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Molecules and Cells

Neuron-Specific Expression of *Scratch* Genes during Early Zebrafish Development

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***Scratch* (*scrt)* genes are neural-specific in mammals, but their homologues have not been well studied in non-mammalian vertebrates. In this report, we isolated three zebrafish *scrt* genes, *scratch1a* (*scrt1a)*, *scratch1b* (*scrt1b)*, and *scratch2* (*scrt2)*, which belong to the Snail superfamily of zinc finger transcription factors. Spatiotemporal expression analysis revealed that *scrt1a* and *scrt2* were initially detected in the central nervous system (CNS) during early somitogenesis while *scrt1b* was first detectable in neuronal clusters in the brain during late somitogenesis. Interestingly, *scrt*-expressing cells largely overlapped with *huC*-positive differentiating neurons and partially with *neurogenin1*-positive neuronal precursor cells. In addition, *scrt*-expressing cells were dramatically increased in *mind bomb*, a neurogenic mutant. Taken together, these results suggest that each zebrafish *scrt* gene is specifically expressed in neuronal cells and may be involved in differentiation of distinct neuronal populations in the vertebrate nervous system.**

INTRODUCTION

The Scratch (Scrt) family is a member of the Snail superfamily of zinc finger transcription factors which are involved in regulating cell movement processes both during embryonic development and tumor progression (Barrallo-Gimeno and Nieto, 2005; Nieto, 2002). Like the Snail family proteins, the Scrt family contains one N-terminal basic amino acid-rich domain (SNAG), which is present only in vertebrates, and five zinc finger motifs acting as sequence-specific DNA-binding motifs. However, the Scrt family proteins are distinguished from other Snail family proteins by the presence of a Scratch domain. The SNAG domain of SCRT proteins is required for transcriptional repression and nuclear localization activities of the rat Gfi1 proto-oncoprotein (Grimes et al., 1996), although the SNAG domain of human SCRT is not necessary for such activities (Nakakura et al., 2001a). Currently, the exact function of the Scratch domain remains unclear.

Among *Scratch* homologues, the nematode *scrt* homologue ces-1 is important in controlling the death of specific neurons (Metzstein and Horvitz, 1999). In flies, *scrt* is expressed in most neuronal precursors and is involved in promoting neuronal cell fates (Roark et al., 1995). However, the mouse and human *SCRT* are expressed in newly differentiating, post-mitotic neurons and the mouse *Scrt* induces neuronal differentiation in P19 embryonic carcinoma cells (Nakakura et al., 2001b), implying it plays a role in neuronal differentiation. In particular, the human *SCRT* acts as a neural-specific transcriptional repressor *in vitro* by antagonizing the function of the basic helix-loop-helix (bHLH) proteins MASH-1 and E12 (Nakakura et al., 2001a; 2001b).

In this study, we characterized the expression of three zebrafish *scratch* paralogs during development. These genes were specifically expressed in distinct sets of neuronal populations in the central nervous system (CNS) during early embryogenesis. Interestingly, the *scrt*-expressing cells overlapped with *huC*-positive neurons and the number of *scrt*-expressing cells was significantly increased in the *mind bomb* mutant embryos, suggesting that *scrt* genes are expressed in differentiating neurons, similar to their mammalian counterparts.

MATERIALS AND METHODS

Zebrafish maintenance

Adult fish were maintained at 28.5°C in a 14 h light/10 h dark cycle. Wild-type and *mib*^{ta52b} embryos (Itoh et al., 2003) were raised as described previously (Westerfield, 1995). Embryonic stages were determined by the hours post-fertilization (hpf) and microscopic observation. Embryos were treated with 0.2 mM PTU (1-phenyl-2-thiourea) to prevent pigment formation.

Cloning of the zebrafish *scratch* genes

To isolate the zebrafish *scratch* genes, total RNA was isolated from wild-type zebrafish embryos at 24 hpf using TRIZOL (MRC, Inc.) and reverse-transcribed. Full-length *scratch1a* (Genbank Acc. No. BC154637), *scratch1b* (BC091823), and *scratch2* (BC135018) were generated by PCR amplification using the following primers: *scrt1a* forward primer, 5′-GTTCTCCAGC GTTGCACTTGCATG-3′; *scrt1a* reverse primer, 5′-CACCCCA AAACTCAGAGGACATTG-3′; *scrt1b* forward primer, 5′-GCA

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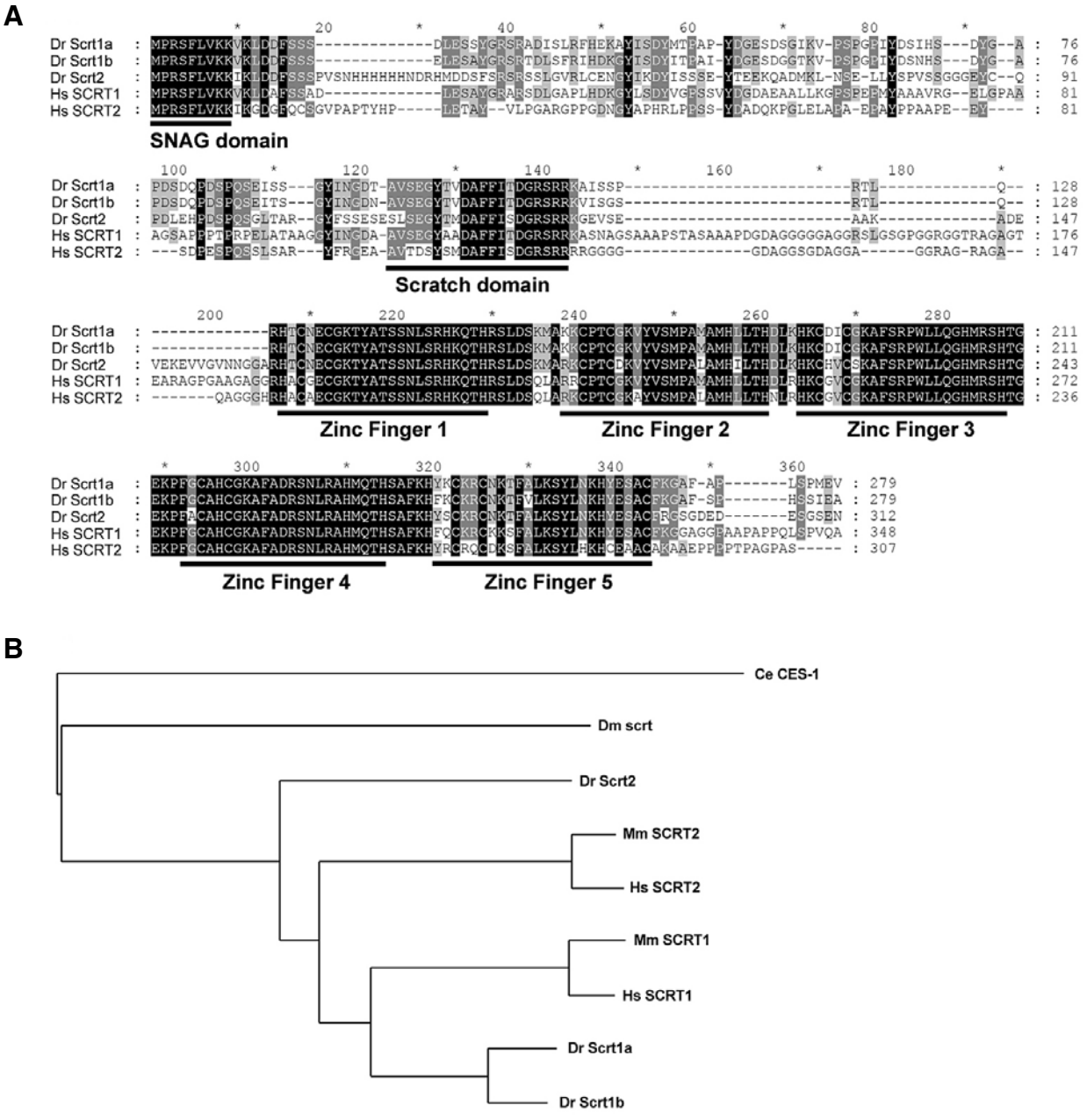

Fig. 1. Comparison of the amino acid sequences of Scratch homologues. (A) Multiple sequence alignments were performed using Clustal X. Scratch homologues contain one SNAG domain in the N-terminus, one Scratch domain in the central region, and five C2H2 zinc finger motifs in the C-terminus. Identical residues are shown in black. (B) The phylogenetic tree of zebrafish Scrts and their putative orthologues from other species. Human (Hs) SCRT1 (Genbank Acc. No. NP_112599), human SCRT2 (NP_149120), mouse (Mm) SCRT1 (NP_570963), mouse SCRT2 (NP_001153882), zebrafish (Dr) Scrt1a (NP_001107073), zebrafish Scrt1b (NP_001014369), zebrafish Scrt2 (NP_998802), Drosophila (Dm) scrt (AAA91035), and *C. elegans* (Ce) CES-1 (AAF01678).

GGATCAACTCTTCTATTGTGCGG-3′; *scrt1b* reverse primer, 5′-CTCACTTCCCTGTCACGTGTTAGGATGA-3′; *scrt2* forward primer, 5′-GCGGGTCAGTCTGCGACCGATG-3′; *scrt2* reverse primer, 5′-GCTCGCAAAAATCCCCACAAGACT-3′. The amplified PCR products were inserted into pEasy-T3 cloning vectors (BioPrince), and sequenced using an automated sequencer.

Whole-mount *in situ* hybridization

To synthesize RNA probes, pEasy-T3 plasmids containing *scrt1a*, *scrt1b*, and *scrt2* full-length cDNAs were linearized with *Pst*I, *Sal*I, and *Hin*dIII, respectively. Anti-sense RNA probes were transcribed *in vitro* using T7 RNA polymerase and digoxigenin or fluorescein-labeled UTP. Antisense digoxigenin-labeled RNA probes for *ngn1* (Kim et al., 1997) and *huC/elavl3* (Kim et al., 1996) were produced using a digoxigenin-RNA labeling Kit (Roche, Germany) according to the manufacturer's instructions. Whole-mount *in situ* hybridization was performed as previously described (Gwak et al., 2010).

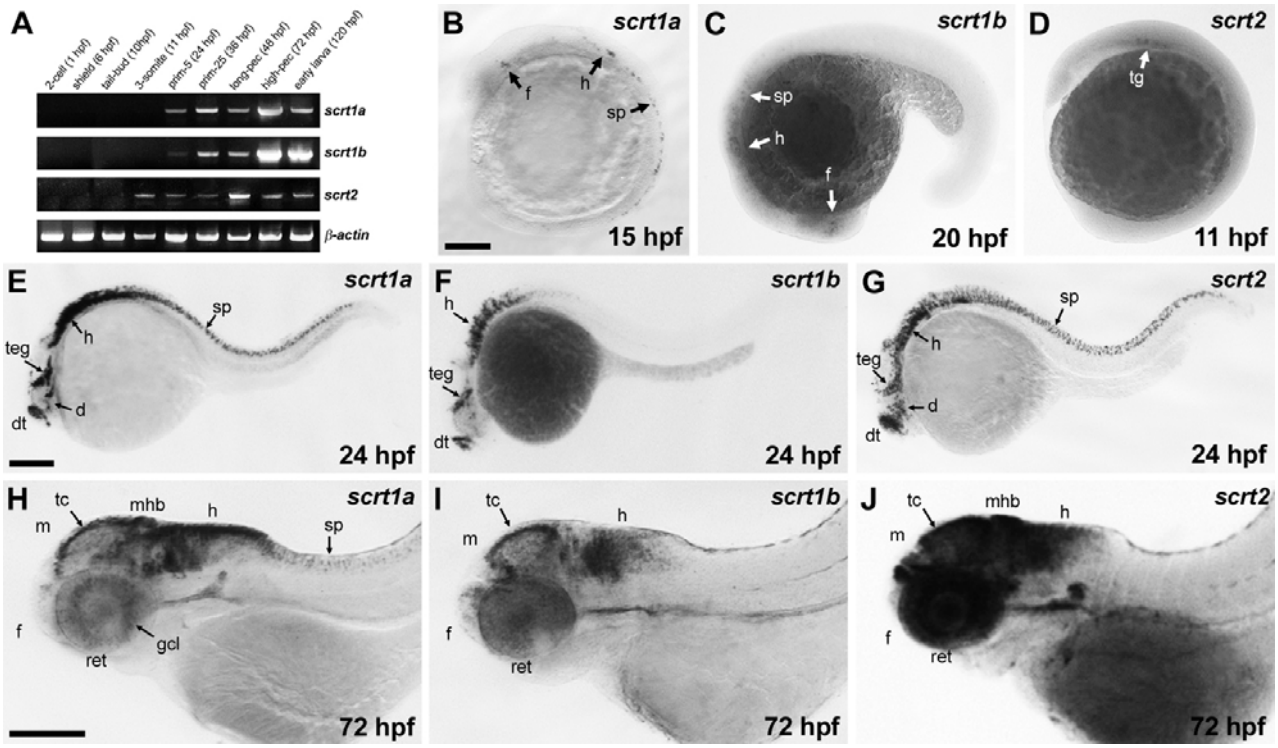

Fig. 2. Expression profile of *scratch* genes during early development. (A) The three *scratch* genes are expressed from somite-stages to the early larval stage but not the cleavage- and gastrula-stages. *Actin* was used as a loading control. (B) At the 12-somite stage (15 hpf), *scrt1a* expression was detected in subsets of neurons in forebrain, hindbrain, and spinal cord. (C) At the 22-somite stage (20 hpf), *scrt1b was* expressed in subsets neurons in the forebrain, hindbrain and spinal cord. (D) Expression of *scrt2* was first detectable in the trigeminal ganglia at the 3-somite stage (11 hpf). (E) At 24 hpf, *scrt1a* was expressed in the dorsal telencephalon, diencephalon, tegmentum, hindbrain and spinal cord neurons. (F) At 24 hpf, expression of *scrt1b* was detected in the dorsal telelecephalon, tegmentum, and hindbrain. (G) At 24 hpf, *scrt2* was specifically expressed in the dorsal telencephalon, diencephalon, tegmentum, hindbrain and spinal cord neurons. (H) At 72 hpf, *scrt1a* was detected in the ganglion cell layer in the retina, and the tectum, midbrain-hindbrain boundary, hindbrain, and spinal cord neurons. (I) At 72 hpf, *scrt1b* was expressed in the tectum and hindbrain. (J) At 72 hpf, expression of *scrt2* was strongly detected in the retina, forebrain, midbrain, midbrain-hindbrain boundary, and hindbrain. Abbreviations: d, diencephalon; dt, dorsal telencephalon; f, forebrain; gcl, ganglion cell layer; h, hindbrain; m, midbrain; mhb, mid-hindbrain boundary; ret, retina; sp, spinal cord; tc, tectum; teg, tegmentum; tg, trigeminal ganglia. Scale bars = 200 μm.

RESULTS AND DISCUSSION

Isolation of zebrafish *scratch* genes

To analyze the expression patterns of the zebrafish *scrt* family genes in developing embryos, we isolated three *scratch* genes based on the similarity of amino acid sequences to human *SCRATCH*. The amino acid alignment of zebrafish and human SCRT revealed highly conserved regions, such as the N-terminal SNAG domain, which is not present in invertebrates, the Scratch domain in the central region, and five zinc finger motifs in the C-terminus (Fig. 1A). Based on comparison of the putative amino acid sequences and phylogenetic relationships, we designated these genes *scratch1a*, *scratch1b*, and *scratch2* (Fig. 1B).

Expression profiles of *scratch* mRNA in developing embryos

In mammals, *Scrt* expression appears in newly differentiating, post-mitotic neurons in the brain and spinal cord, as well as in the retina and in lung neuroendocrine cells (Marín and Nieto, 2006; Nakakura et al., 2001a). To determine *scrt* expression profiles during early development of zebrafish, we performed RT-PCR and whole-mount *in situ* hybridization. Expression of *scrt1a* and *scrt1b* was first detected at 24 hpf and of *scrt2* at the 3-somite stage, coinciding with the onset of primary neurogenesis which begins at 10.5 hpf and is complete before 24 hpf (Figs. 2A, B, B′, and B″) (Mueller and Wullimann, 2003; Park et al., 2003). At the 2-cell and shield stages, no transcripts were detected, indicating that the three *scratch* genes are only expressed zygotically.

To further examine temporal and spatial expression of scratch genes during early development, whole-mount *in situ* hybridization was employed. Consistent with the RT-PCR results, transcripts of the three scratch genes were not detected in the cleavage and gastrula stages (data not shown). Expression of *scrt1a* was first detected in clusters of neurons in the forebrain, hindbrain and spinal cord at 15 hpf (Fig. 2B). At 24 hpf, *scrt1a* was expressed in the dorsal telencephalon, diencephalon, tegmentum, hindbrain and spinal cord neurons (Fig. 2E). As development proceeded, strong expression of *scrt1a* was maintained in the CNS, including the forebrain, tectum, midbrain-hindbrain boundary, hindbrain, and spinal cord at 72 hpf; *scrt1a* was also specifically expressed in the ganglion cell layer of the retina (Fig. 2H). Expression of *scrt1b* was first detected in clusters of neurons in the forebrain, hindbrain, and spinal cord at 20 hpf (Fig. 2C). At 24 hpf, *scrt1b* was expressed in the dorsal

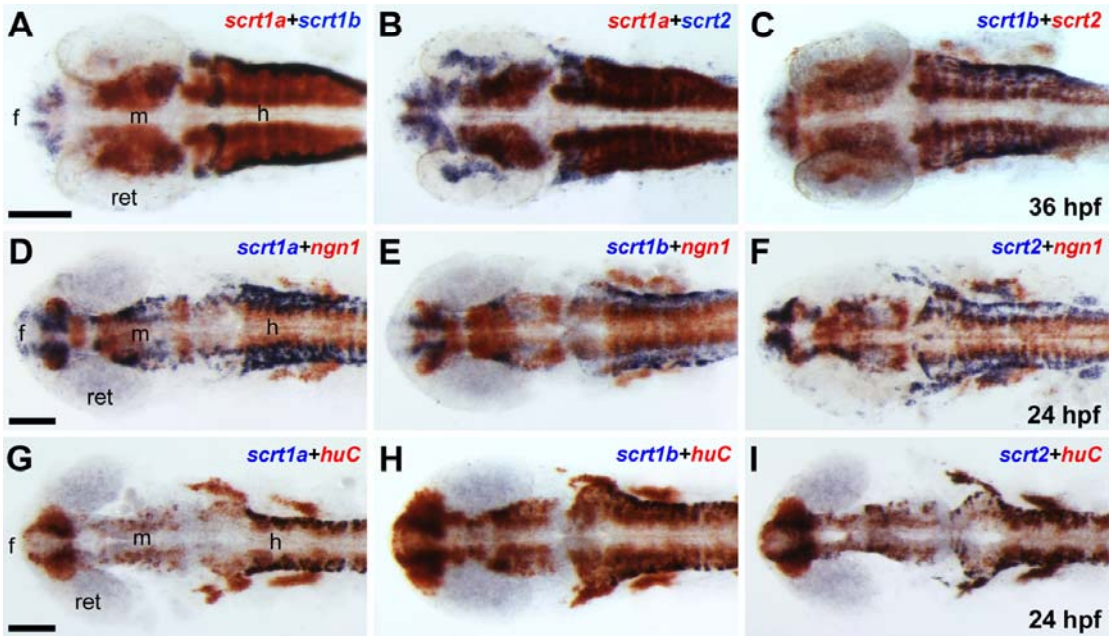

Fig. 3. Expression of *scratch* genes during early neurogenesis. Images are dorsal view with anterior to the left for all panels. (A-C) 36 hpf, (D-I) 24 hpf. (A, B) Expression of *scrt1a* partially overlapped with that of *scrt1b* (A) and *scrt2* (B) in the CNS at 36 hpf. (C) Expression of *scrt1b* overlapped predominantly with that of *scrt2* at 36 hpf. (D-F) *scrt1a*- (D), *scrt1b*- (E), and *scrt2*-expressing cells (F) partially overlapped with *ngn1*-positive neuronal precursor cells at 24 hpf. (G-I) *scrt1a*- (G), *scrt1b*- (H), and *scrt2*-expressing cells (I) are all *huC*-positive, differentiating neurons in the CNS at 24 hpf. Abbreviations: f, forebrain; h, hindbrain; m, midbrain; ret, retina. Scale bars = 100 μm.

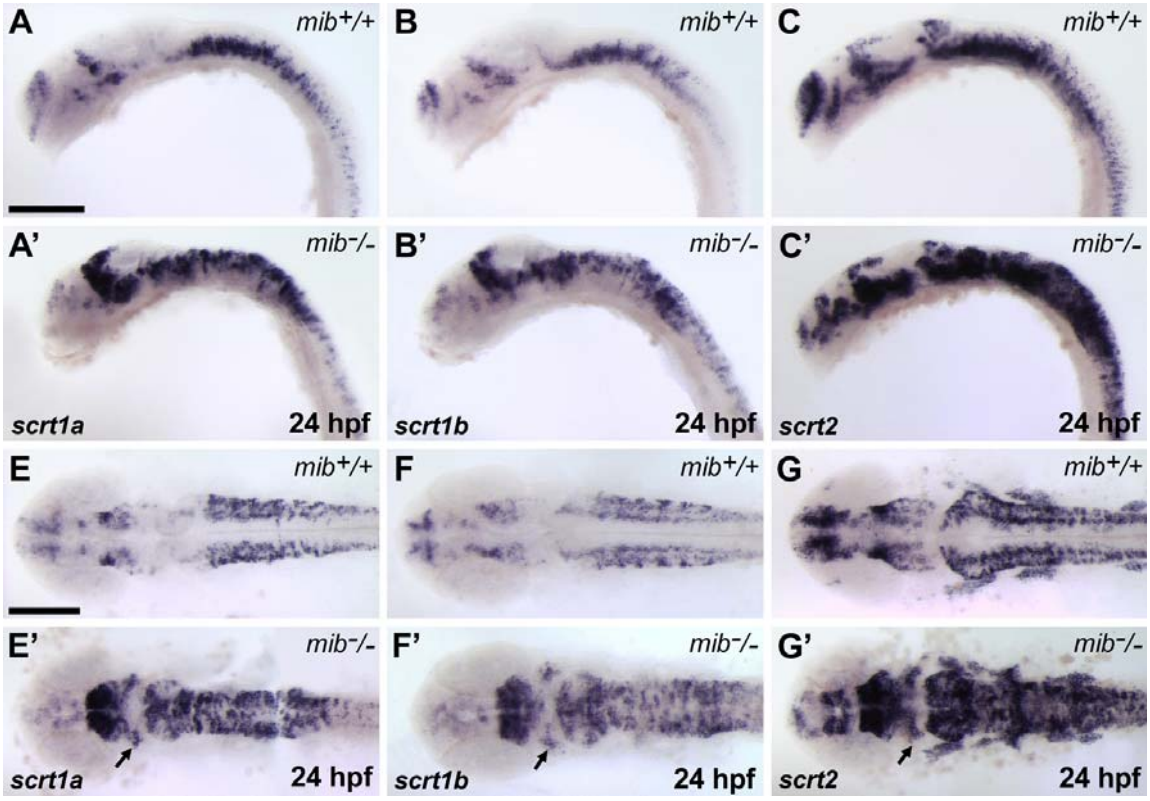

Fig. 4. Increase in *scratch*-expressing cells in *mind bomb/mib,* a neurogenic mutant. (A-C′) Lateral view with anterior to the left. (E-G′) Dorsal view with anterior to the left. The numbers of *scrt1a* (A, A′, E, and E′), *scrt1b* (B, B′, F, and F′), and *scrt2* (C, C′, G, and G′)-expressing cells increased markedly in *mib* mutant embryos at 24 hpf. In *mib* mutant embryos, ectopic *scrt*-positive cells in the midbrain were detected compared to wild-type embryos (arrows in E′, F′, and G′). Scale bars = 200 μm.

telencephalon, tegmentum, and hindbrain, as well as weakly in spinal cord neurons (Fig. 2F). At 72 hpf, *scrt1b* was detectable in the forebrain, tectum, and hindbrain, but not at the midbrain-hindbrain boundary, or in the retina or spinal cord (Fig. 2I).

Expression of *scrt2* was first detected in the trigeminal ganglia at 11 hpf (Fig. 2D). At 24 hpf, *scrt2* was expressed in the dorsal telencephalon, diencephalon, tegmentum, hindbrain, and spinal cord neurons (Fig. 2G). Expression of *scrt2* was detected in the forebrain, tectum, midbrain-hindbrain boundary, and hindbrain, as well as in the retina, at 72 hpf. This expression pattern partially overlapped with *scrt1a* and *scrt1a* (Fig. 2J). To further investigate the expression patterns of the three *scrt* paralogs, we performed double-labeling whole-mount *in situ* hybridization. At 36 hpf, the expression domains of *scrt1a-scrt1b*, *scrt1a-scrt2*, and *scrt1b-scrt2* partially overlapped with each other at clusters of neurons in the forebrain, midbrain, and hindbrain (Figs. 3A-3C). In summary, the expression of the three *scrt* genes was restricted to the CNS, including the brain, spinal cord, and retina, and was similar in terms of temporal and spatial expression, implying that these genes might play similar roles during early neurogenesis.

Scratch genes are expressed in differentiating neurons

To identify whether *scrt*-expressing cells are neuronal precursors or differentiating neurons, double-labeling whole-mount *in situ* hybridization was carried out with neuronal markers. *neurogenin1*/*ngn1*, a marker for neuronal precursors, is required for the development of cranial sensory ganglia precursors (Andermann et al., 2002; Kim et al., 1997). The *scrt*-expressing cells partially overlapped with *ngn1*-positive neuronal precursors at 24 hpf (Figs. 3D-3F). However, *scrt*-expressing cells completely overlapped with *huC/elavl3*-positive differentiating neurons in the brain as well as cranial ganglia, indicating that *scrt* is expressed in most differentiating neurons (3G-I) (Kim et al., 1996). These results suggest that the expression of zebrafish *scrt* genes is specifically restricted to differentiating neurons during early neurogenesis.

We next examined *scrt* expression in the *mind bomb* mutant (*mib*), which has increased levels of differentiating neurons and reduced levels of neuronal precursors in the CNS (Itoh et al., 2003). Since *scrt*-expressing cells completely overlap with *huC*-positive differentiating neurons, we expected that *scrt*-expressing cells would be increased in *mib* mutant embryos. We found that the number of *scrt*-expressing cells was dramatically increased and the bilateral expression domains of *scrt* were fused across the midline in *mib* mutants at 24 hpf (Figs. 4A-4G′). Furthermore, ectopic *scrt*-positive cells were detected in the midbrain of the *mib* mutant embryos (Figs. 4E′-4G′). This ectopic expression indicated that neuronal precursor cells in the *mib* mutant undergo premature differentiation.

In conclusion, zebrafish *scratch* genes are specifically expressed in a subset of differentiating neurons in the CNS during early development, similar to their mammalian counterparts. The analysis of zebrafish *scrt* expression provides a first step in understanding the functional roles of these genes during vertebrate neurogenesis.

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