A reporter-assisted mutagenesis screen using α1-tubulin-GFP transgenic zebrafish uncovers missteps during neuronal development and axonogenesis

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Abstract

α1-tubulin expression occurs in a neural-specific, temporally regulated, and regeneration-inducible fashion in zebrafish. A GFP reporter driven by the α1-tubulin promoter in transgenic zebrafish acts as a stable, in vivo molecular tag that follows neuronal development from birth/specification through postmitotic differentiation to axonal outgrowth and synaptogenesis. We exploited this transgenic system in a reporter expression-dependent (morphology-independent) mutagenesis screen to identify disruptions in genetic loci essential for neuronogenesis and axon elaboration, which would manifest as visually appreciable perturbations in GFP fluorescence. Thirty-two such recessive mutations were obtained, a subset of which was screened through a secondary RNA quantification-based assay to eliminate housekeeping gene defects. Three representative loci, when characterized in detail, were found to exhibit missteps in discrete, sequential stages of embryonic neuronal development. Mutation in sookshma panneurally diminishes the neural precursor pool by affecting cell proliferation in the developing embryo while patterning along the neuraxis remains unperturbed. Disruption of drishti on the other hand ameliorates the mitotic neural population by affecting cell cycle exit of progenitors and stalling their progression to the postmitotic neuronal stage, without impairing subsequent cell fate determination or differentiation. Finally, dhruva is required during neuronal differentiation for axonal branching and terminal innervation in spinal motoaxons and the retinotectal projection. Molecular identification of these loci and analysis of the remaining mutational repertoire will offer unique insights into the genetic inputs that go on to make a mature, differentiated neuron.

Keywords: Tubulin; Transgenic; Mutagenesis; Neurogenesis; Cell cycle exit; Axonal branching; Zebrafish

Introduction

Neuronal development in vertebrates such as zebrafish is a complex, multistep process beginning with the irreversible commitment of naïve ectodermal cells to a neural fate followed by proliferative precursors exiting the cell cycle, migrating and undergoing morphological differentiation to finally establish axonal connections (Blader and Strahle, 2000; Chitnis, 1999; Chitnis and Dawid, 1999; Wilson et al., 2002). On its path to becoming a mature neuron, an uncommitted progenitor is constantly faced with cell fate decisions, each determined by its unique, intrinsic code of expressed genes and the extrinsic microenvironment it resides in. Deciphering the genetic blueprint of neuronal development requires identification of these genes, operative within the cell or as part of the extracellular signaling system. Over the past decade, forward genetic screens in zebrafish, relying on examination of nervous system morphology, histology, or behavior, have offered a powerful approach to identify such loci (Knapik, 2000; Patton and Zon, 2001; Schier et al., 1996; Schier, 1997). In parallel, transgenic technology has been extensively exploited to drive reporter gene expression in select regions of the nervous system using tissue-specific promoters (Udvadia and Linney, 2003). With the objective of identifying genes essential for neuronogenesis and axonal outgrowth in the zebrafish embryo, we present a reporter-based screen that combines the specificity of a neural promoter with the unbiased potential of mutagenesis.

α1-tubulin is a neural-specific isoform of the α-tubulin family, which forms an essential part of the microtubule cytoskeleton in developing axons and dendrites (Baas, 1997; Lewis et al., 1985;...
Its expression spans the entire embryonic development of a neuron; transcription initiates in committed progenitors, continues through the process of proliferation and cell cycle withdrawal, and persists in the postmitotic progeny as they undergo migration and terminal differentiation (Gloster et al., 1994, 1999; Goldman et al., 2001). Once neurons complete axonogenesis and form synapses, transcription is down-regulated, only to be recalled during the regenerative response to injury in adults (Borman et al., 1998; Hieber et al., 1998). A green fluorescent protein (GFP) reporter driven by the 1.696-kb goldfish α1-tubulin promoter fragment (and first intron) faithfully recapitulates endogenous gene expression in our α1-T-GFP transgenic zebrafish (Goldman et al., 2001). Various cis-acting DNA elements have subsequently been found responsible for distinct modes of promoter regulation (Goldman and Ding, 2000; Senut et al., 2004). The α1-T-GFP zebrafish therefore harbors a stable, genetically encoded fluorescent indicator of neuronal development, mutation-induced alteration of which can forecast interrupted events and help identify genes involved in specific stages therein; using this basic premise we have undertaken a large-scale mutagenesis screening effort.

What kinds of mutant phenotypes and genetic loci do we expect to uncover? Because the initiation, maintenance, and down-regulation of α1-tubulin expression demands the presence of multiple signaling pathways impinging upon its promoter, disruption of an upstream regulatory gene or signaling molecule will manifest as a quantitative increase or decrease in reporter expression; therefore, such mutants will be identified. Secondly, the GFP-expressing population marks precursors, nascent mitotic neurons, as well as mature differentiating neurons; therefore, defects in generation, maintenance, or relative abundance of any of these distinct GFP-positive neuronal pools are predetermined to be selected. Further, because α1-tubulin exhibits a stringent neural-specific expression, ectopic or abnormal expression of the reporter will highlight defects in genes maintaining tissue specificity of expression. Finally, if mutants survive until adulthood, the optic nerve regeneration model will be employed to elucidate the role of the disrupted locus in injury-induced repair, which will help dissect molecular requirements for neuronal development versus regeneration (Senut et al., 2004).

In this report, we present a two-step screening strategy that first identifies candidates exhibiting altered reporter expression followed by elimination of defects in cell-essential, housekeeping genes. Mutants demonstrate a diverse array of GFP perturbations, varying from panneuronal quantitative changes to subtle alterations in specific CNS regions and in a few cases, abnormal expression patterns. We pursued the detailed characterization of three representative loci to illustrate the usefulness of this screen in dissecting neuronal development and understanding concomitant α1-tubulin promoter regulation. As described below, the sookshma gene is important for generation of a sizeable neural progenitor pool, drishìi regulates the transition from proliferation to the postmitotic differentiation whereas dhruva directs differentiating neurons during their axonal branching phase. The α1-T-GFP screen therefore offers a focused yet unbiased approach to identify genes involved in sequential cellular events between birth of a neural precursor to morphological differentiation of a neuron.

**Materials and methods**

**Fish breeding**

*Danio rerio* animals were raised in our breeding colony at 28°C observing a 14:10 h light/dark photoperiod. Embryos were produced by natural crosses, developmentally staged according to Kimmel et al. (1995), and their ages expressed as hours postfertilization (hpf) or days post fertilization (dpf). To block pigment production, 1-phenyl-2-thiourea (PTU, Sigma) at a final concentration of 0.003% was added to egg water at 8–10 hpf.

**Mutagenesis and screening**

α1-T-GFP transgenic adult males were mutagenized using N-ethyl-N-nitosourea (ENU, Sigma) according to published protocols (Mullins et al., 1994; Solnica-Krezel et al., 1994) followed by a three-generation breeding scheme to identify recessive, zygotic mutants. Screening was carried out in F3 embryos (24–60 embryos/breeding pair) from 1 to 7 dpf at 24-h intervals by examining GFP using a fluorescence stereomicroscope (MZFLIII, Leica). Onset and characteristics of the GFP defect, its temporal progression, and accompanying morphological abnormalities were carefully cataloged and heterozygous F2 carriers reared for confirmation. RT-PCR was used to assay transgene (GFP) and endogenous α1-tubulin expression in mutants displaying overall changes in fluorescence whereas in situ hybridization for the same genes used to characterize mutants exhibiting local changes in reporter expression. Complementation analysis was carried out only for mutant alleles displaying similar morphological/GFP defects.

**RNA isolation and semiquantitative RT-PCR**

Two to five embryos from age-matched wild-type and mutant groups were frozen in liquid nitrogen and stored at −70°C. Total RNA was isolated, subjected to reverse transcription (RT) followed by radioactive polymerase chain reaction (PCR), and autoradiography as described in Senut et al. (2004). Transcripts analyzed corresponded to the endogenous α1-tubulin gene (324-bp product, primer sequence listed in Senut et al., 2004) and two normalizing controls, e-1F4 (400-bp product, primer sequence listed in Senut et al., 2004) and β-actin (249-bp product, forward primer: 5’ CATCGTTCAACGGAAGTGCTTC 3’, reverse primer: 5’ GGTAAACGCTTCTGGAATGAC 3’) for normalization. All three cDNAs were amplified in the same PCR reaction, which was typically conducted at three previously identified, nonsaturating cycles (between 15 and 20 cycles) to ensure linearity of amplification. Signal strength for each PCR product was quantified using the NIH Image 1.62 software (http://rsb.info.nih.gov/nih-image) and expressed as “mean density × area”.

**BrdU incorporation**

Dechorionated embryos were soaked in 10 mM 5-bromo-2’-deoxyuridine (BrdU, Sigma) diluted in embryo medium with 15% dimethylsulfoxide (DMSO) at 6–8°C for 20 min. After incubation, they were allowed to recover in fresh medium at 28°C for 15 min and fixed in 4% paraformaldehyde (PFA), made in 0.1 M phosphate buffer (PB), pH 7.4, for immunostaining.

**Immunostaining and cell counts**

Whole-mount immunostaining was carried out using standard protocols described in Westerfield (2000). Briefly, embryos were fixed in 4% PFA at 4°C overnight, manually dechorionated, washed in PB, and permeabilized in water at room temperature (RT), 5 min to 1 h, and acetone at −20°C, 7 min. After blocking in 2% goat/horse serum, embryos were incubated with appropriate primary antibody (diluted in PB, 1% DMSO) at 4°C overnight. For embryos older than 24 hpf, 0.2–0.4% Triton X-100 was included in all solutions. Extensive washes using blocking solution were followed by incubation with biotinylated secondary antibody at 4°C
overnight repeat washes and subsequently ABC (avidin and biotinylated–
horseradish peroxidase complex, Vector Laboratories) incubation for 2–4 h, RT. The immune-complex was visualized using the chromogenic substrate 3,3’-
diaminobenzidine (DAB, Hach Company) with hydrogen peroxide. For cross-
sectional analysis, fixed embryos were cryoprotected in a gradient of sucrose (Barthel and Raymond, 1990), embedded in O.C.T mounting medium (Sakura Finetek USA), stored at –70°C, and later sectioned on a cryostat (CM3050S, Leica) at 8–12 μm thickness. Representative sections were processed for routine or BrDU immunostain-
ing as described in Senut et al. (2004).

The following primary antibodies and dilutions were used: rat anti-BrdU (1:250, Harlan, Sera-Lab); rabbit anti-cleaved caspase-3 (1:100, Cell Signaling Technology); mouse FRET11 (zpr-3) and Fret43 (zpr-1) (1:250 each, Zebrafish International Resource Center, ZIRC, Eugene, OR); mouse anti-huC/D (1:500, Molecular Probes); rabbit anti-phosphohistone H3 (1:500, Upstate Biotechnology); rabbit anti-
protein kinase C (1:250, Santa Cruz Biotechnology); rabbit anti-sox3 (1:500, gift from Dr. Jack Parent, University of Michigan, Ann Arbor); mouse zn-5 (1:500, ZIRC); mouse znsp-1 (1:500, ZIRC); mouse zrf-1 (1:250, ZIRC); and mouse zn-12 (1:200, ZIRC). Secondary antibodies were anti-mouse or anti-rabbit biotinylated antibodies (1:250, Vector Laboratories) for whole mounts and anti-mouse, anti-rat, or anti-rabbit antibodies conjugated to cyanin3 (1:250, Jackson Laboratories) for cross-
sectional analysis. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) at 10 ng/ml to visualize nuclei. For corresponding bright-field images, sections were counterstained with 1% cresyl violet (Eastman Kodak) in 1% glacial acetic acid.

For total cell counts, DAPI-stained nuclei in the wild-type or mutant neural tube (at equivalent mid-brain or spinal cord levels) were manually counted in serial sections and their averages presented.

In situ hybridization

In situ hybridization (ISH) was carried out using antisense digoxigenin-
or fluorescein-labeled probes (ISH protocol for crossections; Barthel and Raymond, 1993; Jowett, 2001). Whole-mount ISH was carried out using the same method as for crossections with the following modifications: fixed embryos were dechorionated and stored in methanol at –20°C, later cleared in methanol-xylene (1:1), 5 min, RT; xylene, 30 min, RT, and methanol, 30 min, RT. Embryos were rehydrated in gradients of methanol (90%, 70%, 50%), proteinase K digestion carried out for 5 min, 37°C for embryos older than 24 hpf; rinsed in PBST (phosphate-buffered saline, 0.1% Tween 20), refixed in 4% PFA, 20 min, RT, and rinsed again in PBST for 30 min, RT. Incubation in prehybridization solution for 2 h at 56°C was followed by hybridization with appropriate probe(s) at 56°C overnight. Subsequent washes and antibody incubations using anti-digoxigenin antibody conjugated to alkaline phosphatase (Dig-AP, Roche Diagnostics) at 1:5000 dilution or anti-fluorescein antibody conjugated to horseradish peroxidase (FI-POD, Roche Diagnostics) at 1:100 dilution, followed the crossection ISH protocol. Enzymatic substrates NBT/BCIP (tetrazolium salt/5-bromo-4-
chloro-3-indolyl phosphate, Roche Diagnostics) for Dig-AP and fluorescent tyramide substrate (TSA-fluorescein kit, PerkinElmer Life Sciences) for FI-
POD were used according to manufacturer’s recommendations, which produced colored and fluorescent products, respectively.

The following cDNAs were used: α-tubulin and GFP (Matthew B. Veldman, University of Michigan, Ann Arbor), asl-1h (Allende and Weinberg, 1994), ath5 (Masai et al., 2000), deltaA (Appel and Eisen, 1998), her6 (Pasini et al., 2001), huC’ (Kim et al., 1996), islet-1 (Inoue et al., 1994), krox-20 (Oxtoby and Jowett, 1993), p27b (Masai et al., 2005), patched1 (Concordet et al., 1996), pax2.1 (Brand et al., 1996), stx3.1 and stx3.2 (Seo et al., 1998), and sonic hedgehog (Krauss et al., 1993).

Results

The α1-T-GFP mutagenesis screen generates a diverse repertoire of mutations exhibiting altered transgene and endogene expression

As a first step towards designing an efficient screening strategy, we monitored the developmental progression of GFP fluorescence in transgenic, nonmutagenized embryos and corresponding changes in α1-tubulin mRNA through the first week of development (Figs. 1A and B). GFP first becomes visible at 12 hpf in the neural plate, rapidly spreads through the entire CNS by 24 hpf, highlighting the developing retina, brain segments, and spinal cord and peaks between 24 and 72 hpf. Temporal progression of α1-tubulin mRNA occurs in close concordance, the only deviation being a more obvious down-
regulation, beginning at 5 dpf while a strong GFP fluorescence signal is appreciable at this time (Figs. 1A and B), which persists well beyond 7 dpf (not shown). This difference can be explained by the relative stability of the GFP protein, causing fluorescence to outlive the α1-tubulin mRNA. Using this background analysis, we developed a two-step screening strategy aimed at selecting mutants exhibiting concomitant alterations in transgene and endogene expression. Following ENU mutagenesis, a “primary visual screen” involved examination of GFP fluorescence in F3 embryos each day (until 7 dpf). A vast majority of isolated mutations exhibited panneural alterations in relative GFP levels without changes in expression patterns. Such mutants were subjected to a “secondary RT-PCR screen” to quantify α1-tubulin mRNA, and e1-f4α and β-actin control transcripts. This step was especially incorporated to ascertain that genomic α1-tubulin expression is perturbed in addition to the transgene and also eliminate mutations causing posttranscriptional defects (compromised translation or protein stability), which can affect reporter expression and slip in during the visual screen. A smaller number of mutant alleles displayed deviations in expression pattern, manifested as localized increase or decrease in neural GFP or in a few cases, abnormal/ectopic expression of the transgene. Mutants harboring localized changes were subjected to ISH using an α1-tubulin riboprobe, whereas alleles displaying misexpression of the transgene were deemed inherently interesting and automatically saved for further characterization.

In the first round of screening, we examined approximately 300 F2 families at an average of 6 to 8 incrosses per family. A total of 32 primary mutants were isolated (Table 1; Fig. 1), which display the following distinct transgene phenotypes: (i) panneural GFP reduction without changes in expression pattern, which constitutes the largest mutant class (20/32; Fig. 1C). Affected embryos frequently exhibit CNS morphology defects accompanying or following the GFP decrease; (ii) panneural increase in fluorescence while maintaining the neural-restricted GFP pattern (3/32; Fig. 1D); and (iii) localized alterations in reporter expression, which constitutes the most heterogeneous class of mutants (9/32). Altered phenotypes in this group range from reduced fluorescence in the mid-brain (Fig. 1E) to ectopic

 Imaging

GFP fluorescence in live embryos, immunostained embryos mounted in 3% methylcellulose, and ISH embryos mounted in 50% glycerol, PB were captured using a digital camera adapted onto a Leica stereomicroscope (MZFLIII). Images from flat-mounted immunostained embryos (using 3% methylcellulose) as well as sections were captured using Zeiss Axiohot fluorescence microscope with a similar attached camera. Exposure parameters were kept constant throughout each experiment and images consolidated using Adobe Photoshop 6.0.
expression in the ventral spinal cord region (Fig. 1F); characteristics of specific mutants are listed in Table 1. As part of the secondary screening of panneural GFP mutants (classes i and ii), α1-tubulin transcripts were quantified in age-matched wild-type (WT) siblings; (E) localized decrease in mid-brain whereas fluorescence in other CNS regions only mildly affected; (F) ectopic GFP-positive projections (arrows) from the spinal cord (SC) into ventral myotome (VM). Panels G and H show representative RNA profiles of two mutants (displaying panneural GFP reduction at 1dpf), analyzed using the secondary RT-PCR screen. RT-PCR using age-matched wild-type and mutant embryos at 27hpf (G) and 32hpf (H) were carried out. Upon normalization with e-1F4α and β-actin (β-act), reduction in α1-tubulin mRNA is observed only in panel H; hence, the corresponding mutant is selected for further characterization. Mutant in panel G is discarded as a possible housekeeping gene defect. Scale bars: 25μm in panel F, 250μm in all other figures.

expression in the ventral spinal cord region (Fig. 1F); characteristics of specific mutants are listed in Table 1. As part of the secondary screening of panneural GFP mutants (classes i and ii), α1-tubulin transcripts were quantified in age-matched wild-type and mutant embryos by semiquantitative RT-PCR at the earliest time when GFP defects become discernible. This timing is especially important because later events such as neural degeneration can falsely contribute to differences in α1-tubulin transcript levels. Alleles displaying a consistent and specific α1-tubulin mRNA increase or decrease (of at least twofold) were saved for further analysis (Figs. 1G and H). This selection criterion eliminated a majority of putative mutants while retaining five independent alleles for further characterization (Table 1; RT-PCR-positive alleles). To unambiguously demonstrate the specificity of our screening strategy, we assayed endogenous α1-tubulin levels in four mutants displaying morphological defects (neural degeneration or reduced brain size), not accompanied by GFP changes and as expected, α1-tubulin mRNA was not significantly altered (Table 1, control mutants).

Three complementing mutants representing each phenotypic class were selected for detailed molecular characterization. sookshma (“small/minute” in Sanskrit) displays panneural GFP reduction, drishti (“sight”, Sanskrit) represents enhanced panneural GFP expression, whereas dhruva (“immovable”, Sanskrit) exhibits abnormal GFP expression in the spinal cord. All three alleles are inherited in a recessive Mendelian fashion showing complete penetrance in subsequent generations. The strategy for mutant characterization involved analyzing the spatiotemporal expression pattern of developmental markers in regions displaying the most dramatic GFP perturbations, followed by examining related structures to elucidate common or region-specific gene requirements. Next, a blind assay was carried out for each mutant to identify the developmental error in a group of phenotypically normal embryos, the underlying premise being that the actual molecular block will precede the visually appreciable morphological and GFP defects. This analysis also served to better provide a time window for onset of molecular defects in mutants.

sookshma mutants display reduced cell proliferation resulting in diminution of the neural progenitor pool

shm\textsuperscript{m158} mutants become identifiable as early as 28–30 hpf exhibiting a marked panneural decrease in GFP expression, especially obvious in the mid- and hind-brain (MB, HB) regions
of the developing nervous system (Figs. 2A and B). RT-PCR analysis around this time using whole embryos confirms that expression of the endogenous α1-tubulin gene in mutants is reduced to 12–25% of the wild-type levels (Fig. 2B, inset). Coincident with fluorescence and mRNA changes, subtle morphological defects such as reduced brain-size and smaller brain ventricles appear (Table 1). To decipher the underlying basis of this GFP loss, we first asked if primary neurogenesis does not affect regional patterning in the developing nervous system (Figs. 2G–J). However, a perceptible reduction in the number of islet-1-positive cells in these clusters occurs, especially evident in the FB region. These two markers therefore forecast a broad neurogenesis defect in shm mutants whereas the regional patterning of the neuroepithelium appears grossly unperturbed. Normal patterning was further confirmed by examination of spatial landmarks in the mutant neuraxis. Formation of a normal mid–hind-brain boundary (MHB, pax2.1) as well as rhombomeric segments (krox-20, segments r3 and r5) illustrates that sookshma does not affect regional patterning in the developing nervous system (Figs. 2G–J).

To corroborate the neurogenesis defect, we examined expression of ashlb, a proneural basic helix–loop–helix (bHLH) gene, expressed exclusively in cycling neural progenitors (Allende and Weinberg, 1994; Mueller and Wullimann, 2003). Because both islet-1-positive cells and a significant proportion of the deltaA-expressing cells represent postmitotic neurons, this analysis would resolve if the reduction of these two markers occurs due to paucity of committed proliferating

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Table 1

<table>
<thead>
<tr>
<th>GFP phenotype</th>
<th>Mutant alleles&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RT-PCR-positive alleles</th>
<th>Representative alleles</th>
<th>Additional phenotypes of selected alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panneural decrease</td>
<td>mi5, mi14, mi21, mi46, mi51, mi53, mi68, mi71, mi104, mi107, mi108, mi120, mi125, mi158, mi191, mi198, mi235, mi236, mi264, mi266</td>
<td>mi21, mi68, mi158, mi198</td>
<td>sookshma (shm&lt;sup&gt;mi158&lt;/sup&gt;)</td>
<td>sookshma: Reduced head size and decreased ventricular enlargement, slight turbidity in cerebellum, hind-brain and tectal ventricle, and dorsally curved body axis at 1 dpf whereas somite morphology, heart, circulation and tactile sensitivity are normal; severe neural degeneration at 2 dpf causing a shrunken head and lethality by 3–4 dpf.</td>
</tr>
<tr>
<td>Panneural increase</td>
<td>mi39, mi98, mi304</td>
<td>mi98</td>
<td>drihti (drt&lt;sup&gt;mi49&lt;/sup&gt;)</td>
<td>drihti: Small eyes and enlarged hind-brain ventricle at 2 dpf. Somite and fin morphology, heart, circulation, tactile sensitivity and free swimming behavior all unaffected; slight edema and jaw defect by 5 dpf and lethality by 7–8 dpf.</td>
</tr>
<tr>
<td>Control mutants</td>
<td>mi9, mi103/166, mi218, mi247</td>
<td>none</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Localized GFP changes</td>
<td>mi41, b mi93, e mi99, d mi144, e mi184, f mi199, g mi251, h mi262, h mi294</td>
<td>NA</td>
<td>dhruva (dhr&lt;sup&gt;mi144&lt;/sup&gt;)</td>
<td>dhruva: Reduced motility at 2 dpf; slight dorsal body curvature, decreased heart beat and circulation and dark yolk at 3 dpf; progressive paralysis, hemorrhage in heart, posterior lateral line defect and lethality by 4 to 5 dpf.</td>
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<sup>a</sup> Mutants subjected to subsequent RT-PCR screen are indicated in bold.
<sup>b</sup> GFP fluorescence decrease in mid-brain.
<sup>c</sup> Overall decrease except around ventricles.
<sup>d</sup> Decrease in fore-brain and mid-brain.
<sup>e</sup> Increase in ventral spinal cord.
<sup>f</sup> Slight increase in jaw area.
<sup>g</sup> Decrease in optic primordium.
<sup>h</sup> Decrease in olfactory pit, fore-brain, and mid-brain.
neural precursors or is caused by defects in subsequent steps of differentiation. Analysis of ash-1b expression at various times in fact mirrored the decrease in neuronal populations in shm embryos described above. Figs. 2K and L illustrate that although the general pattern of ash-1b expression remains unchanged, its mRNA levels are markedly diminished throughout the nervous system, especially in the posterior MB, presumptive cerebellum and rhombomeres. This analysis was carried out in a group (n = 25) of phenotypically normal embryos at 21–22 hpf based on the premise that the molecular defect should precede the visually appreciable morphological and GFP changes. As expected, a genetically significant proportion (25%) of total embryos exhibited this compromised pattern of ash-1b expression. This defect can be reliably identified even at 19.5 hpf, establishing that diminution of the neural precursor pool is one of the very early identifiable defects in shm embryos.

The size of the proliferating neural precursor pool in a developing embryo depends on general influences such as cell proliferation and cell death and more specific developmental events such as commitment of neuroepithelial cells to the neuronal pathway. The Notch-Delta signaling pathway restricts the neurogenic potential of unspecified neuroepithelial cells by directing a subset to the neuronal lineage while maintaining neighboring cells in an uncommitted state for future/alternate fates (Artavanis-Tsakonas et al., 1999; Campos-Ortega, 1993).

Expression of bHLH proneural genes such as ash-1b, neurogenin, and neuroD is up-regulated in presumptive neuronal cells, whereas Notch-mediated signaling in their neighbors targets the transcriptional repressor, her6, to down-regulate transcription of these proneural genes. To examine if the compromised ash-1b profile in shm embryos reflected a perturbation of the Notch-dependent neurogenesis pathway, we analyzed expression of its repressor, her6. Figs. 2M and N show two regions of her6 de-repression in the shm brain, an appreciable expansion in the diencephalon and anterior mid-brain boundary (arrowhead) accompanied by a modest increase at the mid–hind-brain boundary (arrow). Whereas the former expanded domain could contribute to the loss of neurogenic markers in the FB/anterior-MB region, the increase in her6 expression at the MHB was too meager and restricted to account for the massive loss of ash-1b positivity in the mid-brain and the hind-brain domains. An interesting comparison can be drawn.
with the zebrafish \textit{hdac1} mutant exhibiting a similar loss of neurogenic markers compensated by a striking compensatory up-regulation of \textit{her6} in complementary regions (Cunliffe, 2004). Because the \textit{ash-1b} and \textit{her6} domains in the \textit{shm} mutant failed to exhibit a similar compelling complementarity of expression, especially in the most affected CNS regions (MB, HB), we asked if neural cells therein were born in fewer numbers or selectively eliminated by apoptosis. Vital acridine orange staining at early time points (19–22 hpf) showed no clear increase in the dying cell population. Instead, the number of mitotic figures marked by phosphohistone H3 (PH3) is significantly reduced at 28 hpf and can be detected as early as 19.5 hpf throughout the developing embryo (retina, brain, spinal cord, and somite segments). Interestingly, this decrease is very impressive around the presumptive ventricles, which form regions of high neurogenic activity in wild-type siblings. We counted mitotic cells in the MB ventricular region (outlined area; Figs. 2O and P) of 20 embryos, one fourth of which clearly exhibit a reduction by approximately 35% (75.3 ± 5.1 in wild-type embryos, \(n = 4\); 52.4 ± 6.6 in \textit{shm}, \(n = 4\)). It is interesting that this proliferation defect, although not limited to neural tissues, is followed by CNS-restricted apoptosis at around 28 hpf (not shown). Whereas one could envisage neural specificity for \textit{sookshma} function, a more likely explanation is that the CNS being one of the most proliferative organs at this time is extremely sensitive to such developmental insults.

Gross visual examination of the mutant neural tube (Figs. 2O and P) as well as cross-sectional analysis at the level of the hindbrain and mid-brain ventricles at 19.5 hpf (Figs. 2Q and R) showed that it was perceptibly thinner than its wild-type counterpart, presumably due to the proliferation defect. In fact, total cell counts of six consecutive sections spanning the MB region (corresponding to the outlined region in Figs. 2O and P) confirmed a significant reduction in \textit{shm} mutants (329.2 ± 17.2 cells per section in wild-type, \(n = 2\); 244.7 ± 29.1 cells per section in \textit{shm} embryos, \(n = 3\)). The normal zebrafish CNS undergoes a phase of rapid growth and cell proliferation around this time; however, significantly fewer (GFP-positive) neuronal

Fig. 3. Mutation in \textit{drishti} increases GFP and \(\alpha_1\)-tubulin mRNA in the developing CNS. Bright-field (A) and fluorescent (B) images of \textit{dri} mutant and a sibling at 3 dpf. Note the panneurally increased GFP expression and reduced eye size. Mutant retinas display a significant increase in fluorescence, clearly visible in PTU-treated embryos (C). RT-PCR at 2dpf confirms a concomitant increase in \(\alpha_1\)-tubulin (\(\alpha_1T\)) mRNA, e-1F4\(\alpha\) (e1F) is the normalizing control (D). Transverse sections of 2-day-old embryos show that the \textit{dri} spinal cord has slightly reduced cell number, 107 ± 8, \(n = 3\), in wild-type and 83 ± 11.3, \(n = 3\), in mutants (DAPI staining, E and K); however, ISH analysis using a GFP (fluorescein-labeled) riboprobe (F and L) and \(\alpha_1\)-tubulin (fluorescein-labeled) riboprobe (G and M) demonstrate that these transcripts are remarkably higher in mutants (panel J shows a cresyl violet-stained representative section for the spinal cord level at which above analysis was performed). A similar increase in mRNA expression is observed in \textit{dri} brain at 2dpf (panels H and N, \(\alpha_1\)-tubulin ISH using a digoxigenin-labeled riboprobe; GFP ISH not shown). \textit{dri} retina at 2dpf lacks the typical laminar organization of its wild-type counterpart (cresyl violet staining, panels I and O’); whereas the strongest expression in WT retina is restricted to the GCL (and central INL), Ellipses (E–G, J–M) outline the spinal cord region in which total cell counts were performed. Scale bars: 250 \(\mu\)m in panels A and B; 100 \(\mu\)m in panel C; and 25 \(\mu\)m in panels J, M (for E–G and K–M), N (for H,N) and O’ (for I, I’ and O’).
precursors are added to shm brains, initiating a disparity in overall fluorescence and portending the appearance of visually discernible low-GFP mutants within a few hours.

Based on the above analysis, we conclude that shm^mil38 causes a general cell proliferation deficit leading to severe attenuation of the neural precursor pool and consequently an appreciable decline in GFP fluorescence.

α1-tubulin and GFP mRNA expression is increased in neural cells of drishti mutants

drishti^mo98 represents a small group of mutants exhibiting a panneural increase in fluorescence while maintaining neural specificity of reporter expression. Mutants become discernible at 2–3 dpf, displaying enhanced GFP, especially appreciable in retina, hind-brain, and spinal cord with fluorescence persisting at comparably high levels throughout the mutant’s life span (Figs. 3A–C). The only morphological defect that occurs concomitantly or immediately thereafter is a smaller eye and an enlarged HB ventricle (Table 1). As development progresses, eye size arrests at the 2-day stage and edema develops (5 dpf), causing subsequent lethality. RT-PCR analysis using 2-day-old mutant and normal siblings determined the α1-tubulin transcript in dri embryos to be 167–200% of the wild-type levels (Fig. 3D). The increase in GFP fluorescence with coordinate enhancement of α1-tubulin mRNA suggested that mutation in drishti enhanced transcription from the α1-tubulin promoter.

However, we first sought to eliminate the possibility that mutants simply harbored a greater number of neural (GFP-positive) cells, causing a gross increase in fluorescence/mRNA. DAPI staining of 2-day-old spinal cord sections at mid-trunk level indicated that the average cell number is instead reduced in dri embryos (107 ± 8, n = 3, in wild-type and 83 ± 11.3, n = 3, in mutants; Figs. 3E and K). To confirm an enhancement in relative α1-tubulin promoter activity in dri neural cells, we analyzed transgene (GFP) and endogene (α1-tubulin) mRNA in spinal cord, brain and, retinal sections by ISH. The overall levels of both transcripts are uniformly higher in all CNS regions examined while maintaining the neural-specific expression pattern, asserting that the α1-tubulin promoter is upregulated (Figs. 3F–H and L–N). Another interesting finding is the complete absence of the characteristic laminar cytoarchitecture in the retina (Figs. 3I, O and 5A, I). Neural retina in the wild-type embryos is organized into distinct layers with the innermost layers comprised of differentiating cells (ganglion cell layer and central cells of the inner nuclear layer) exhibiting the highest levels of α1-tubulin mRNA. In sharp contrast, the mutant retina is composed entirely of cells bearing a characteristic neuroepithelial morphology, expressing α1-tubulin at a uniformly high level (Figs. 3I′ and O′). Because α1-tubulin expression serves as a hallmark of neuronal development, does its enhancement in dri embryos imply a disturbance in progression of neuronogenesis? Further, what role can we envisage for the cognate gene in retinal development? The
following sections describe the characterization of the spinal cord/hind-brain neurogenesis and retinal development to elucidate a possible common cellular role for *drishti*.

*drishti* regulates neurogenesis by blocking the “proliferation to differentiation” switch

To explore the role of *drishti* in neurogenesis, we examined the cellular composition of a 2-day-old mutant spinal cord using neural stage-specific markers. HuC/D, a marker for postmitotic neuronal cells, is expressed in fewer cells, restricted to the more peripheral boundary of the mutant spinal cord, leaving a larger proportion of postmitotic cells in the central cavity compared to wild-type counterparts (Figs. 4B, G and C, H). The proportion of postmitotic cells in mid-trunk sections (Hu-positive cells/DAPI-stained nuclei) decreased from 70.5 ± 3.7% (*n* = 3) in wild-type to 53.9 ± 1.9% (*n* = 3) in mutants. A complementary pattern emerged with Sox3 immunostaining. A transcription factor expressed in self-renewing neural progenitors in vertebrates, Sox3 plays a role maintaining neural cells in an undifferentiated state (Bylund et al., 2003). Sox3-positive cells surround the central canal in an approximately one-cell thick layer in wild-type embryos but the mutant shows a significant increase with undifferentiated precursors populating a large central region of the spinal cord (Figs. 4D and I). Does *drishti* affect cell cycle exit of neural progenitors causing a diminished Hu-positive neuronal population? To address this issue, we examined two general markers of cell cycle progression. A BrdU pulse, which provides an instant snapshot of cells engaged in S-phase of the cell cycle, shows the dividing cell number to be indisputably higher in mutants (Figs. 4E and J). It was important to eliminate the likelihood of cells getting arrested in the S-phase; therefore, we determined cells actively undergoing mitosis by PH3 immunostaining. The overall number of mitotic figures is substantially smaller than S-phase cells with only a few PH3-positive (1–3) cells per section; however, we noted a definite increase in the *dri* spinal cord. Quantification of this difference was achieved by counting the number of sections with 1–3 PH3-positive figures. 80.7% of all mutant sections show at least 1 mitotic figure as opposed to only 30.7% of such wild-type spinal cord sections, which confirmed that the *dri* neural progenitor pool is actively enriched by mitosis (Figs. 4F and K). In parallel, a marked increase in these cell cycle markers is also observed in the hind-brain of mutant embryos. Figs. 4M–P show that the proliferating ventricular zone of the mutant HB, which harbors a majority of PH3-positive mitotic cells, is expanded at the cost of the Hu-positive differentiating cell population. In agreement, cells in the large Hu-negative ventricular zone bear a pseudostratified columnar appearance (not shown). Based on this analysis, we inferred that *drishti* plays a role in the neural cell fate decision between self-renewal and cell cycle exit in the spinal cord and HB.

*drishti* is required for cell cycle exit of retinoblasts but not for subsequent cell fate specification or differentiation

Progression of retinal development from a morphologically homogenous, neuroepithelial organ to an organized, laminated structure involves a well-orchestrated series of events: cell cycle exit of retinoblasts, commitment to particular cell fates, migration to appropriate layer positions, and finally morphological differentiation. Two- to three-day-old *dri* retinas assume an immature, pseudostratified appearance, completely devoid of the characteristic laminated architecture (Figs. 5A and I). Although microphthalmia persists, some regions with plexiform appearance and rudimentary lamination become evident at 4 dpf (not shown). The presence of immature neural cells in the retina is reminiscent of progenitor enrichment in

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**Fig. 5.** The *dri* retina is enriched for proliferating retinoblasts, which when unable to withdraw from the cell cycle, undergo premature death. DAPI-stained retinal sections at 3 dpf illustrate the loss of lamination in *dri* mutants (A, I). Bracketed regions mark the three cellular and two plexiform layers in wild-type retinas replaced by a contiguous neuroepithelial structure. Panels B and J show the localization of BrdU-positive cells in the CGZ of wild-type retinas at 2 dpf whereas a majority of cells in mutant retina are cycling. Albeit restricted to retinal periphery, an expanded S-phase cycling population is still maintained in mutant retinas at 3 dpf (C, K). Arrowheads indicate an accompanying abundance of PH3-positive mitotic figures at 2 dpf (D, L) and 3 dpf (E, M). Developmental progression of retinoblasts to Hu-positive postmitotic cells is severely impaired at 2 dpf, partial recovery observed at 4 dpf with signs of primitive lamination (F, N; G, O, brackets). Excess cells are eliminated by apoptosis in 2-day-old *dri* retinas illustrated by cleaved-caspase-3 (casp) immunostaining (H, P). Cell death is very rarely detected at later time points examined. Scale bars: 25 μm in all panels.
the mutant spinal cord, we therefore probed if *dri* affected retinal neurogenesis in a similar manner, by BrdU pulse labeling and PH3 immunostaining. A short BrdU pulse at 54 hpf labels cycling retinoblasts restricted to the marginal proliferative zone in wild-type retinas (Circumferential Germinal Zone, CGZ; Fig. 5B). In sharp contrast, the BrdU-labeled population is dramatically expanded with S-phase cells populating the entire central retina in *dri* embryos (Fig. 5J). Does the predominantly proliferative nature of the 2-day-old *dri* retina simply reflect a block in progression of retinal development? BrdU incorporation at 77 hpf showed that similar to wild-type retina, proliferating cells in the mutant eye are now restricted to the more peripheral region with the central retina mostly BrdU-negative, highlighting a normal transition from embryonic to postembryonic pattern of proliferation (Marcus et al., 1999). However, even at this time point, the number of cycling BrdU-positive cells in peripheral zones is substantially higher than their wild-type counterparts (Figs. 5C and K). This establishes that the mutant retina does not simply represent a developmentally arrested, immature eye and is in fact actively biased towards proliferation. The mitotic status of *dri* retinas at both 54 and 77 hpf mirrors the increased BrdU incorporation, evidenced by a larger number of PH3-positive cells in mutant retinal sections (Figs. 5D, L and E, M). Based on the spinal cord analysis, we next asked if *drishti* plays a role in the decision of retinoblasts to quit the cell cycle and initiate postmitotic migration and differentiation. To explore this “proliferation-to-differentiation” block, newly postmitotic neuronal population marked by HuC/D expression was examined. Hu-positive cells, destined to adopt ganglion and amacrine cell identities, are detected in the ganglion cell layer (GCL) and the inner nuclear layer (INL) in accordance with their ultimate cell fates in wild-type retinas. Surprisingly, the 2-day-old mutant retina is almost completely devoid of Hu-positive cells with few retinal sections showing isolated neuronal cells (Figs. 5F and N). At later time points however (4 dpf), the *dri* retina tries to recover with postmitotic cells emerging and even attempting to organize into rudimentary layers, interrupted by plexiform patches (Figs. 5 G and O). Although the *dri* retina is stalled in a predominantly proliferative state, no morphological evidence of hyper-proliferation or multifolding exists, and in fact eye size is reduced. We suspected that extra cells are eliminated by a compensatory cell death mechanism. This was confirmed by the presence of cell corpses, indicated by pyknotic nuclei and acellular holes towards the vitreal surface. This massive cell death occurs by an apoptotic mode, evidenced by increased activated caspase-3 staining, and is restricted primarily to 2 dpf (Figs. 5H and P), diminishing to undetectable levels at 3–4 dpf. We conclude that the 2-day-old *dri* retina has an expanded pool of undifferentiated, self-renewing retinoblasts, which when unable to achieve their appropriate fate by withdrawing from the cell cycle, undergoes apoptosis.

The mature neural retina is composed of a diverse array of cell types, each occupying a predetermined radial position in the multilayered structure. Because cell fate specification in the developing eye is intimately related to the timing of cell cycle withdrawal and not the past lineage, an obvious question was the role of *drishti* in commitment and differentiation of the six retinal cell types (Cepko et al., 1996; Livesey and Cepko, 2001). We selected markers indicative of overt morphological differentiation in cell types representing each retinal layer and compared their expression between mutant and wild-type embryos. Figs. 6A–J show that all cell types examined are clearly represented in the mutant retina, albeit in reduced numbers, presumably due to fewer cells undergoing terminal mitosis and embarking on the differentiation pathway. DM-GRASP (zn-5), a marker for differentiating retinal ganglion cells (RGC), is expressed in fewer cells close to the vitreal surface of the *dri* retina and a thin optic nerve is also identifiable in some sections. Similarly, bipolar cells and their processes, marked by protein kinase C (pkC) expression,
and double cone and rod photoreceptors (zpr-1 and zpr-3 immunoreactivity, respectively) represent the inner and outer nuclear layers (INL and ONL), respectively, in dri retinas. Further, Müller glia (highlighted by zrf-1 immunostaining), the only nonneuronal cells in the retina reside in the mutant INL with their processes emanating radially in a manner similar to their wild-type counterparts. Also, none of the cell types examined is mis-patterned or present in inappropriate layer positions, asserting that retinal cells are able to migrate to their final positions and undergo terminal differentiation. We conclude that although the pool of available postmitotic neurons is diminished, later cellular events such as fate determination, migration, and morphological differentiation remain unaltered in the dri retina.

Propagation of the first neurogenic wave is blocked in drishti retina

The neurogenesis program in zebrafish retina begins at 28 hpf with retinoblasts in a small ventronasal cluster exiting the cell cycle following which neurogenic activity spreads towards the peripheral retina in a stereotyped, wavelike fashion and new cells get rapidly recruited into the postmitotic pool (Hu and Easter, 1999). Could an early retinal neurogenesis block forecast the developmental arrest seen at 2 dpf? ath5, a proneural gene expressed transiently in the first retinal cells undergoing terminal mitosis and their postmitotic progeny, serves as a good indicator for activation of this program (Masai et al., 2000). Because dri mutants become identifiable at 2 dpf, we examined ath5 expression in a group of phenotypically normal embryos at 34 hpf (n = 20). Figs. 7A and B show ath5-expressing cells poised to generate the future RGC layer present in wild-type retinas. In sharp contrast, a genetically significant proportion (one-fourth) of embryos exhibit markedly diminished ath5 expression with the only detectable expression restricted to the ventronasal patch. This indicates a block in propagation of the neurogenesis wave, which quite possibly presages the retinal defects observed later. To exclude the likelihood of an earlier patterning defect, we examined two homeodomain genes, six3.1 and six3.2, known to be important for initial retinal field specification (Seo et al., 1998). Figs. 7C and D illustrate a normal retinal primordium at the 15-somite stage and at 32 hpf (not shown). Next, we examined expression of the p27b/kip1 cyclin-dependent kinase inhibitor (CDKI), a component of the “retinal neurogenic timer” that prevents progenitors from re-entering the cell cycle (Masai et al., 2005; Nakayama et al., 1996). Wild-type retinas show strong p27b expression in cells presumably undergoing their final mitosis whereas mutant retinas display a near-complete abolishment of this signal, corroborating that cell cycle exit of retinoblasts is compromised (Figs. 7E and F). It is interesting to note that even at this early time point, the Hu-positive postmitotic population is severely reduced in the entire CNS, especially noticeable in the tectum and hind-brain (Figs. 7G and H). This undoubtedly indicates that the dri-induced defect in cell cycle withdrawal and subsequent postmitotic differentiation is not restricted to the retina, even at this early time point. Hedgehog signaling has previously been shown to be crucial for triggering the transition of retinal primordium (and the neural tube) from proliferative to neurogenic phase (Neumann and Nuesslein-Volhard, 2000; Rowitch et al., 1999). To probe if it is disturbed in dri mutants, we examined expression of patched1 receptor, which is autoregulated by Hedgehog signaling as well as sonic hedgehog itself (Concordet et al., 1996). Both genes are unperturbed by the mutation (Figs. 7I and J). To summarize this characterization, derva08 reveals a component of the gene regulatory hierarchy that directs cell cycle progression/exit of neuroblasts in the zebrafish CNS.
Mutation in dhruva blocks branching of spinal motor axons

\( dhv^{mi144} \) is a representative member of the third, most diverse class of mutants manifesting localized disturbances in GFP expression pattern. Mutants appear between 54 and 60 hpf exhibiting altered motility and morphological irregularities follow at 3 dpf (Fig. 8A; Table 1). Spontaneous swimming behavior is affected, varying in severity from slight reduction to complete paralysis. Its most attractive feature that prompted its selection is a striking increase in GFP fluorescence in fibers extending from the ventral spinal cord into the ventral myotome (Figs. 8B and C). Unlike previous mutants exhibiting defects in general levels of GFP fluorescence, \( dhv^{mi144} \) (and other mutants displaying abnormal “patterns of transgene expression”) was automatically selected for further characterization without any subsequent screening steps. The underlying premise here was that the likelihood of such specific disturbances (e.g., abnormal/ectopic GFP expression) occurring due to disruption of general housekeeping genes was extremely low; thus, obviating the need for secondary RT-PCR screening. Further, a subtle pattern defect as this would not be expected to yield an appreciable \( \alpha_1 \)-tubulin RNA difference in whole-embryo RT-PCR assays.

The presence of segmentally iterated, GFP-positive projections from the ventral spinal cord suggested that \( dhv \) affects spinal motor axons, which was examined using znp-1, a monoclonal antibody that labels axons, branches, and terminals of both primary and secondary spinal motor neurons (PMN, SMN). During normal development, a motor neuron innervates its target myotomal field by initially extending an unbranched pioneer axon that delimits its spatially exclusive territory and once it reaches its terminal extent, side branches emerge from the central axonal shaft and synapse with...
underlying fibers (Liu and Westerfield, 1990). In wild-type embryos at 57 hpf, analysis of the caudal (spinal segments 20 through 22; Fig. 8D) and rostral (spinal segments 10 through 12, Fig. 8F) regions of the spinal cord reveals that the ventral myotome is overlaid with an intricate meshwork of secondary, tertiary, and higher order branches extending laterally from the central nerve. In the corresponding segments of the mutant, however, although the ventral root of the spinal motor nerve is pioneered correctly, only a few short, abortive branches sprout from branch points (Figs. 8E and G). Interestingly, the central axonal shaft adopts a thickened appearance with swollen varicosities. This phenotype is identifiable by znp-1 immunostaining early on, in a pool of phenotypically normal 48-h-old embryos \((n = 12)\) and \(dhw\) embryos never recover from this defect (immunostaining at 4 dpf, not shown), thus eliminating the possibility that branching is simply delayed. We conclude that \(dhw\) is essential for motor axonal branching, loss of which forestalls terminal synapse formation causing paralysis in mutants.

Pathfinding by the primary growth cone is intimately related to its ability to elaborate collateral branches later on; defects in axonal extension by experimental manipulation or mutagenesis have in fact been shown to cause abnormal branching (Becker et al., 2003; Bernhardt and Schachner, 2000). To delineate the branching defect from any conceivable prior pathfinding errors, extension of the unbranched spinal nerve was examined at 32 hpf. A group of embryos \((n = 12)\) from a carrier pair mating examined by znp-1 immunostaining reveals no instance of inappropriate stalling and pausing or lack thereof, which could prompt the later branching failure, thus restricting \(dhw\) requirement to a later time point (Fig. 8H). Also, general axonogenesis and establishment of the primary axonal scaffold, examined by zn-12 immunostaining, is normal at this time (not shown).

Finally, in an attempt to illustrate the role of \(dhw\) in axonal outgrowth specifically from secondary motor neurons, we examined the expression of DM-GRASP (neurolin) in wild-type and mutant animals. DM-GRASP is a cell surface protein expressed by SMNs, which typically localizes to cell bodies as well as the fasciculated segments of their axons (Fashena and Westerfield, 1999). Interestingly, expression of this protein is transient; it builds up during the phase of axon outgrowth and is rapidly down-regulated once axonal branches reach their myotomal targets and establish synaptic connections. Immunostaining with the corresponding antibody (zn-5; Fashena and Westerfield, 1999) highlighted somata of SMNs and their fasciculated spinal segments but its restricted expression pattern precluded visualization of the corresponding branches, yielding no direct clue about the involvement of \(dhw\) in secondary motor axonal branching. However, an indirect evidence stemmed from the intriguing observation that at all time points examined (2–4 dpf), expression of this protein remains substantially higher in mutant cell bodies and fasciculated axons compared to their normal counterparts (Figs. 8I and J). How do

Fig. 9. Mutation in \(dhw\) disrupts the retinotectal architecture without affecting RGC differentiation and optic nerve formation. znp-1 immunostaining of 3-day-old embryos highlights tectal innervation, which is dramatically reduced in mutants (A–C). Higher magnification images indicate unbranched (E) or stalled retinal axons (F) in mutant tecta whereas the wild-type tectum displays an intricate arborization pattern (D). Brackets mark the fasciculated segments of the optic tract as it approaches the tectum. Immunostaining using zn-5 antibody in panels G and H confirms that RGCs elaborate axons and form a normal optic tract in \(dhw\) mutants at 57 hpf. Subsequently, impaired arborization and innervation probably leads to loss of DM-GRASP down-regulation in 3-day-old \(dhw\) retinas (zn-5 immunostaining of retinal sections; I, J). All images are oriented such that anterior is towards left. Scale bar: 100 µm in panels A–C, 50 µm in panels D–F, G and H, and I and J.
we explain this enhancement in light of the known characterization of *dhruva*? An increase in DM-GRASP levels indicates that axons of mutant SMNs are somehow unable to establish terminal synapses and subsequently signal to their cell bodies to down-regulate its expression. Inferring from the above-mentioned role of *dhruva* in primary motor axon branching, it is reasonable to speculate that a similar disruption of branching and terminal synaptogenesis in secondary motor axons forestalls down-regulation of DM-GRASP expression while the protein is simultaneously being turned down in their wild-type counterparts. This therefore serves as indirect evidence for *dhruva* requirement during axonal elaboration from SMNs as well.

**dhruva function is required for retinotectal arborization**

Is the *dhruva* gene function restricted to spinal axons or does it play a more ubiquitous role in axonogenesis? One of the most well characterized axonal systems, which requires branching and arborization for terminal synapse formation, is the retinotectal projection. Axons elaborated by RGCs reach the tectal boundary at 46–48 hpf and over the next 2 days invade the future synaptic field by extensive branching and arborization, forming the stereotyped retinotopic map (Kaethner and Stuermer, 1992; Stuermer, 1988). To investigate the role of *dhruva* in formation of the retinotectal projection, we examined zn-1 staining in the tectum at 3 dpf. Fig. 9A shows the staining covering a large part of the wild-type tectum in its typical innervation pattern. A closer examination reveals that RGC axons approach the tectal boundary as distinct fasciculated segments, begin invading their territory by axon extension and branching and ultimately form an intricate mesh of terminal arbor (Fig. 9D). Interestingly, staining in all age-matched mutants is significantly reduced, growth cones extend to a limited distance in the tectum in an almost unbranched fashion (Figs. 9B and E). In a few cases, mutant axons appear to stall near the tectal boundary and form a few rudimentary arbors (Figs. 9C and F). To eliminate the possibility that the mutation affects RGC differentiation and axon outgrowth, we examined wild-type and mutant retinas by zn-5 immunostaining. Figs. 9G and H reveal that the differentiating ganglion cell layer (GCL) is comparable between the two. Both form thick, fasciculated optic nerves and visually identifiable optic chiasma. Therefore, requirement of *dhruva* appears to be crucial for the later tectal innervation stage. In accordance with its role in spinal motor axons, it is reasonable to speculate that it regulates retinotectal axonogenesis by affecting its branching phase. It is also interesting that similar to its expression phenotype in the spinal cord, DM-GRASP remains consistently higher in mutant RGCs (3–4 dpf; Figs. 9I and J), yet another indication of impaired arborization and subsequent loss of tectal synaptogenesis.

**Discussion**

Using a reporter-dependent, forward genetic approach, we identified mutations in three distinct sequential events in the zebrafish neurogenesis and differentiation pathway. These genetic loci represent our larger screening effort that generated 32 mutants, each member perturbing α1-tubulin-driven GFP expression and conceivably blocking an essential step in the neuronal development cascade.

**Does the α1-T-GFP screen offer any selective advantages?**

In the recent spate of mutagenesis screens dissecting CNS development, does this approach present any unique, advantageous features? Yes. First, the use of a fluorescent reporter to monitor neuronal development vastly simplifies the identification of mutants. Disturbance in GFP fluorescence being the sole screening criterion, mutants can be selected in absence of – or prior to onset of – CNS-related dysmorphologies, obviating the need for morphological landmarks or staining patterns, typical of phenotype-based screens. In fact, reporter expression changes preceded phenotypic/behavioral defects in a number of primary mutant alleles. Secondly, owing to the panneural nature of α1-tubulin expression, a gene does not require functional restriction to particular CNS organs or cell types to be selected. An oft-repeated strategy in earlier screens relies on focusing on specialized structures (retina, MB-HB boundary, motor neurons/axons, etc.), we however analyzed fluorescence in the entire developing CNS, increasing our screening potential and making it a particularly unbiased approach. This is exemplified by the primary mutant pool displaying reporter defects in regions as varied as the tectum, spinal cord, brain ventricles, etc. (Table 1). In the same vein, mutant characterization is not restricted to particular organs, helping uncover common developmental missteps in the retina/tectum and spinal cord of the dri and dhv mutants. The third point of distinction derives from the neuronal stage-independent pattern of α1-tubulin expression. α1-T-GFP spans neuronal progression from specification to synaptogenesis, offering the unique opportunity to identify genes anywhere within this developmental hierarchy, a point validated by the selected loci functioning either at the neural progenitor stage, when cells are poised to undergo terminal mitosis or in postmitotic differentiating neurons. Finally, in a significant departure from some recently published reporter-based screens, tissue-specific promoter-driven expression here is not used solely as an in vivo imaging tag to visualize specific structures. For example, a recently published, elegant mutagenesis study used the *brn3c*-driven GFP to highlight the retinotectal projection and identify mutants affecting the temporal or spatial pattern of the axonal architecture (Xiao et al., 2005). The α1-T-GFP screen, although designed to identify such altered patterns of reporter expression, is also preordained to recognize changes in relative GFP levels, the underlying premise being that mutation-induced alteration of the α1-tubulin promoter activity will manifest as a quantitative increase/decrease in reporter levels. A subset of such mutations should therefore occur in transcription factors or signaling molecules upstream of the α1-tubulin gene and thus help understand promoter regulation in an in vivo context. This is corroborated by the high-GFP *dri* mutant, which shows an enhancement in transgene and endogene mRNA in neural cells, suggesting altered expression of the α1-tubulin promoter.
Screen design and mutant selection

Based on the premise that potentially interesting mutants should exhibit coordinate changes in the GFP transgene and endogenous α1-tubulin expression, we employed a sequential screening strategy that examined the two criteria. Thirty-two primary mutants displaying GFP disturbances were obtained and classified solely on their reporter expression characteristics as panneural, localized, or ectopic defects. Most mutants were accompanied by widely disparate morphological characteristics ranging from dramatic CNS defects to subtle disturbances. As a preliminary elimination step, mutants manifesting developmental defects prior to fluorescence changes were rejected to exclude the possibility that GFP expression was compromised. However, candidates exhibiting CNS phenotypes concurrently – or after reporter changes set in – were retained because disruption of potentially essential neurogenic genes (and α1-tubulin) is likely to cause such catastrophic developmental consequences. The initial mutant recovery rate (11%) is comparable or higher than some recent ENU-based screens, although numerous factors including mutagenesis efficiency and individual selection criteria determine the number of alleles identified (Kim et al., 2006; Xiao et al., 2005). None of the mutants is adult viable, precluding examination of their cognate gene functions in the regenerative response, which will await cloning of the disrupted loci.

A majority of primary mutations affected panneural GFP levels, prompting the introduction of a secondary RNA-based selection step. An insertional mutagenesis screen recently concluded that a large proportion of embryonic-lethal mutations reside in cell-essential genes, approximately one-third of them causing defective protein production (Amsterdam et al., 2004). This necessitated weeding out such posttranscriptional defects, which was achieved by the RT-PCR screen and resulted in elimination of 70% of such loci examined. We believe that this unexpectedly high percentage of RT-PCR-negative alleles is attributable to our stringent selection criteria, which rejects individuals exhibiting small and more subtle α1-tubulin mRNA changes. Mutants exhibiting localized GFP changes, most of which disrupted tectal expression, are currently being analyzed and will be published separately. Finally, alleles displaying abnormal/ectopic expression patterns such as dhv underwent no secondary screening because the likelihood of such specific developmental defects by housekeeping gene disruption is relatively scarce.

Where do the representative loci reside in the neurogenesis regulatory hierarchy?

Using appropriate developmental markers, we uncovered cellular events that were most likely interrupted by the three selected mutations.

sookshma

In the embryonic brain, co-existing cellular processes such as proliferation, cell cycle exit and differentiation, and apoptosis determine the size of the neural precursor pool; shm disrupts one of these processes causing severe attenuation of progenitors. This decrease is not attributable to premature cell cycle exit as postmitotic neuronal markers exhibit no compensatory increase. In fact, the neuronal precursor marker profile is reminiscent of the hdc1 mutant where loss of ash-1b positivity is caused by disturbance in the Notch-dependent neurogenesis pathway, indicated by a complementary expansion of the downstream target, her6 (Cunliffe, 2004). The her6 expression domain in shm is expanded at the diencephalon-anterior mid-brain region, which may contribute to the loss of neurons and their precursors in a local, restricted fashion. In fact, the islet-1-positive neuronal clusters in the fore-brain region exhibit a marked reduction at 28 hpf. However, how does one explain the severe paucity of ash-1b-positive precursors in the rest of the brain structures with her6 only mildly de-repressed at the mid–hind-brain boundary and the most affected mid-brain and hind-brain domains remaining her6 negative? The answer stems from a significant reduction in PH3-positive mitotic figures; a proliferation deficit probably engendering a defect in the generation of a sizeable cycling neuronal population. Especially striking is the mitotic reduction in the ventricular region of the mid- and hind-brain, which constitutes a zone of high proliferative activity in wild-type embryos. A recent study showed that ventricular proliferation increases dramatically between 18 and 21 hpf, compared to the remaining neuraxis and is crucial for initial brain ventricle formation (Lowery and Sive, 2005). In agreement, shm mutants exhibit the cell proliferation defect at around 19 hpf, causing dramatically thinner neuroepithelial tissue, reduced brain size and ventricles. It is interesting that the overall phenotype and morphological characteristics of shm mutants are closely reminiscent of the curly fry mutation (Song et al., 2004). cfy causes hyper-proliferation in neural and other tissues, resulting in premature cell death and embryonic lethality; reiterating the stringent control of the cell cycle that embryonic development demands, disturbance in either direction causing disastrous consequences. Although a detailed analysis of cell cycle progression is still required to identify the stage-specific block, we suspect that sookshma encodes an essential cell cycle function, loss of which may initially be compensated by the maternal gene product.

drishti

A very consequential event in the life of a neural progenitor, after it undergoes the requisite cell divisions, is a growth phase-exit and initiation of differentiation. Mutation in drishti affects this transition, enriching the developing CNS for cycling progenitors at the expense of postmitotic neurons. Whereas neural composition is altered in the spinal cord and hind-brain, the proliferative population in dri retina is much more dramatically expanded, compensated by premature apoptosis leading to microphthalmia. This difference between CNS regions can be attributed to varying proliferative potentials at the time of analysis or slightly different spatial requirements for drishti. Previous studies have identified retinal mutants displaying similar developmental blocks at a proliferative stage, including the recently published mutation in hdc1
(add mutant) (Stadler et al., 2005; Yamaguchi et al., 2005). A vital difference in *dhruhti* gene function however is its panneural requirement, evidenced by mitotic enrichment and differentiation block seen throughout the CNS. Despite the seemingly catastrophic cellular defect at 2 dpf, the *dri* retina makes a genuine effort to recover, illustrated by generation of postmitotic neurons, peripheral migration, and terminal differentiation of cell types examined. The retinal marker analysis unequivocally differentiates it from known lamination mutants, some of which are defective in morphological differentiation of particular cell types, for example, *young* mutant (Link et al., 2000); or which manifest migration and retinal patterning anomalies; *oko meduzy* (Malicki and Driever, 1999), *mosaic eyes* (Jensen et al., 2001), *glass onion* (Pujic and Malicki, 2001), etc.

Neurogenesis is intimately related to cell cycle progression, which in turn is exquisitely regulated by cell-intrinsic and -extrinsic factors (Ohnuma and Harris, 2003). A fine balance between intracellular cyclins/cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs) orchestrates cell cycle reentry/exit, whereas upstream signaling pathways (Wnt, Notch, Hedgehog) and intracellular mediators (protein kinase A) target them, biasing them in particular directions (Dyer and Cepko, 2001; Levine and Green, 2004; Masai et al., 2005; Yamaguchi et al., 2005; Yang, 2004). The neurogenesis block seen in the 2-day-old *dri* retina is pressured by gene expression changes in the proneural *ath5* as well as a CDKI, *p27b/kip1* at 34 hpf. This suggests a very early disturbance in neural cell cycle exit, possibly involving one of abovementioned signaling cascades. Their epistatic relationship with *drishti* awaits further characterization; however, we could confirm that expression of two Hh signaling genes, *shh* and *ptc1*, was intact, distinguishing *dri* from retinal mutants *sonic you* (Stenkamp et al., 2002) and *slow muscle-omitted* (Stenkamp and Frey, 2003). Based on its characterization, a molecular candidate for *drishti* should direct early cell cycle exit and neurogenesis in a panneural fashion but is expected to be dispensable for subsequent events of cell fate specification, migration, and differentiation.

**dhruva**

Axonogenesis occurs in two distinct phases, the primary axon initially travels long distances and demarcates its target territory followed by side branches emerging from the main axonal shaft to synapse with their targets. Branching is a highly dynamic process with continuous extension and retraction of growth cones establishing the final arborization pattern. In two distinct axonal structures, the spinal motoraxons and the retinotectal projection, secondary and higher-order branching, occurs in a stereotyped, temporally regulated fashion, both of which are disrupted in *dhv* embryos. Neuronal differentiation (PMNs, SMNs, and RGCs), and primary axonal pathfinding proceed normally; however, the switch from extension to branching/arborization mode is completely blocked resulting in stunted, abranching axons in the spinal cord and stalled retinal axons with reduced arborization in the tectum. It is interesting that the *dhv* mutation elucidates a common genetic requirement for axonal structures that were recently the focus of two independent mutagenesis screens (retinotectal cytoarchitecture, Xiao et al., 2005; neuromuscular synaptogenesis, Panzer et al., 2005). In fact, a complementation analysis performed during the preparation of this manuscript showed that the *xavier* mutant isolated in the latter screen is allelic to *dhruva*.

Branching is a specialized form of axonogenesis depending on several factors; primary growth cone behavior such as pausing and internal cytoskeletal reorganization, target- and substrate-derived signals as well as functional activity/stability of synaptic connections (Acebes and Ferrus, 2000; Kalil et al., 2000). Based on this information, what predictions can we make about the molecular nature of *dhruva*? It could encode either a component or an intracellular regulator of the cytoskeletal machinery, affecting the intrinsic ability of an axon to initiate lateral branches. In such a case, *dhv* will elucidate distinct molecular mechanisms for the pioneer growth cone extension and for secondary branching. Alternatively, lack of target-derived cues or changes in the permissive/repulsive nature of the underlying substrate could impair branch initiation and extension. Neurotrophic factors, guidance cues as well as cell adhesion molecules have been known to affect branching; a candidate for *dhruva* will however need to fulfill the criteria of being shared by tectal and the spinal targets/environments (Babb et al., 2005; Cohen-Cory and Fraser, 1995; Feldner et al., 2005; Wang et al., 1999; Yates et al., 2001). Another possibility is that after initial extension, secondary axons collapse prematurely due to a defect in stable synapse formation. If this were true, it will be interesting to examine the requirement of *dhruva* for terminal synapses versus *en passant* synapses. Finally, we would like to investigate if *dhruva* has additional roles in primary axon elongation because some mutant retinal axons stall at the tectal boundary or in fasciculation/defasciculation because the spinal nerve appears thicker and DM-GRASP is up-regulated. Interestingly motoraxon behavior in *dhv* bears striking resemblance to the *beat* mutants in *Drosophila* in which motor axons fail to defasciculate from the nerve bundle and bypass their synaptic targets due to increased adhesion between axons (Fambrough and Goodman, 1996; Pipes et al., 2001). Whereas in zebrafish spinal segments, the three primary motor axons traverse as a common bundle for a very short distance before adopting cell-specific trajectories, it will be interesting to examine if adhesion/repulsion between *dhv* axons is in any way related to the branching defect.

**Mutations and α1-tubulin promoter regulation**

The basic premise for mutant selection in this screen is that affected genes directly or indirectly alter the α1-tubulin-driven GFP expression, which begs the question, how does each disrupted locus affect α1-tubulin promoter regulation? The *dri* mutant offers the most direct correlation because it displays an increase in α1-tubulin (and GFP) mRNA without increasing total cell number or affecting tissue specificity of its expression. Is it possible that the shift in neural composition seen in *dri* embryos, causing an abundance of precursors indirectly contributes to this increase? Examination of α1-tubulin distribution in distinct neural cell classes of the retina.
showed no obvious bias in its expression favoring undifferen-
tiated precursor cells, and in fact, the postmitotic differenti-
tiating RGCs form one of the strongest expressing cell types. 
This hints at the possibility that in addition to directing the 
neuronogenesis switch, dhrishti negatively regulates (directly or 
indirectly) expression from the α1-tubulin promoter. It will be 
interesting to delineate the dhr- dependent intracellular pathway 
that impinges upon the α1-tubulin promoter once its 
molecular identity is revealed. However, another interesting 
possibility is that the block in cell cycle exit causes precursors 
to be stalled/arrested at one particular developmental stage 
leading to increased accumulation in each cell of the α1-
tubulin (and GFP) gene products. A careful analysis of 
kinetics of cell cycle progression and exit of precursors could 
help explore this option. Mutation in sookshma causes a 
reduction in the neuronal progenitors suggesting that attenu-
ation of this GFP-positive precursor pool is the primary event 
that prompted its selection in the visual screen. It will 
however be interesting to examine if sookshma has an 
additional role in maintaining α1-tubulin expression in the 
nervous cells. Finally, α1-tubulin regulation by dhrava is most 
intriguing. The presence of GFP-positive spinal fibers in the 
dhv embryos is unexpected because the transgenic cassette 
harbors a nuclear localization signal, restricting GFP to the 
nucleus with smaller amounts leaking into axonal projections. 
Based on the branching defect, we proposed two hypotheses 
to explain this fluorescence defect. Physical thickening of 
spinal nerves and varicosities make the axonal GFP visually 
appreciable, distinguishing it from the wild-type spinal cord. 
The second possibility is that loss of branching and terminal 
synaptogenesis prevents down-regulation of α1-tubulin pro-
moter in motoneurons (similar to DM-GRASP regulation), 
causing an increase in nuclear as well as diffused axonal GFP. 
Examination of α1-tubulin mRNA (α1-tubulin ISH at 3– 
4 dpf, not shown) in mutant spinal motoneurons and RGCs 
did not show a significant enhancement at a time when DM-
GRASP is increased (Figs. 8I, J and 9I, J). It is possible that 
we missed the time window for α1-tubulin down-regulation, 
which could occur at an earlier time point (2 dpf) resulting in 
accumulation of the stable GFP protein until 3–4 dpf. On the 
other hand, it could imply differences in target-dependent 
down-regulation of axonal growth-associated genes such as 
α1-tubulin and DM-GRASP, making identification of the 
dhrava gene even more interesting.

In conclusion, uncovering the molecular identities of 
sookshma, drishti, and dhrava as well as developmental 
characterization of the remaining mutants will contribute 
significantly to our understanding of complex neurogenic 
pathways in zebrafish.

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