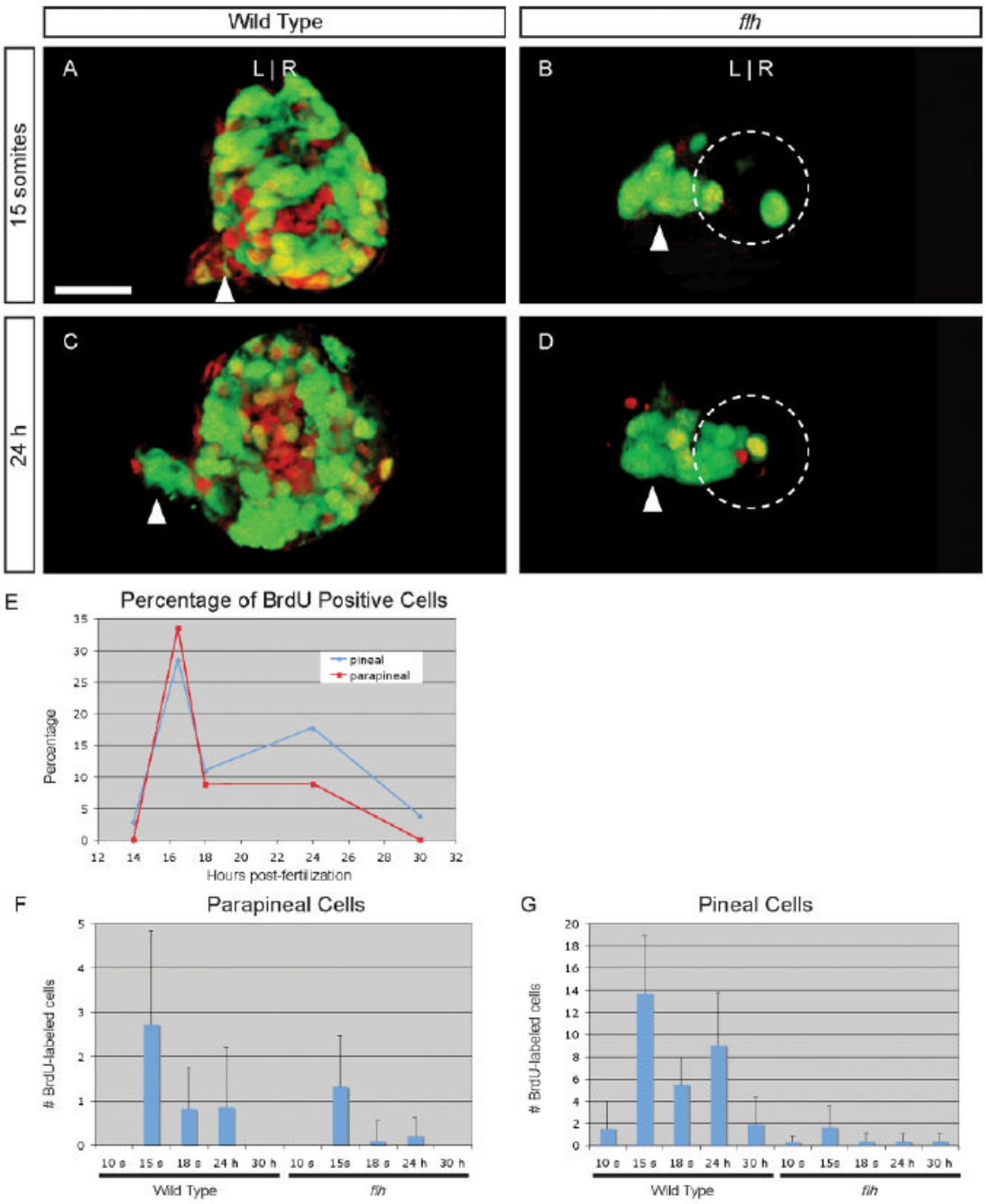

Fig. 1.

Cell division of parapineal precursors is largely complete by 18 s. **A–D:** Dorsal views of 4 d larvae, with BrdU (red) and *foxd3:gfp* (green) labeling in the epithalamus, following a BrdU pulse at (A, B) 15 s or (C, D) 24 hr stage in (A, C) WT or (B, D) *flh* mutant embryos. Dashed circles in B, D indicate the pineal cells. Scale bar = 25 μM. **E:** Line graph of the percentage of WT parapineal (red line) or pineal (blue line) cells that are labeled at 4 d, following a BrdU pulse at the indicated developmental stages. **F:** Bar graph showing the absolute number of BrdU-positive parapineal cells in WT or *flh* embryos. **G:** BrdU-positive pineal cells in WT or *flh* embryos.

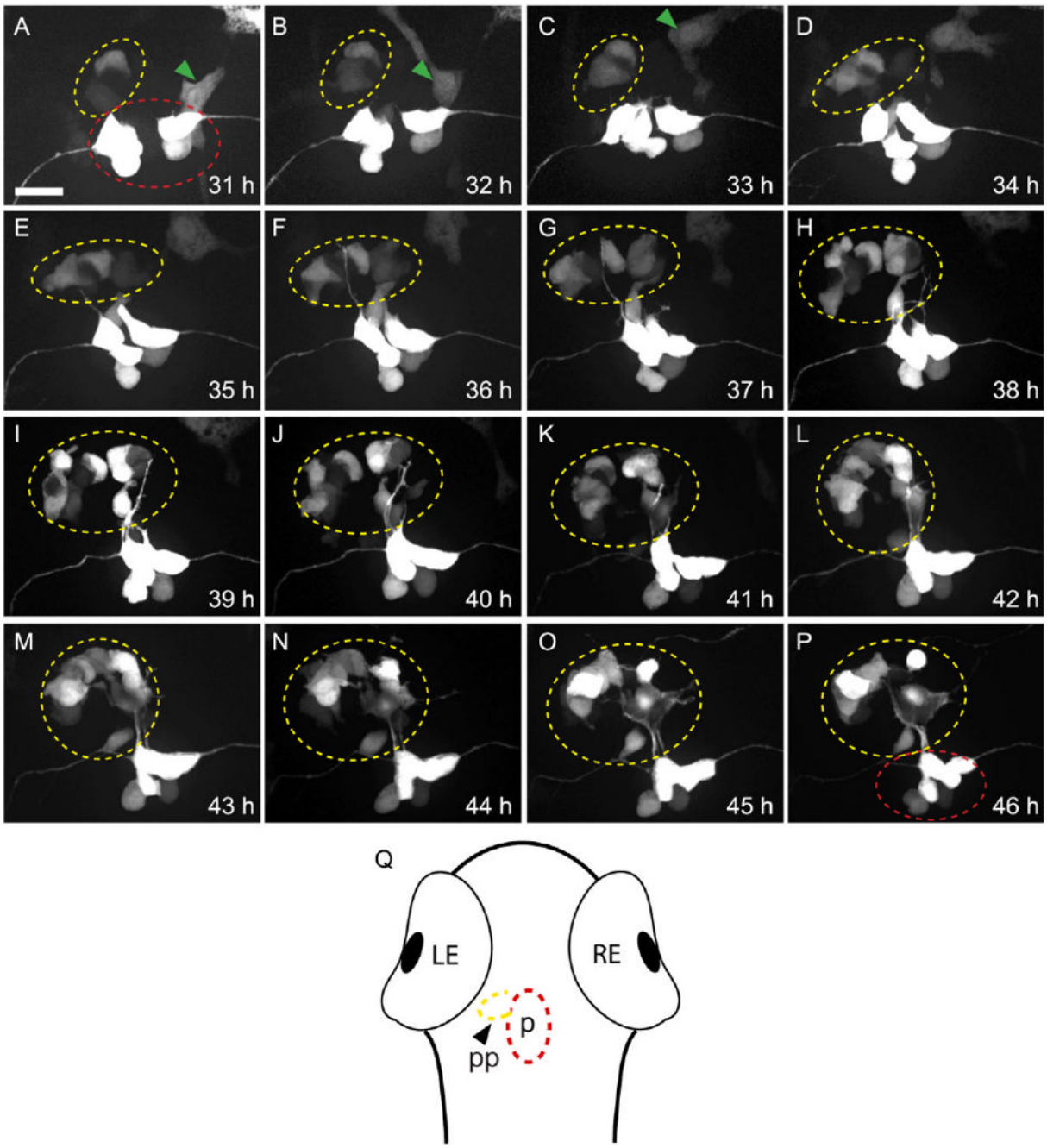

Fig. 2.

Parapineal cells migrate asymmetrically in *flh* mutants. All panels are dorsal views of *foxd3:gfp* labeling in the epithalamus of a single embryo at the times indicated. Parapineal cells are circled in yellow, pineal cells in red; a neural crest cell (which also expresses *foxd3:gfp*) in A–C is indicated by a green arrowhead. **A:** The first labeled parapineal cells are apparent near the midline at 31 hr. **B–E:** They migrate leftward. **F–P:** They are joined by more leftward-migrating parapineal cells during the subsequent 10 hr. Scale bar = 25 μm. **Q:** Diagram of a zebrafish larvae, showing the relative position of the pineal (p, red oval) and parapineal (pp, yellow oval) within the head.

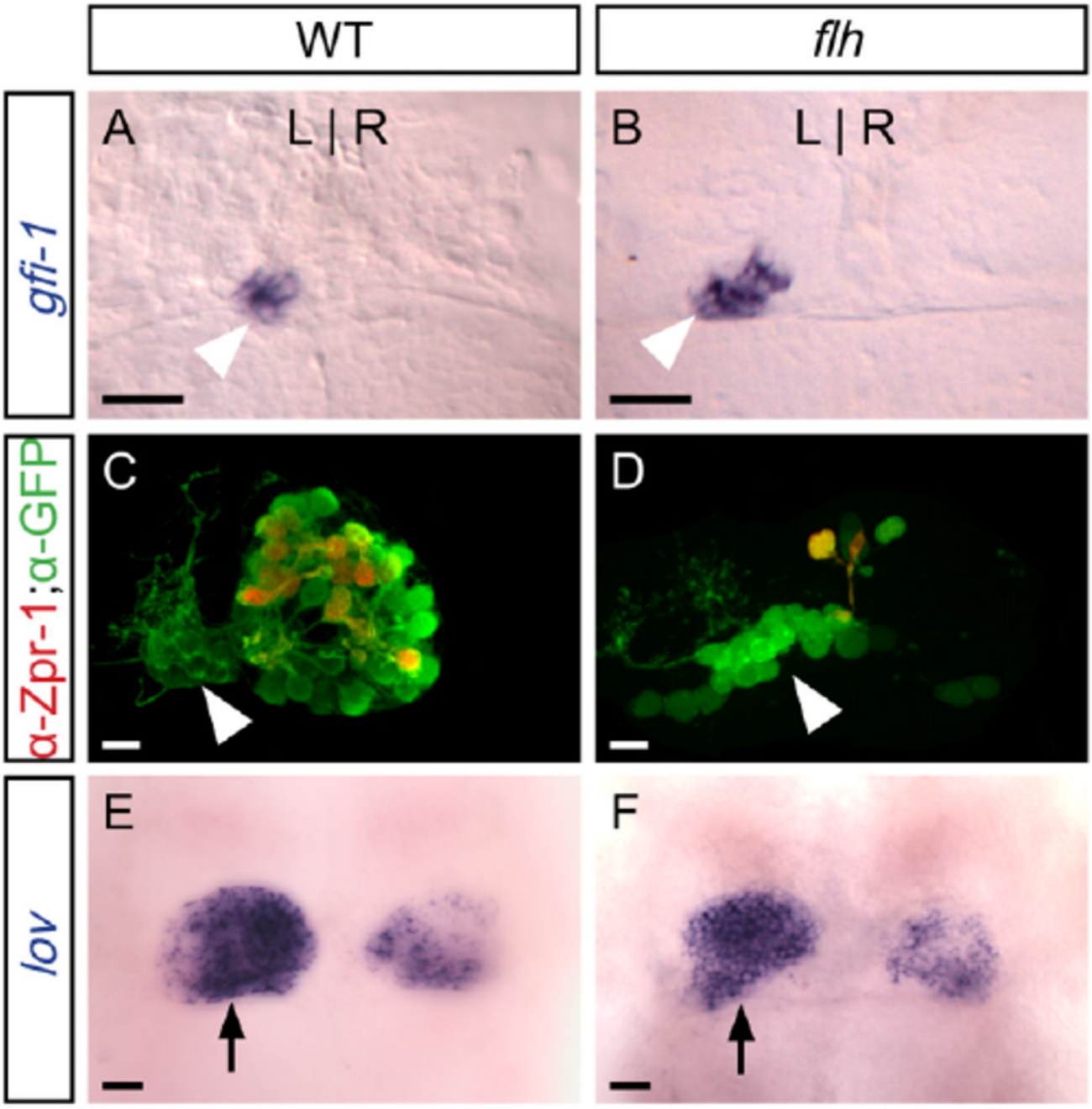

Fig. 3.

In *flh* mutants, parapineal specification is normal, but the pineal organ is reduced in size. All panels are dorsal views of the epithalamus of 4 d larvae. **A:** Expression of *gfi-1* (blue), which is restricted to the parapineal organ (arrowhead), reveals ~10 left-sided cells in WT and (**B**) *flh* larvae. **C:** In WT larvae, both the parapineal (arrowhead) and pineal express *foxd3:gfp* (green); many cells of the pineal organ are also labeled by the red-green cone marker Zpr-1 (Fret43) (red), but no parapineal cells are Zpr-1-labeled. **D:** In *flh* mutants, the pineal organ is drastically reduced in size, including fewer Zpr-1-labeled cells. As in WT, no Zpr-1-labeled cells are detected in the parapineal. **E:** At 4 d, *lov* expression is asymmetric in the habenular

nuclei; the habenula adjacent to the parapineal (arrow) expresses *lov* extensively in WT and (**F**) *flh* mutants. Scale bars = 25 μm.

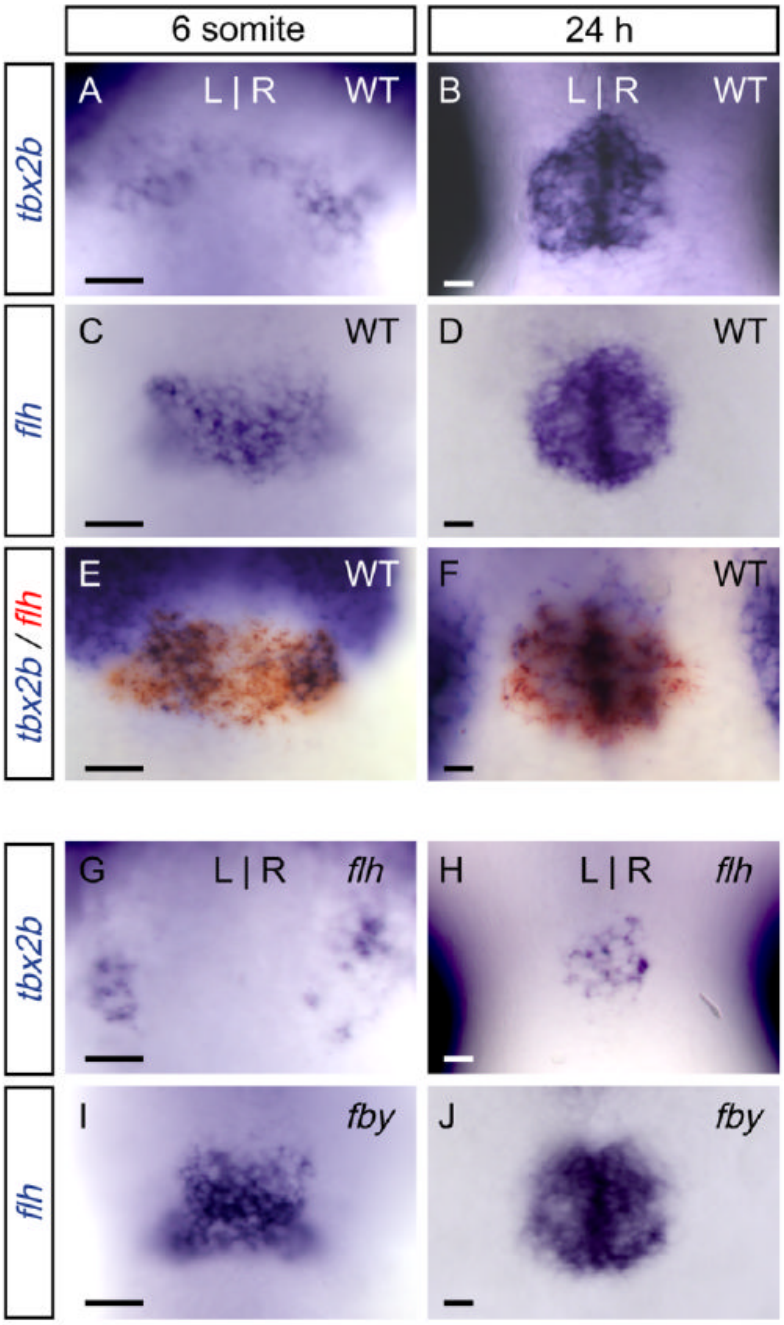

Fig. 4.

Flh and Tbx2b do not regulate one another's expression. All panels are dorsal views of the epithalamus at the stage indicated. (**A,B**) *tbx2b* and (**C,D**) *flh are* expressed in the pineal complex anlage at the 6-somite and 24-hr stages. **E,F:** Double labeling for *tbx2b* (blue) and *flh* (red) reveals that they overlap in expression at the 6-somite and 24-hr stages; *flh* is expressed further medially than *tbx2b* at the 6-somite stage. **G:** In *flh* mutants, expression of *tbx2b* initiates on time and (**H**) is maintained, albeit in a reduced number of cells. **I,J:** In *fby* mutants (a lesion in *tbx2b*), expression of *flh* is unaltered relative to WT. Scale bars = 25 μm.

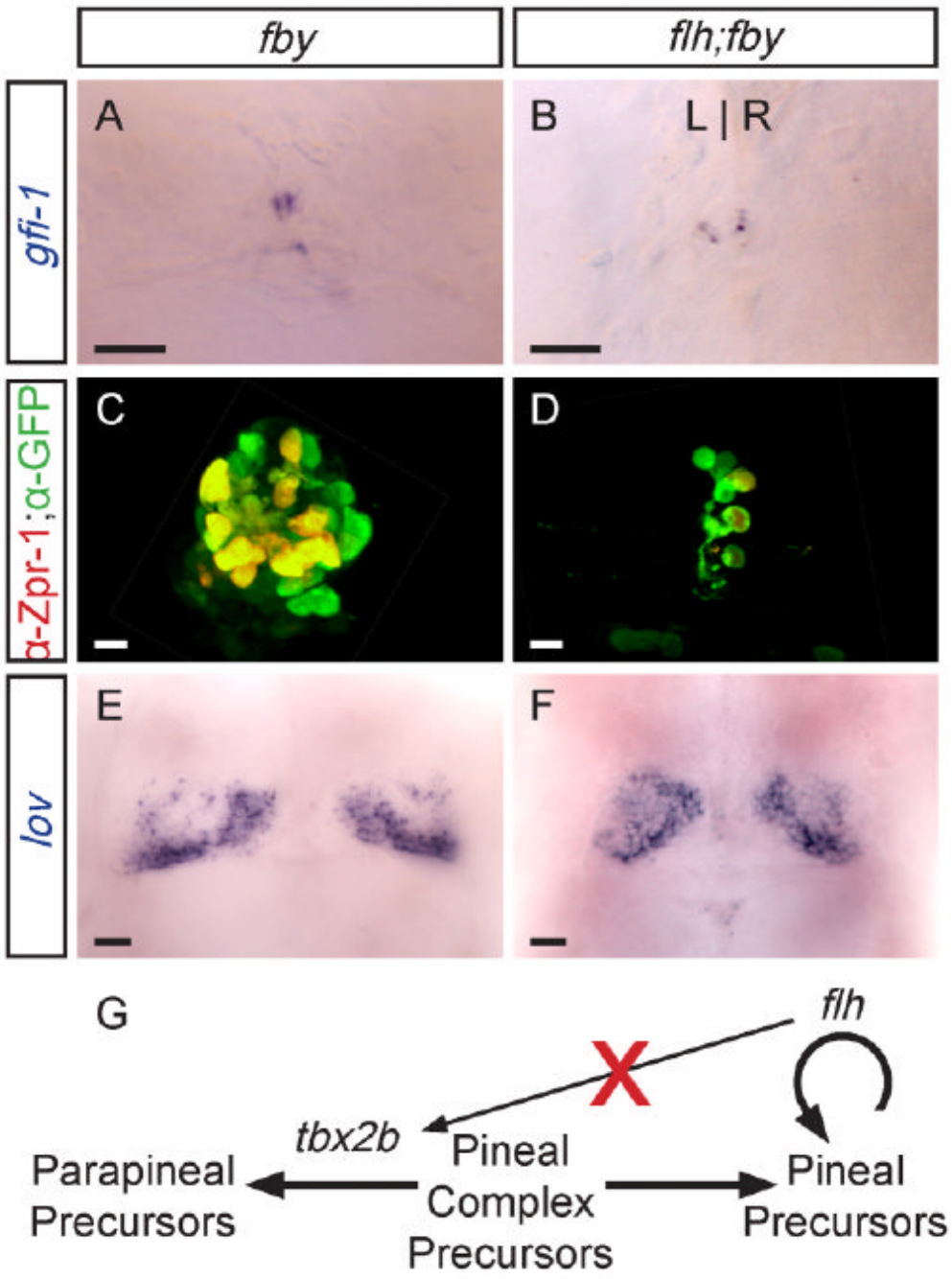

Fig. 5.

Double homozygous *flh;fby* mutants exhibit an additive phenotype. All panels are dorsal views of the epithalamus of 4 d larvae. **A:** In *fby* single mutants or (**B**) *flh;fby* double mutants, only a few disorganized parapineal cells are specified, and they fail to migrate to the left side of the brain. **C:** *fby* single mutants form a pineal organ. **D:** *flh;fby* double mutant larvae have both a reduced pineal, similar to *flh* single mutants (compare to Fig. 3D). **E:** In *fby* single mutants and (**F**) *flh;fby* double mutants, reduced *lov* expression is detected in the left habenula. Scale bars = 25 μm. **G:** Summary of *tbx2b* and *flh* activity in pineal complex development. The activity of *tbx2b* gives parapineal precursors their identity, while *flh* is needed for proliferation of pineal precursor cells. Expression of *tbx2b* does not require *flh* activity.

TABLE 1

Fate of Pineal Complex Cells Labeled with BrdU Between 10 s and 30 hr

Genotype	Stage[a]	GFP$^+$ pineal cells	BrdU$^+$ pineal cells	GFP$^+$ parapineal cells	BrdU$^+$ parapineal cells	N
WT	10 s	47 +/− 6	1 +/− 3	8 +/− 2	0	11
	15 s	50 +/− 4	14 +/− 5	8 +/− 2	3 +/− 2	25
	18 s	50 +/− 4	5 +/− 2[b]	9 +/− 2	0.8 +/− 0.9[b]	21
	24 h	51 +/− 5	9 +/− 5[b,c]	8 +/− 2	1 +/− 1[b,d]	13
	30 h	48 +/− 7	2 +/− 2	10 +/− 2	0	7
flh$^{-/-}$	10 s	17 +/− 6	0.3 +/− 0.5[e]	8 +/− 2	0[b,e]	10
	15 s	16 +/− 5	2 +/− 2[b]	11 +/− 2	1 +/− 1[b]	25
	18 s	15 +/− 4	0.3 +/− 0.8[f]	7 +/− 2	0.1 +/− 0.4[b,f]	21
	24 h	14 +/− 5	0.3 +/− 0.7[b]	8 +/− 2	0.2 +/− 0.4[b]	15
	30 h	15 +/− 6	0.4 +/− 0.7	10 +/− 3	0	17

[a] Developmental stage when BrdU was first applied to embryos during a 0.5 hr pulse; all samples were fixed at 4 d for analysis.

[b] Significantly different from WT 15 s by 2-tailed T-test, $p < 0.03$

[c] Significantly different from WT 18 s by 2-tailed T-test, $p < 0.01$

[d] Not significantly different from WT 18 s by 2-tailed T-test, $p > 0.7$

[e] Not significantly different from WT 10 s by 2-tailed T-test, $p > 0.17$.

[f] Significantly different from WT 18 s by 2-tailed T-test, $p < 0.01$

TABLE 2
Number of Labeled Cells Present in the Pineal Complex of Single and Double Mutants

Gene/protein (cell types labeled)	Genotype	Number of cells[a]	N
gfi-1 (parapineal cells)	WT	10 +/− 1	12
	flh	10 +/− 1	14
	fby;flh	3 +/− 2[b]	24
GFP in Tg(*foxd3:gfp*) (pineal and parapineal cells)	WT	52 +/− 10	5
	flh	28 +/− 7[c]	5
	fby;flh	24 +/− 10[c]	7
Zpr-1 antibody (red-green double cone cells)	WT	30 +/− 5	5
	flh	5 +/− 5[c]	4
	fby;flh	7 +/− 1[c]	4

[a] Average number of cells labeled per larvae at 4 d, plus or minus one standard deviation.

[b] Significantly different from WT and *flh* by 2-tailed T-test, $p < 0.01$.

[c] Significantly different from WT by 2-tailed T-test, $p < 0.03$.