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LETTER

Motor Neuron-Expressed MicroRNAs 218 and Their Enhancers Are Nested Within Introns of *Slit2/3* Genes

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Summary: *miR218-1* and *miR218-2* are embedded in introns of *SLIT2* and *SLIT3*, respectively, an arrangement conserved throughout vertebrate genomes. Both *miR218* genes are predicted to be transcribed in the same orientation as their host genes and were assumed to be spliced from *Slit2/3* primary transcripts. In zebrafish *miR218* is active in cranial nerve motor nuclei and spinal cord motor neurons, while *slit2* and *slit3* are expressed predominantly in the midline. This differential expression pattern suggested independent regulation of *miR218* genes by distinct enhancers. We tested conserved noncoding elements for regulatory activity by reporter gene transgenesis in zebrafish. Two human enhancers, 76 kb and 130 kb distant from *miR218-2*, were identified that drove GFP expression in zebrafish in an almost complete *miR218* expression pattern. In the zebrafish *slit3* locus, two enhancers with identical activity were discovered. In human *SLIT2* one enhancer 52 kb upstream of *miR218-1* drove an expression pattern very similar to the enhancers of *miR218-2*. This establishes that *miR218-1/-2* regulatory units are nested within *SLIT2/3* and that they are duplicates of an ancestral single locus. Due to the strong activity of the enhancers, unique transgenic lines were created that facilitate morphological and gene functional genetic experiments in motor neurons. genesis 53:321–328, 2015. © 2015 Wiley Periodicals, Inc.

Key words: genomic regulatory blocks; GRB; long-range gene regulation; conserved synteny; gene duplication; microRNA

RESULTS AND DISCUSSION

The concept of genomic regulatory blocks (GRBs) posits that a gene with long-range regulation is driven by enhancers at great distances that are often found in introns of neighboring genes and beyond (Kikuta *et al*., 2007a). Because essential enhancers cannot break away from the gene they regulate without adverse consequences for the organism, nested units of enhancers, target gene, and bystander genes were kept together during evolution, resulting in large regions of conserved synteny in all vertebrate genomes (Becker and Lenhard, 2007). Through an enhancer detection screen performed in zebrafish, we discovered that miRNAs are can be GRB target genes (Ellingsen *et al*., 2005; Kikuta *et al*., 2007b).

SLIT2/3 orthologs encode secreted proteins that interact with ROBO receptors and provide guidance cues for cell migration and axonal projections (Dickson and Gilestro, 2006). This signaling is crucial in the midline and by repelling growth cones prevents axons from crossing to the other side of the CNS (Keleman *et al*., 2002; Rasband *et al*., 2003). In the developing forebrain slit/robo signaling is essential for formation of

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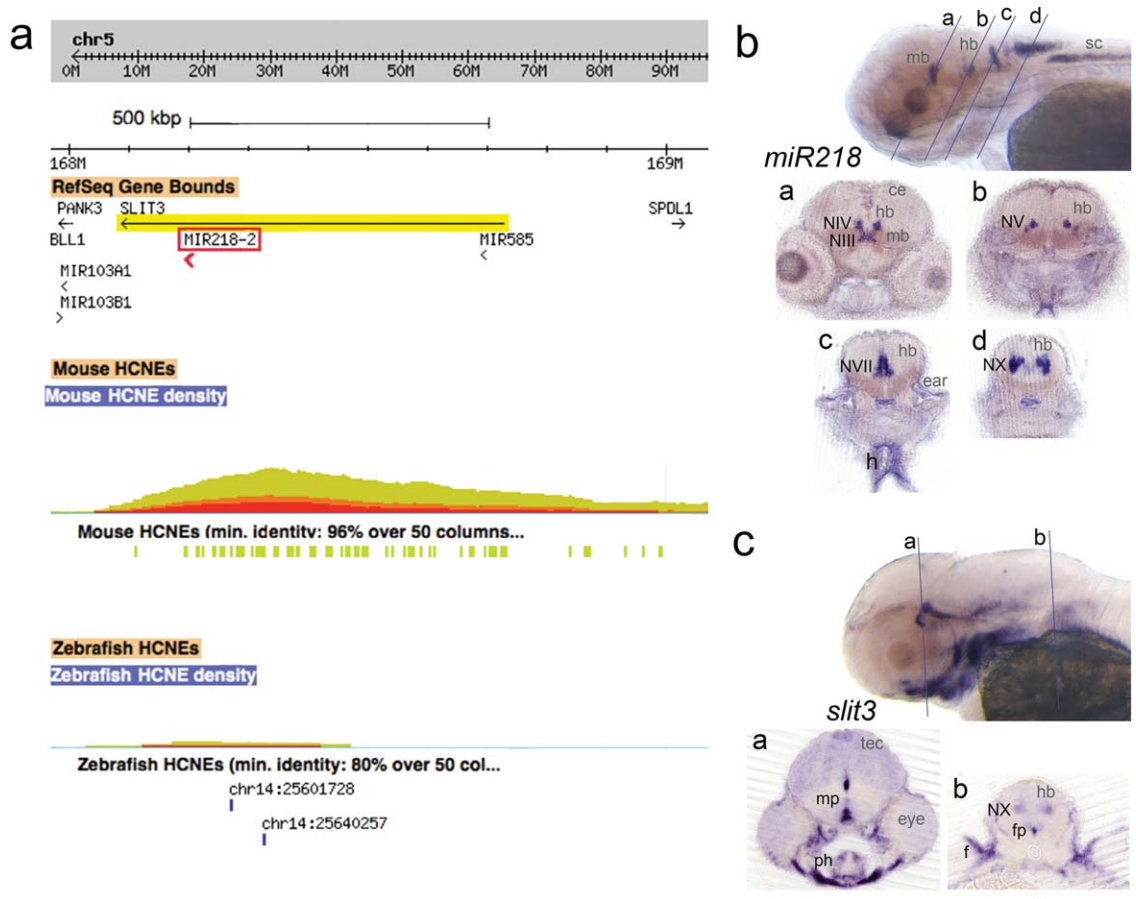

FIG. 1. **(a)** Ancora Genome browser window of the human *SLIT3* gene on chromosome 5, harboring *miR218-2* in intron 14. The red-yellow colored hill represents the density of noncoding sequences that are conserved with the mouse genome (minimum identity 96% over 50 bp; illustrated as yellow bars). The highest density is ~100 kb distant of *miR218-2*. Conservation with the zebrafish genome is shown below. Only two HCNEs (80% identity over 50 bp) are present (illustrated by the blue bars). **(b,c)** *miR218* LNA and *slit3* in situ hybridization, shown are 4 dpf zebrafish larvae. Labeled structures are indicated unilaterally with black abbreviations. Abbreviations in grey are used to describe anatomical features. Abbreviations: ce, cerebellum; f, fin; fp, floorplate; h, heart; hb, hindbrain; mb, midbrain; mp, midbrain basal plate; NIII, oculomotor nerve nucleus; NIV, trochlear nerve nucleus; NV, trigeminal nerve nucleus; NVII, facial nerve nucleus; NX, vagal nerve nucleus; ph, pharyngeal arches skeleton; tec, tectum; vn, vagal nucleus.

the commissures (Barresi *et al*., 2005; Zhang *et al*., 2012).

Consistent with this function zebrafish *slit2* is expressed in the axial mesoderm, floorplate and prechordal plate (Yeo *et al*., 2001), whereas *slit3* is expressed in pharyngeal arches, pectoral fin buds, midbrain basal plate, floorplate and also in cranial motor nuclei as well as weakly in spinal cord motor neurons (Pan *et al*., 2012; Yeo *et al*., 2001).

By contrast, much less is known about *miR218* function. The expression pattern of *miR218* was discovered in an in situ hybridization screen in zebrafish (Wienholds *et al*., 2005). In heart development, miR218 targets the slit2 receptor robo1 for repression (Fish *et al*., 2011). We have analyzed the expression pattern of *dre-miR218* in detail by locked-nucleic-acid (LNA) in situ hybridization (Fig. 1b). Transcripts were localized in the nuclei of cranial motor nerves, in spinal cord motor neurons and in the heart.

We used the Ancora genome browser, which has been developed to identify GRBs (Engstrom *et al*., 2008). Ancora uses high sequence conservation thresholds (see methods) to identify highly conserved noncoding elements (HCNEs) in human/zebrafish synteny blocks. For *SLIT2* and *SLIT3*, we found that human/zebrafish conserved synteny does not extend beyond *SLIT2/3* gene boundaries. This indicates that HCNEs essential for *SLIT2/3* and *miRNA218* genes are contained within *SLIT2/3* genes, suggesting a nested "microRNA-GRB" within a "SLIT-GRB" arrangement.-*miR218-1* is located in intron 14 of *SLIT2* (chr4, gene span: 380 kb), whereas *miR218-2* is found in intron 14 of *SLIT3* (chr5, 640 kb). The zebrafish has three members of the miR218 family, *dre-mir-218a-2* is located in

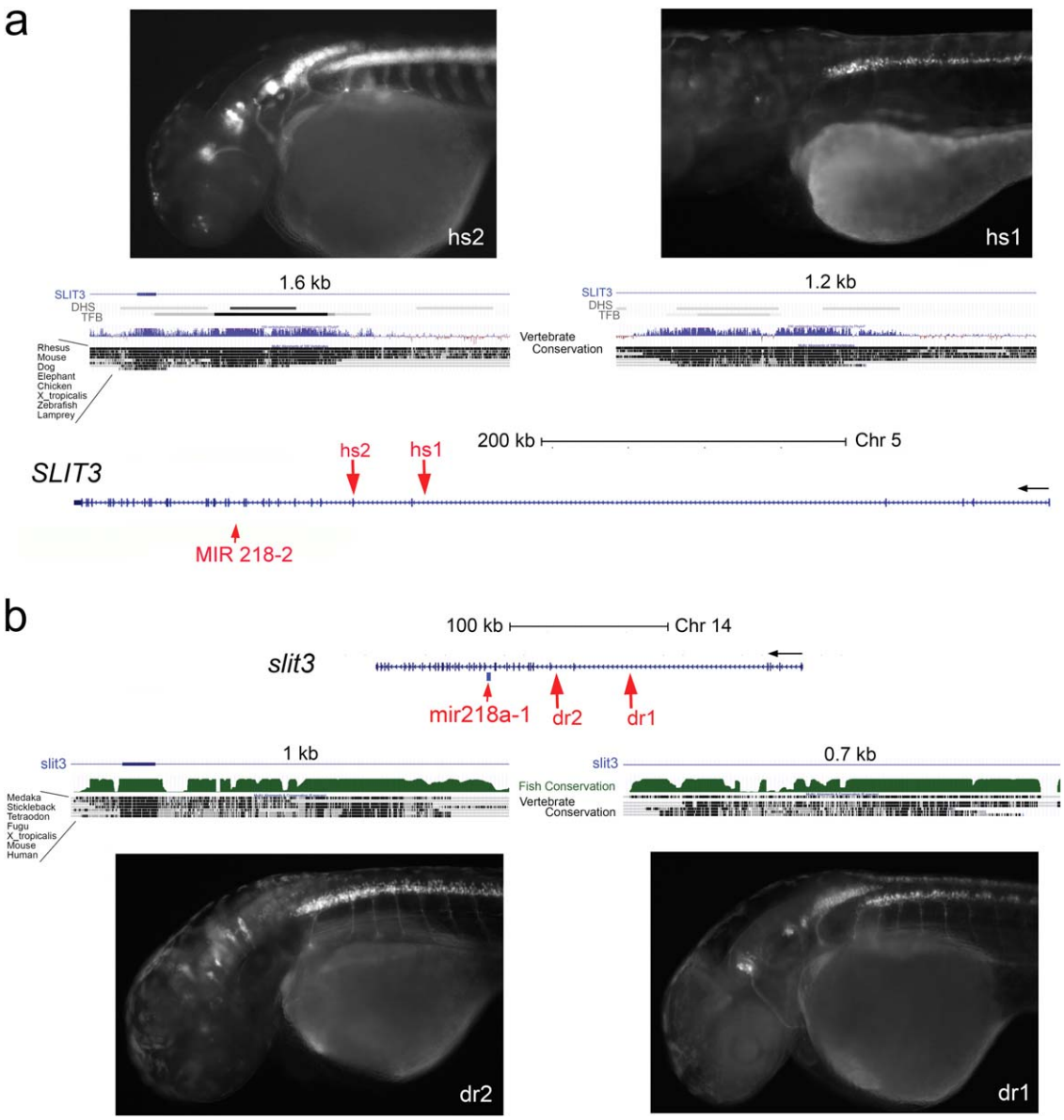

FIG. 2. Elements amplified from human (hs) and zebrafish (dr) sequences within *SLIT3/slit3* had specific enhancer activity that drove expression in a *miR218-2* like pattern in brainstem- and spinal cord motor neurons. **(a)** Modified UCSC genome browser window showing the human *miR218-2/SLIT3* locus. *SLIT3* as well as the *miRNA 218-2* are located in reverse orientation in the genome sequence. The 3′ end of *SLIT3* is to the left. *miR-218-2* is located in the intron 14 of *SLIT3*. Two noncoding sequences conserved between human and zebrafish genomes were identified as enhancers of *miR218-2*. While hs2 drove GFP in most or allof the neuronal expression domain of *miR218*, hs1-driven GFP was seen only in spinal cord motor neurons. **(b)** In zebrafish the *slit3* gene is much smaller than in the human genome. Two sequences that show conservation between human and zebrafish have enhancer activity in a pattern very similar to the enhancers identified in human *SLIT3*. Abbreviations: DHS, DNase 1 hypersensitive sites; TFB, transcription factor binding identified by ChIP seq – both tracks are provided by ENCODE.

intron 14 of *slit2* (chr1, 124 kb), *dre-mir-218a-1* is within intron 14 of *slit3* (chr14, 270 kb) and *dre-miR-218b* (chr10) appears to be fish specific (e.g., fugu, stickleback, zebrafish) and is intergenic, suggesting a loss of the corresponding slit host gene after the fish-specific whole genome duplication. The human *SLIT3* locus is illustrated in Ancora genome browser view (Fig. 1a), showing the extent of the conserved synteny block and HCNEs in vertebrate and mammalian genomes. The density of conserved non-coding sequences is highest towards *miR218-2*.

Because of genomic co-localization, but divergent expression of *miR218* and *SLIT3*, we asked whether enhancers could be found within *SLIT3* introns that drive expression patterns of *miR218-2*, and analyzed HCNEs in this locus using zebrafish reporter

Table 1
Tested Elements in Human and Zebrafish Genomes

Gene loci	Distance to miR218	Element coordinates	miRNA 218 coordinates
Human			
SLIT3			*miR218-2*
hs1 1252 bp	130 kb upstream	chr5:168,324,966-168,326,217	chr5:168,195,151-168,195,260
hs2 1590 bp	76 kb upstream	chr5:168,271,410-168,272,999	
SLIT2			*miR218-1*
hs1 1165 bp	52 kb	chr4:20,481,479-20,482,643	Chr4:20,529,898-20,530,007
Zebrafish			
slit3			*miR218a-1*
dr1 700 bp	78 kb upstream	chr14:25,640,158-25,640,857	chr14:25,562,465-25,562,547
dr2 968 bp	38 kb upstream	chr14:25,601,116-25,602,083	

Size and genome sequence coordinates of the tested elements. Human coordinates relate to hg19 release, while zebrafish coordinates are provided for zv9.

transgenesis. In contrast to published data (Yeo *et al*., 2001), our hybridization probe did not reveal *slit3* transcripts in cranial motor nuclei or in the spinal cord (Fig. 1c). To test individual enhancers, noncoding sequences conserved between human and zebrafish genomes were cloned into a GFP-reporter gene construct and the constructs were injected into fertilized zebrafish eggs to establish transgenic lines through fluorescent screening as described (Ishibashi *et al*., 2013; Kawakami, 2004; Navratilova *et al*., 2009).

Enhancers were identified in both human and zebrafish genomes (Table 1). In the human genome one element, namely hs2, 76 kb distant from *miR218-2*, drove GFP expression in cranial nerve nuclei and in spinal cord motor neurons, while hs1, 130 kb distant, regulated the GFP only in spinal cord motor neurons (Fig. 2a). This is not surprising, as overlapping, but nonidentical, activity of enhancers driving the same gene has been observed previously and appears to be common, as was shown, for example, for *fgf8* (Komisarczuk *et al*., 2009). Two conserved sequences cloned from the zebrafish genome, dr1 at 78 kb- and dr2 at 38 kb distance from *miR218a-1*, were found to have highly similar if not identical activity to hs1 (Fig. 2b). The GFP expression patterns in the transgenic lines identify the tested sequences as enhancers of *miR218* genes, although, since *slit3* was also shown to express in cranial nerve nuclei and in spinal cord motor neurons (Yeo *et al*., 2001), some enhancer function might be shared between the two genes.

To test whether the enhancers could have evolved as enhancers of *SLIT3* or of the embedded *miR218-2*, we next analyzed the *SLIT2* locus harboring *miR218-1* (Fig. 3). For direct expression pattern comparisons, we performed *slit2* transcript in situ hybridization (Fig. 3a). *slit2* expression is similar to *slit3* in the floorplate and midbrain basal plate. In addition, *slit2* is expressed in the eye, in cell clusters in the forebrain and in the lateral hindbrain, but not in cranial nerve nuclei or spinal cord. Genomic fragments for enhancer testing in transgenic zebrafish were defined by conservation in noncoding sequences between human and zebrafish genomes. No HCNE was found in alignments shown in Ancora genome browser (80% over 50bp), but conservation at a lower level was visible in UCSC (Fig. 3b). Element hs1, in the fifth intron of *SLIT2*, 52 kb upstream of *miR218*, had consistent *cis*-regulatory activity (Fig. 3b,c). Transgenic larvae expressed GFP in spinal cord motor neurons and in the vagal nerve nucleus, similar to hs1-, hs3-, dr1-, and dr3- driven GFP (Fig. 2). The fact that the expression pattern relates to miR218, but not to *slit2* indicates that we have identified an enhancer of *miR218-1*. Genome sequencing of the amphioxus *Branchiostoma* and comparisons with the human genome have shown that four human paralogs correspond to one gene in amphioxus, strongly supporting the model of two rounds of whole genome duplications in the vertebrate lineage (2R hypothesis) [for review see (Kasahara, 2007; Putnam *et al*., 2008)]. Thus, our data support the notion that *miR218-1/2* and *SLIT2/3* genes have coevolved in animal evolution from a single ancestral *slit/mir218* locus.

In conclusion, we have shown that the expression patterns of the two *miR218* paralogs, which are different from that of their slit host genes, are regulated by distinct enhancers. Identified human and zebrafish enhancers drove GFP expression exclusively in cranial and spinal cord motor neurons. Our findings indicate that *miR218* genes are not simply spliced out of the mRNA of their host genes, but have their own long-range *cis*-regulatory domains within the bounds of their host genes.

Our transgenic zebrafish lines reveal exquisite morphological details of motor neurons expressing miR218 (Fig. 4). Cranial nerve nuclei had been transgenically labeled in zebrafish by *isl1* and pectoral fin motor neurons were visualized in an enhancer detection line as *hoxb4a* expression domain, respectively (Higashijima *et al*., 2000; Punnamoottil *et al*., 2008). In future, the use of the enhancers in Gal4 driver constructs (Fig. 4e,g,h) will facilitate specific manipulation of motor neurons with miR218 identity through crossing into *UAS:effector* gene lines.

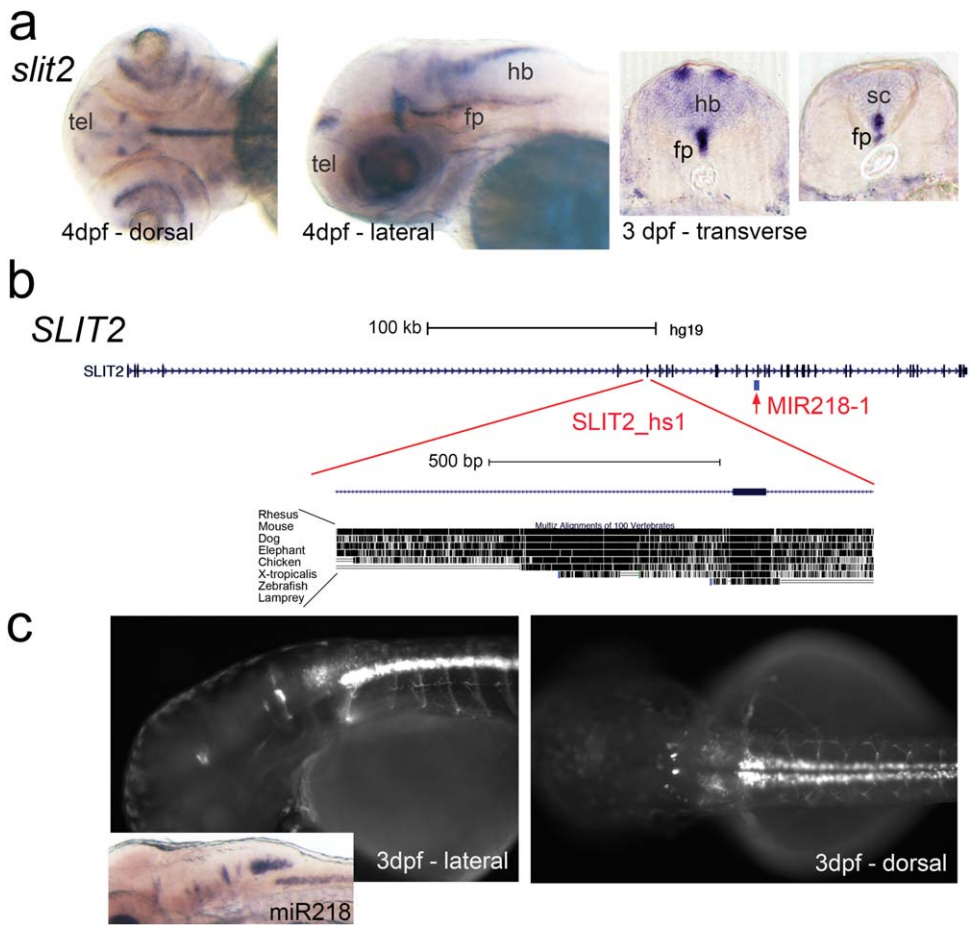

FIG. 3. **(a)** zebrafish *slit2* in situ hybridization. *slit2* is expressed in the telencephalon, in the dorsal hindbrain, the midbrain basal plate and floorplate and the ganglion cell layer in the eye. Two sections, on the level of the hindbrain and of the spinal cord, show that cranial nerve nuclei and motor neurons are negative for *slit2* transcripts. **(b)** Human *SLIT2* gene with *miRNA218-1* located in intron 14. The HS1 element (1165 bp) is 52 kb upstream including intron 5 and exon 6 sequences. UCSC genome browser indicates sequence conservation in all vertebrate genomes, including the basal vertebrate lamprey. **(c)** Transgenic larvae with hs1-driven GFP. The expression pattern is that of *miR218*, but when compared to the enhancers identified within *SLIT3* (Fig. 2), the expression in the vagal nerve nucleus is much weaker and expression in anterior cranial nerve nuclei is missing.

METHODS

Animal Experiments

Work with zebrafish and juveniles had been performed in compliance with institutional and national animal welfare laws approved by the animal ethics committee (Zebrafish facility at the Victor Chang Cardiac Research Institute, reference number 09/06; Zebrafish facility at University of Sydney, approval number K00/1-2010/1/5241). Zebrafish were maintained in a zebrafish facility at 28.5 °C. Maintenance, breeding and egg collection were performed according to standard protocols.

Computational Analyses

Human and zebrafish *Slit3* loci sequences were analyzed in Ancora and UCSC genome browsers. Highly conserved non-coding sequences were defined in the mouse genome as 96% conservation over 50 bp and in the zebrafish genome as 80% over 50 bp. HCNEs conserved between human and zebrafish were chosen for testing.

Molecular Cloning, Microinjection, and Zebrafish Transgenesis

Elements including the HCNEs were designed for testing. The sequences were extracted from the genome browser and DNA fragments were PCR amplified with following primer sequences ("hs" stands for *Homo sapiens* and "dr" for *Danio rerio*):

SLIT3_hs1: *forward* CCTAAGCTCCCATCTCCAG, *reverse* TGCACCTGTCTAGAAGTTCTCTT;

SLIT3_hs2: *forward* CTGCCTGCAAACACTTTGAC, *reverse* AGAGGGTTCCCAAAGGTAGC;

slit3_dr1: *forward* GAACGAACGGCGATCACTCT, *reverse* AAGAGCTTGTTGTACGTCTCAGG;

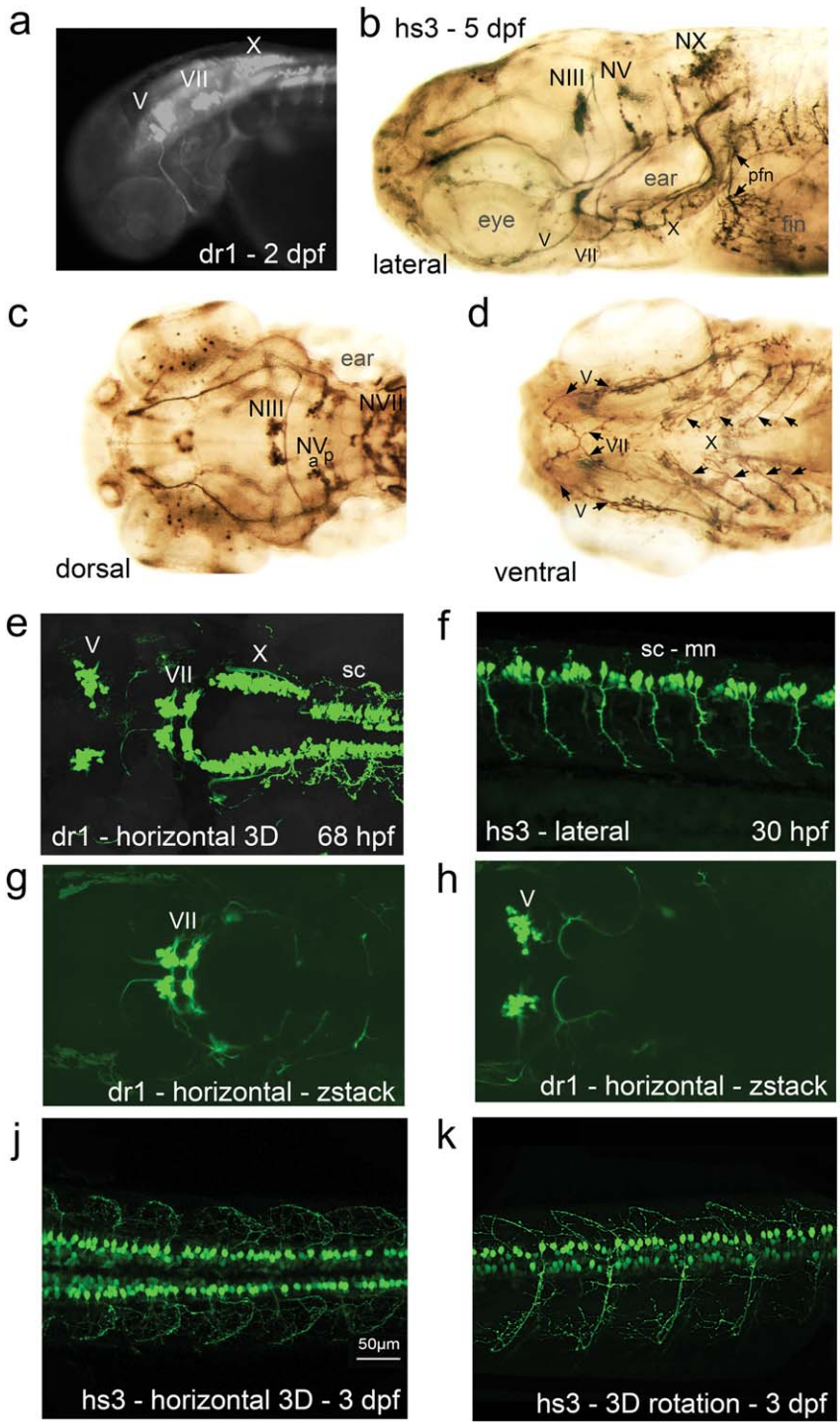

FIG. 4. Two enhancers regulating zebrafish *miR218a-1* (dr1) and human *miR218-2* (hs3) drive GFP in transgenic embryos and larvae. GFP expression is found in cranial nerve nuclei and in spinal cord motor neurons and distributed in the axons. **(a)** Overview fluorescent microscope image. **(b–d)** GFP expression pattern was enhanced through anti-GFP immunostaining. The images are from different larvae and in different orientations. **(e–k)** Confocal live imaging. (e) z-stack image through the whole expression domain as scanned in this specimen. (f) Optical section showing spinal cord motor neurons in 30 hpf embryos. **(g,h)** Two z-stack images of the scan shown in "e"; one is further dorsal with focus on the facial nerve nucleus (g), while the trigeminal nerve nuclei appear further ventral in this scan (h). (j,k) 3D projections of spinal cord motor neurons. Abbreviations: hpf, hours post fertilization; mn, motor neurons; sc, spinal cord; NIII, oculomotor nerve nucleus; NV, trigeminal nerve nucleus (a, anterior; b, posterior); NVII, facial nerve nucleus; NX, vagal nerve nucleus; pfn, pectoral fin nerve. Note that V, VII, X are the corresponding nerves and X has several branches underneath the ear as seen in the lateral view.

slit3_dr2: *forward* TCCTTCAGGAACAATCAGGA, *reverse* CCTGTCATGGGGTCAGATGT.

Slit2_hs1: *forward* TTCACTGATACAGCTTTTAGGC, *reverse* AAGGAGAACATTTGAGTGACCT

After subcloning into pCR8® (Invitrogen), the fragments were recombined into a Tol2 GFP destination vector. All steps, microinjection of the purified DNA into fertilized eggs and screening for positive founder fish have been described before (Ishibashi *et al*., 2013). Lines from at least three different founders were analyzed for each tested construct.

Live Microscopic Analyses

Embryos were screened, sorted, and imaged using Leica fluorescence microscopes (M165 FC and DM IL LED Fluo). Detailed imaging was performed by confocal microscopy (Zeiss LSM 710).

In Situ Hybridization

slit2 and *slit3* sequences have high similarities and fragments were designed that exclude the similar regions. *slit3* probe was designed from cDNA as published in GenBank (NCBI accession number CU638814, clone cssl:d0764). A 2.1 kb fragment covering the end of the gene (exons 30-35) was amplified with the following primers: forward 5′-ACTGTACCAGGGACACAô TCC-3′, reverse 5′-GGTGCACACAAGCTCAGTTA-3′. *slit2* probe was designed from GenBank entry CU638813. A 259 bp sequence at the 3′ end of the gene was amplified with the following primer pair: forward 5′-TGTCAGTGTCTGCCAGGTTA-3′, reverse 5′-CTGTAGATTGCAGATGGAGGA-3′.

miR218 LNA-enhanced probe was ordered from Exiqon (www.exiqon.com).

In situ hybridization was performed after standard protocols as published previously (Thisse and Thisse, 2008; Oxtoby and Jowett, 1993). In brief, embryos for hybridization were collected and dehydrated in a series of Methanol/PBT (0.1% Tween 20) to be stored at −20 °C. On the day of hybridization, the embryos were hydrated in a reverse series, washed, permeabilized with 10 mg/ml Proteinase K for 25 min and then post fixed in 4%PFA for 20 min to be washed again and transferred into hybridization buffer. Prehybridization occurred at a temperature of 70 °C and the probes were added to a final concentration of 1 ng/μl after two hours. The hybridized probe was detected with anti DIG-AP Fab fragments (1:5000; Roche) at 4 °C overnight. The BCIP/NBT staining reaction was performed after extensive washes. For miR218 LNA probe hybridization, the protocol was slightly modified as described in (Wienholds *et al*., 2005). Most importantly in this protocol, the hybridization temperature was 22 °C below the melting temperatures of the LNA-modified probes.

Sectioning

For vibratome sectioning the embryos were embedded in in silicon embedding molds in a mixture of 0.5% gelatine/30% albumin solution (frozen aliquots) and a 10% volume of glutaraldehyde. The mixture set quickly and required immediate positioning of the embryos. Sections were cut at 30 μm.

Immunohistochemistry

Anti GFP and anti tubulin immunohistochemical staining was performed as described previously with the modification that Collagenase (2 mg/ml; Sigma, C9891) was applied 20–25 min for tissue permeabilization (Punnamoottil *et al*., 2008; Wilson *et al*., 1990). In brief, GFP was detected with a Rabbit polyclonal anti-GFP antibody (1:1000; AMSBio TP401). The secondary antibody was biotinylated and Avidin was used for signal amplification (Vectastain ABC Kit, Elite, Rabbit IgG, PK-6101). The Avidin had a horseradish peroxidase conjugate that used DAB as a substrate in the staining reaction. DAB (Sigma D5637) was kept frozen in aliquots (25 mg/500 μl dH_2O) and one aliquot was dissolved in 30 ml PBS. To start the reaction 0.3%H_2O_2 was added into the wells to a final concentration of 1 μl/ml staining solution. A dark brown staining product developed within 20–40 min.

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