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TISSUE-SPECIFIC STEM CELLS

Bone morphogenetic protein signaling regulates Id1-mediated neural stem cell quiescence in the adult zebrafish brain via a phylogenetically conserved enhancer module

Gaoqun Zhang1 | Marco Ferg1 | Luisa Lübke1 | Masanari Takamiya1 | Tanja Beil1 | Victor Gourain1 | Nicolas Diotel2 | Uwe Strähle1 | Sepand Rastegar1

1Institute of Biological and Chemical Systems-Biological Information Processing (IBCS-BIP), Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany
2Université de La Réunion, INSERM, UMR 1188, Diabète athérothrombose Thérapies Réunion Océan Indien (DéTROI), Saint-Denis de La Réunion, France

Correspondence
Uwe Strähle, PhD, and Sepand Rastegar, PhD, Institute of Biological and Chemical Systems-Biological Information Processing (IBCS-BIP), Karlsruhe Institute of Technology (KIT). Postfach 3640, 76021 Karlsruhe, Germany. Email: uwe.straehle@kit.edu (U. S.) and sepand.rastegar@kit.edu (S. R.)

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Abstract
In the telencephalon of adult zebrafish, the inhibitor of DNA binding 1 (id1) gene is expressed in radial glial cells (RGCs), behaving as neural stem cells (NSCs), during constitutive and regenerative neurogenesis. Id1 controls the balance between resting and proliferating states of RGCs by promoting quiescence. Here, we identified a phylogenetically conserved cis-regulatory module (CRM) mediating the specific expression of id1 in RGCs. Systematic deletion mapping and mutation of conserved transcription factor binding sites in stable transgenic zebrafish lines reveal that this CRM operates via conserved smad1/5 and 4 binding motifs under both homeostatic and regenerative conditions. Transcriptome analysis of injured and uninjured telencephalas as well as pharmacological inhibition experiments identify a crucial role of bone morphogenetic protein (BMP) signaling for the function of the CRM. Our data highlight that BMP signals control id1 expression and thus NSC proliferation during constitutive and induced neurogenesis.

KEYWORDS
adult neurogenesis, BMP, cis-regulatory modules, id1, neural stem cell, radial glial cell, regeneration, telencephalon, transcription, zebrafish

1 | INTRODUCTION

In contrast to the mammalian adult brain, which contains only two main neurogenic regions that are both located in the forebrain and have rather limited ability to generate new neurons or to repair an injury, the brain of adult zebrafish contains numerous proliferative regions.1-3 These are distributed throughout different subdivisions of the brain and show high reactivation and repair capability upon lesion during adulthood.4-7

The ventricular zone of the adult zebrafish telencephalon is the most extensively studied neurogenic region in this context. This region produces new neurons, which integrate into existing neural networks throughout the lifetime of the animal.1,2,6,9 It is densely populated by the cell bodies of radial glia cells (RGCs), which are the neural stem cells (NSCs) of the adult telencephalon.2,9,10 The cell bodies of the RGCs extend two processes: a short one to the ventricular surface and a long one that crosses the brain parenchyma to reach the pial...
13 days postinjury and a concomitant sustained production of new zebrafish leads to an increase in NSC proliferation from 48 hours to such as glial acidic fibrillary protein (Gfap), brain lipid binding protein (Bbp), and the calcium-binding protein β (S100β). Only a very low percentage of RGCs proliferate (type 2) and express the proliferative cell nuclear antigen (PCNA). This latter population can give rise to new neuroblasts (type 3 cells), either through asymmetric division or through direct conversion.

A stab wound injury inflicted upon the telencephalon of adult zebrafish leads to an increase in NSC proliferation from 48 hours to 13 days postinjury and a concomitant sustained production of new neurons that migrate from the ventricular layer to the injury site to replace the damaged/lost neurons. Remarkably, 3 weeks after the brain injury, the fish feed and breed normally, barely exhibiting histological traces of the traumatic damage. To quickly and efficiently replace dying/dead neurons, the number of RGCs entering the cell cycle and starting proliferation is greatly increased during regenerative neurogenesis. Inflammatory signaling molecules cause expression of Gata3, a zinc finger transcription factor necessary for proliferation of RGCs, neurogenesis and migration of newborn neuroblasts. In order to maintain a continuous supply of new neurons and to simultaneously prevent the exhaustion of the adult NSC pool, a tight control between quiescence, proliferation, differentiation, and self-renewal of the RGCs is crucial. In a screen for transcriptional regulators expressed in the telencephalon of adult zebrafish, we previously identified the helix-loop-helix factor id1 (inhibitor of DNA binding 1) as a key player of both homeostatic and regenerative neurogenesis. Id1 is mostly expressed in quiescent RGCs, and its expression is upregulated in the ventricular zone upon telencephalic injury. Forced expression of id1 causes quiescence of NSCs, while id1 knockdown increases the number of proliferating RGCs. Id1 reduces cycling NSCs both during constitutive neurogenesis and after the initial wave of induction of proliferation in reactive neurogenesis. Our data argue for a role of id1 in maintaining the balance between dividing and resting NSCs by promoting RGC quiescence. We speculated that this might prevent depletion of the NSC pool.

Neither Notch signaling nor inflammatory signals appear to be involved in regulating id1 expression in the adult telencephalon. These data raised the central question of how and by which specific signals id1 expression is restricted to adult neural stem cells and is upregulated after brain injury. To address these questions, we decided to investigate the mechanisms of transcriptional regulation of this gene during constitutive and regenerative neurogenesis.

We identified a phylogenetically conserved id1 cis-regulatory module (CRM) that drives GFP expression in RGCs of the adult brain of transgenic zebrafish. This RGC-specific CRM harbors transcription factor (TF) binding sites conserved between human, mouse, and zebrafish id1 homologues. Deletion mapping, mutations of the binding sites, as well as pharmacological inhibition and transcriptome analysis suggest a role for the BMP pathway in controlling id1 expression in RGCs both during constitutive and regenerative neurogenesis.

### Significance statement

In the adult brain, to maintain a continuous supply of new neurons and to avoid the exhaustion of the neural stem cells (NSCs) pool, a tight control between quiescence and proliferation is crucial. The inhibitor of DNA binding 1 (id1) gene controls the balance between dividing and resting neural stem cells by promoting quiescence. A regulatory sequence of id1 was identified, which mediates the input from the bone morphogenetic protein signaling into the adult NSCs. This regulatory sequence has a high potential to serve as an interface, which will permit to alter the balance between proliferation and maintenance of stem cells in experimental, as well as medical, applications.

### 2. MATERIALS AND METHODS

#### 2.1 Zebrafish strains and maintenance

All experiments were performed on 6- to 12-month-old AB wild-type (wt) fish or on the transgenic reporter line described in this article. Zebrafish housing and husbandry were performed as recommended by Reference 26. Animal experiments were carried out in accordance with the German animal protection standards and were approved by the Government of Baden-Württemberg, Regierungspräsidium Karlsruhe, Germany (Aktenzeichen 35-9185.81/G-272/12 and 35-9185.81/G-288/18 "Adulte Neurogenese").

#### 2.2 Identification and cloning of putative CRMs

Identification of CRMs driving id1 expression in zebrafish was based on their conservation in comparison to other fish species. We utilized Ancora (http://ancora.gene-reg.net) to select sequences for functional analysis. Ancora represents a database of highly conserved noncoding elements (HCNEs) that are identified by scanning pairwise BLASTZ net whole-genome alignments with different similarity parameters. Criteria to be selected for functional testing were 80% sequence identity in a 50 bp window in all of the species Oryzias latipes, Gasterosteus aculeatus, and Tetraodon nigroviridis within a 100 kb window around the id1 locus. Genomic coordinates in putative CRMs chosen to test for regulatory potential were polymerase chain reaction (PCR)-amplified from genomic DNA. Amplicons were subcloned into pCRB/GW/TOPO (Invitrogen) to create entry vectors for subsequent cloning into the Tol2-GFP-destination vector pT2KHGFpzGATA2C1 as described by. These constructs were used to generate stable zebrafish transgenic lines. The sequences of all CRMs are provided in Appendix S1.
2.3 | Mutation and deletion of different binding sites in the id1 core sequence

Individual TF binding sites were mutated by converting the core sequence of the binding site as defined by MatInspector to a stretch of thymidines. The approach was PCR-based by employing primers designed to include the desired change.

Deletions were created using a similar methodology, a PCR-based approach using tailed primers designed to overlap with and anneal to the opposite strand of the adjoining region. The sequences of all primers used in this study can be found in Appendix S2.

2.4 | Injection of plasmids

For the generation of transgenic fish via a Tol2-based approach,29 50 ng/μL plasmid DNA was injected. The injection solution was prepared by adding 1% of phenol red and 30 ng/μL Tol2 mRNA. After injection the embryos were incubated at 28°C until they reached the desired stage. Embryos expressing GFP at 24 hpf were out-crossed with wt fish to obtain stable progenies that express the transgene in the F1 generation. Each reporter construct was tested in at least three independent transgenic lines.

2.5 | Stab wound and chemical treatment of adult zebrafish

The stab wound injury was always inflicted in the left telencephalic hemisphere while the contralateral right hemisphere was kept intact and served as a control. Three to seven animals per transgenic line were tested for GFP induction upon telencephalon injury.

The stab wound procedure was performed as described.21 For the treatment, 9-month-old Tg(id1-CRM2::GFP) fish were bathed in 300 mL fresh fish water containing 600 μL of a 10 mM DMH1 (Tocris, Wiesbaden-Nordenstadt, Germany) stock solution (final concentration 20 μM) for 7 days. As a control, 600 μL of DMSO were added to 300 mL of fresh fish water. Every morning the fish were fed with regular adult fish food and the DMH1 or DMSO solution was changed every 2 days. Stab wounds were inflicted as described on the second day of treatment and the fish sacrificed for analysis 5 days after the injury. All experiments were carried out independently at least three times.

2.6 | Preparation of adult zebrafish brains, in situ hybridization, immunohistochemistry, imaging, and quantification

Brain dissection, in situ hybridization, and immunohistochemistry were performed as described in References 1 and 21. Primary antibodies used in this study include chicken anti-GFP (1:1000, Aves labs, Davis, California), mouse anti-PCNA (1:500, Dako, Agilent, Santa Clara, California) and rabbit anti-S100 (1:400, Dako). Secondary antibodies were conjugated with Alexa fluor dyes (Alexa series) and include anti-GFP Alexa 488, antimonuse Alexa 546 and anti-rabbit Alexa 633 (1:1000, Invitrogen, Waltham, Massachusetts).

Pictures of in situ hybridized sections were acquired with a Leica compound microscope (DM4000B). For imaging and quantification immunohistochemistry brain slices mounted in Aqua-Poly/Mount (Cat No. 18606-20, Polysciences, Inc) with #1.5 thickness coverslips were imaged with a laser scanning confocal microscope Leica TCS SP5. To obtain single-cell resolution images, an HCX PL APO CS x63/1.2NA objective was used with the pinhole size set to 1-airy unit. Fluorescent images for GFP, PCNA, and S100β were acquired sequentially in 16-bit color depth with excitation/emission wavelength combinations of 488 nm/492 to 550 nm, 561 nm/565 to 605 nm, and 633 nm/650 to 740 nm, respectively. Pixel resolution for XY and Z planes are 0.24 and 0.50 μm, respectively. For individual brain samples, at least three transverse sections cut with a vibratome (VT1000S, Leica) at different anterior-posterior levels representing anterior, posterior an intermediate telencephalic regions were imaged.

In order to quantify the changes in bambia, id3, and smad5 mRNAs following brain injury at 5 days postlesion, up to 11 telencephalic pictures at the injury site were taken for each of the genes. Then, using ImageJ, the pictures were processed setting up a threshold for the staining intensity and quantification of the upregulated area was performed along the control and stab-wounded ventricular zone from the dorsomedial to the dorsolateral part of the telencephalon. The fold induction between the injured vs control ventricular zone was subsequently calculated.

2.7 | Image analysis

Confocal brain images were opened with Fiji/ImageJ software30 as composite hyperstacks to manually evaluate colocalization of GFP, PCNA, and S100β proteins. Colocalization of fluorescent signals was assessed by at least two experimenters. For quantifications, three sections per brain were analyzed. Cells were counted in the dorsomedial and the dorsolateral ventricular zone.

2.8 | Statistical analysis

For quantifications of id1-CRM2::GFP and derivative constructs, the number of cells was determined by counting the cells in Z-stacks of 50 μm thickness in 1 μm steps (×40 objective lens). Comparisons between two data sets and between more than two data sets were performed using Welch two sample t-test and one-way ANOVA followed by Tukey’s multiple pairwise-comparison test, respectively. Statistical significance was assessed by using R. Cells were always counted at the dorsomedial and dorsolateral regions of the adult zebrafish telencephalon ventricular zone.
FIGURE 1  Legend on next page.
2.9 | Quantitative real-time PCR

Total RNA was isolated from adult telencephala using Trizol (Life Technology). First-strand cDNA was synthesized from 1 μg of total RNA with the Maxima First-strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer’s protocol. A StepOnePlus Real-Time qRT-PCR system (Applied Biosystems) and SYBR Green I fluorescent dye (Promega) were used. Expression levels were normalized using β-actin (Figure 7). The relative levels of mRNA were calculated using the 2^(-ΔΔCT) method. The primer sequences are listed in Table S2.

2.10 | RNA sequencing and library preparation

For RNA sequencing, total RNA was isolated from three injured hemispheres (5 days postinjury) and three uninjured contralateral telencephalic hemispheres each to create injured and uninjured RNAseq libraries, respectively. Our data were generated from three biological repeats (as described in Reference 25). Two milligrams of total RNA was used to prepare each of the six mRNAseq libraries. RNA sequencing data were retrieved from a previous data set.25 The data analysis was carried out as described21 using the latest version of the zebrafish reference genome, assembly GRCz11 (https://www.ncbi.nlm.nih.gov/assembly/GCA_000002035.4/).

3 | RESULTS

3.1 | Identification of a CRM mediating expression of id1 in adult NSCs

To elucidate the mechanisms underlying id1 expression in adult NSCs and its injury-induced upregulation, we performed a systematic search for CRMs controlling expression of id1 in the ventricular zone of the adult telencephalon. Via phylogenetic sequence comparison of the id1 locus, we identified five conserved putative cis-regulatory modules (CRM1-5) upstream and downstream of the id1 coding sequence (Figure 1A). The identified conserved noncoding sequences were inserted in front of a gata2 minimal promoter,32 coupled to a GFP reporter cassette28 and introduced into the germ line of zebrafish to generate stable transgenic lines. Tg(id1-CRMX: GFP), where X represents one of the five CRMs (Figure 1B-F, H-L and H-L’). All five CRMs mediated expression in 24-hour postfertilization (hpf) zebrafish embryos in somewhat overlapping but distinct and specific patterns (Figure 1B-F). In the adult telencephalon (Figure 1H-L and H-L’), only id1-CRM2 mediated specific GFP expression in the ventricular zone (white arrows, Figure 1K and Movie S1) in a pattern identical to the previously described GFP-tagged id1 BAC (bacterial artificial chromosome) transgenic line, TgBAC(id1:GFP)25 (Figure 1M and M’), while CRM5 (Figure 1H), CRM4 (Figure 1I), CRM3 (Figure 1J), and CRM1 (Figure 1L) drove ectopic GFP expression presumably at the tela choridea and blood vessels (white rectangle, Figure 1H, magnification in 1H’), in neurons (white rectangle, Figure 1I, L and respective magnification in Figure 1I’ and 1L’), and in blood vessels and presumptive oligodendrocytes/neurons (white rectangle, Figure 1J and magnification in 1J’). The ventricular GFP-positive cells resembled RGCs in morphology and coexpressed the RGC marker S100β (96.9% ± 2.3%), (Figure 1K, K’, N, Q, and R) which were shown to be the NSCs in the adult zebrafish telencephalon.8,10 GFP was predominantly expressed in S100β+/PCNA− type 1 cells (82.5 ± 8.4%, n = 3 telencephala; Figure 1N-Q and R-U), while only a small number of GFP+/S100β+ cells represented type 2 cells coexpressing PCNA (17.5 ± 8.4%, n = 3), a marker for cell proliferation. GFP expression was also excluded from the rostral migratory stream (RMS), a highly proliferative domain in the telencephalon composed of type 3 progenitors (Figure 1K, white arrowhead). These data show that GFP+ cells correspond in majority to quiescent type 1 RGCs. Furthermore, both GFP expression and intensity were increased in the left telencephalic hemisphere upon stab injury compared to right uninjured control hemispheres (Figure 1R, U, V-W, and Y-Z). After stab wounding the

![FIGURE 1](https://example.com/figure1.png)
proportion of GFP/5100β−/PCNA− type 1 and GFP/5100β−/PCNA+ type 2 stem cells was not altered in the injured left hemisphere relative to the right uninjured side, which is similar to the endogenous id1 gene and the id1 BAC transgenic line.25 Additionally, GFP was predominately found in quiescent cells (Figure 1W,X). Thus, the id1-CRM2 drives expression of GFP in the RGCs in a pattern identical to the endogenous id1 gene, and this expression is inducible by injury.

3.2 Fine-mapping of the regulatory sequences mediating expression in RGCs

In order to map the core regulatory region of the id1-CRM2 responsible for its specific activity in the RGCs of the telencephalic ventricular zone, a series of 5’ and 3’ overlapping deletion variants of the reporter construct were generated and analyzed in stable transgenic lines (Figure 2A and data not shown). The transcriptional activities of these mutant versions were first investigated by monitoring GFP expression in 24 hpf zebrafish embryos. We observed at least three independent transgenic lines per construct and only constructs driving strong GFP expression resembling id1 embryonic expression were selected for further analysis in the adult brain (Figure 2A and data not shown).

The deletion construct designed as id1-CRM2-core which contains a 157 bp long stretch of the CRM2 sequence (Figure 2A, chr11:18,706,838-18,706,994) drove expression in the ventricular zone of the adult telencephalon (Figure 2B). Double labeling experiments of the transgenic line Tg(id1-CRM2-core:GFP) revealed that this shorter version of the CRM2 drives mainly expression in PCNA+, S100β+ RGCs (Figure 2B-E and J), as observed for the endogenous id1.25 id1-BAC:GFP and id1-CRM2 (Figure 1R,U,V). Moreover, this short sequence responded to injury by increased expression and intensity of GFP (Figure 2F-I,L,M; n = 3 telencephala). Thus, our deletion approach led to the identification of a 157 bp sequence that appears to harbor all relevant sequences to drive expression in NSCs and to respond to injury. Remarkably, this sequence also proved sufficient to drive GFP expression in the brain, eye, somites, midline and urogenital opening in 24 hpf embryos (Figure S1) indicating that both embryonic and adult regulatory signals act through this CRM.

3.3 Id1-CRM2-core is structurally and functionally conserved between fish and human

Sequence analysis of zebrafish id1-CRM2-core with the MatInspector software23 showed that this regulatory module harbors TF binding sites for Forkhead box protein A2 (FoxA2), cyclic AMP response element binding protein (CREB), Homeobox domain transcription factor (Pknox), and Early growth response gene 1 (Egr1), as well as two smad binding motifs (SBMs) (Figure 3A). The entire core sequence displays a high degree of conservation between zebrafish, mouse, and human homologues. We thus tested whether the sequence is also functionally conserved by constructing a transgene harboring the human version of the zebrafish CRM2-core referred to as Hsid1-CRM2-core. After stably introducing this transgene into the germ line of zebrafish, we analyzed expression in the adult telencephalon (Figure 3B-E). The human sequence also mediated expression in the ventricular zone, in type 1 progenitors corresponding to quiescent RGCs (PCNA−, S100β+), similar to the zebrafish sequence (Figures 3J and 2J, respectively). Moreover, when a brain injury was inflicted in one hemisphere of the telencephalon the Hsid1-CRM2-core carrying transgene also responded to the stab wound by increased expression at 5 days postlesion (dpi) (Figure 3F-I,L,M; n = 3 telencephala). Taken together, these results show that the mechanism of id1 regulation appears to be conserved from fish to human, suggesting that the mechanisms underlying the control of neurogenesis are very similar despite the remarkably different abilities to repair lesions in the adult brain among these phylogenetically distant vertebrate species.

3.4 BMP response elements are required for id1-CRM2 activity in the zebrafish adult telencephalon

The central region (chr11:18,706,875-18,706,953) of the id1-CRM2-core, situated between the foxA2 and egr1 binding sites and containing two SBMs (Figures 3A and 4A), is similar to a previously identified BMP response element (BRE) of the mouse and human id1 gene34,35,36. Because id1 is a direct target of the BMP signaling pathway37 and smads transduce the BMP signal from the cytoplasm to the nucleus38 (Figure 6A), we tested whether this BRE is necessary for the function of id1-CRM2 in the zebrafish adult telencephalon. To this aim we generated two id1-CRM2 mutant variants, in which either the conserved 74 bp sequence covering the BRE was deleted (id1-CRM2Δ74) (Figure 4B) or both SBM1 and SBM2 sequences were mutated (id1-CRM2-mut-SBMs) (Figure 4C). GFP expression in the ventricular zone and in RGCs was abolished in transgenic lines carrying the mutant constructs (Figure 4D-H (id1-CRM2Δ74; no GFP, expression in S100β+ cells n = 5)) and (Figure 4I-M [id1-CRM2-mut-SBMs, no GFP expression in S100β− n = 6]) and no induction of GFP expression upon injury could be detected for both constructs (Figure S3). These results show that the BRE located in the id1-CRM2 is critical for the expression of id1 in RGCs. Moreover, they suggest that BMP signaling may play a role in controlling id1 expression in the telencephalon of the adult zebrafish.

We next tested whether the well-characterized mouse id1 BRE34,35 that contains multiple SBMs would control expression in the telencephalon. A construct containing two tandem copies of the mouse BRE was shown to mediate GFP expression in all BMP signaling target tissues of zebrafish embryos.39,40 Surprisingly, however, it did not show any activity in the RGCs of the ventricular zone of the adult telencephalon (Figure 4O), and was unaffected by brain injury (Figure 4P,Q). In the transgenic line Tg(BRE:GFP), GFP expression is
Deletion mapping of id1-CRM2 identified a 157 bp core region, which confers RGC-specific expression in the adult telencephalon. A, 5' and 3' deletions of id1-CRM2 analyzed for expression in zebrafish embryos and adult brains. Results are summarized on the right; + indicates specific GFP expression; ± and − represent weak and absence of expression, respectively. B-I, Immunohistochemistry of telencephalic transverse sections with antibodies against GFP (B, F), S100β (C, G), and PCNA (D, H) (merged panels: E, I). Section levels and areas of magnification are indicated in the upper right-hand corner of each image. B-E, White arrows show two RGCs. E, The upper cell is GFP+/S100β+/PCNA− (type 1 RGC), whereas the lower cell is GFP+/S100+/PCNA+ (type 2 RGC). F-I, Upon stab wound injury, the reporter construct expression is upregulated. The left injured side is labeled with a white asterisk. B-I, Section levels and areas of magnification are indicated in the upper right-hand corner of each image. J, Quantification of PCNA and S100β expression in id1-CRM2:GFP-positive cells. K, Relative population size of type 1 and type 2 RGCs in the control and lesioned hemisphere. L, M, Quantification of GFP-positive cells and GFP intensity upon injury. Graphs showing the number of GFP-expressing cells (L) and the intensity ratio between left uninjured (control) and right injured hemispheres respectively (M). Bars: mean ± SD. Significance is indicated by asterisks: *P < .05; **P < .01; ***P < .001. Boxed image in lower left-hand corner of (F-I) represents entire brain sections. n = 3 animals (B-L), n = 15 sections (M). Scale bar = 20 μm (B-I); 200 μm for Boxed image in lower left-hand corner of (F-I).
FIGURE 3  Conservation of the zebrafish id1-CRM2 core sequences and its function across evolution. A, Sequence comparison of zebrafish id1-CRM2-core (Danio) with human (Homo) and mouse (Mus) orthologous sequences. Conserved nucleotides are indicated with an asterisk. Conserved motifs are outlined by yellow boxes comprising putative DNA recognition sequences for the transcription factors FoxA2, Smad (SBM1 and 2), CRE binding protein (CREB), Pknox, and EGR1. Nucleotide sequences in green, red, or blue correspond to previously identified sequences in the mouse id1 orthologue: Smad binding element (SBE), a Smad 1/5 binding site and a binding site for an unknown binding protein, respectively.

B-I, Immunohistochemistry of telencephalic transverse sections with antibodies against GFP (B, F), S100β (C, G), and PCNA (D, H) (merged panels: E, I). B, Expression of GFP in RGCs at the telencephalic ventricular zone driven by the human id1 regulatory sequences in the zebrafish adult telencephalon. F, Expression of the human Tg(Hsid1-CRM2) driven reporter construct is upregulated upon stab wound injury. The injured telencephalic hemisphere is labeled with a white asterisk. J, Quantification of PCNA and S100β expression in GFP+ cells in the Tg(Hsid1-CRM2) line. K, Relative population size of type 1 and type 2 RGCs in the control and lesioned hemispheres. The proportion of GFP+/S100β+/PCNA+ type 1 and GFP+/S100β+/PCNA+ type 2 stem cells is not altered in the injured hemisphere relative to the control hemisphere of the telencephalon. L, M, Quantification of GFP+ cells upon injury. The number of GFP-expressing cells (L) and the intensity ratio between left and right hemispheres comparing undamaged hemisphere (control) and damaged telencephalic hemisphere (M) are both increased following injury. Bars: mean ± SD. Significance is indicated by asterisks: * P ≤ 0.05; ** P < 0.01. n.s. = not significant. Boxed in image in lower left-hand corner of (F-I) represents entire brain sections. n = 3 animals (B-L), n = 15 sections (M). Scale bar = 20 μm (B-I); 200 μm for boxed image in lower left-hand corner of F-I.
restricted to blood vessels of the adult brain (Figure 4O, n = 7), in contrast to the expressions driven by id1-CRM2 (Figure 4N) and id1-CRM2-core (Figure 2B), which are RGC-specific (compare Figure 4N with Figure 4O). This blood vessel expression was observed with two independent lines of the BRE:

**FIGURE 4** The conserved BMP response element in the id1-CRM2 is crucial for correct expression of the GFP reporter in the ventricular zone and RGCs. A-C, Scheme showing mutated id1-CRM2 reporter constructs: A, id1-CRM2 wt construct with putative TF binding sites indicated in gray. B, id1-CRM2-Δ74 construct which contains a deletion of a 74 bp stretch of the most conserved sequence in id1-CRM2. C, id1-CRM2-mut-SBMs construct with mutations in the 2 SBMs (1 and 2) of id1-CRM2. D, Deletion of the 74 bp stretch in the id1-CRM2 abolished GFP expression in the ventricular zone. E-H, Enlarged micrographs of D, E-H. Immunohistochemistry with GFP (E), S100ß (F), and PCNA (G) antibodies on telencephalic cross sections of the id1-CRM2-Δ74 transgenic line shows no GFP reporter expression in S100ß+ RGCs (H, merged view). J-M, Mutations in Smad binding motifs (SBM1 and 2) abolished GFP expression in the ventricular zone. J-M, Magnification of white-boxed region in I. J-M, Immunohistochemistry with GFP (J), S100ß (K), and PCNA (L) antibodies on telencephalic cross sections of the id1-CRM2-mut-SBMs transgenic line show no colocalization between the RGC marker, S100ß, and GFP (M, merged view). N, GFP expression driven by id1-CRM2:GFP reporter construct in the ventricular zone (control). O, Q, Immunohistochemistry of telencephalic cross sections with GFP (O, Q) and PCNA (P). O, Q, The BRE does not drive GFP expression in the RGCs (Q) and is not inducible by telencephalic injury (Q). The left injured side is labeled with a white asterisk. Anteroposterior positions of transverse sections are indicated in the upper right-hand corner of each image. Scale bar = 20 μm (E, F, G, H, J, K, L, M); 200 μm (D, I, N, O, P, Q).
factors surrounding the SBMs (Figure 3). To test whether these neighboring sequences are necessary in addition to the BRE, each of these sites was individually mutated, and stable transgenic lines were generated with the resulting mutant constructs (Figure 5A-E). While mutation in the foxA2 and egr1 binding sites had no effect on the expression pattern of GFP in the RGCs of the adult brain (Figure 5B,E, P,Q), we observed a reduction in GFP expression for constructs with mutated pknox or cre binding sites (Figure 5C,D,P,Q). Notably, we still observed a strong and specific response to stab injury of the telencephalon in these two mutant lines (Figure 5F-J,P,Q and Figure 5K-O,P,Q). This finding indicates that these sites together with SBMs are necessary for RGC-specific expression. However, since mutations of the individual cre (Figure 5F-J,P,Q) and pknox (Figure 5 K-O,P,Q) sites did not affect the capacity to respond to injury, these sites appear not to be required for injury-induced expression via the BMP pathway.

3.6 | Id1-CRM2 expression is induced by the BMP pathway in response to injury

A crucial question is whether the increase in id1-CRM2 mediated transcription at the ventricular zone upon injury involves BMP signaling,
as suggested by the requirement of the BRE for CRM2 activity. To
address this question, in a first step we analyzed deep sequencing
data sets of the transcriptomes generated from 5 days postlesion (dpl)
hemispheres vs contralateral noninjured adult zebrafish telencephala.25 We discovered an increase in the transcription levels of
several key genes involved in canonical BMP signaling (Figure 6A,B)
when compared to the control, uninjured side. Among those, we iden-
tified the BMP receptor1aa (bmp1aa), the BMP receptor specific signal
transducers smad1 and smad5 as well as several direct target genes of
BMP signaling, including id1, id3, izts2a and bambia (Figure 6B).
For genes that are significantly upregulated (Figure 6B) and that display a
restricted pattern of expression in the telencephalon under homeo-
static conditions (bambia, smad5 and id3), the results were further ver-
ified by in situ hybridization (ISH) on telencephalic cross sections at
5 dpl (Figure 6C-E). A distinct upregulation of bambia, smad5, and id3
at 5 dpl in the injured left telencephalic hemisphere was detected on
the sections when compared to the right uninjured hemisphere, where
expression did not change (Figure 6C-E; n = 4 telencephala per gene).

The RNA sequencing and ISH data were confirmed by quantifying the
staining intensity of bambia, smad5 and id3 following brain injury at
5 days postlesion at the injury site in comparison to the same region
in the intact contralateral control hemisphere (Figure 6C'-E').

To functionally manipulate BMP signaling during the response to
injury, telencephala of adult fish were injured on the second day of
treatment with 20 μM of the BMP signaling pathway inhibitor DMH1
dorsomorphin homologue 142,43). The fish were analyzed at 5 dpl. As
visualized by in situ hybridization (Figure 7A,B,D,E) and by qPCR anal-
ysis (Figure 7C,F), suppression of BMP signaling by exposure to
DMH1 led to a significant loss of induction of the endogenous
id1 gene (Figure 7B) and id1-CRM2:GFP reporter gene (Figure 7E) upon
injury of the telencephalon. We also observed a strong reduction in
the basal expression of the gfp transgene and id1 endogenous gene in
the uninjured telencephalic hemispheres upon DMH1 treatment
(Figure 7C,F).

In summary, our investigation of the transcriptional regulation
mediated by id1-CRM2 suggests that BMP signaling positively regu-
lates the expression of id1 in the adult zebrafish brain during constitut-
ive and regenerative neurogenesis.

FIGURE 6 Genes involved in canonical BMP signaling are induced in response to telencephalic injury. A, Scheme of the BMP signaling
pathway. B, RNAseq analysis of injured telencephalic hemispheres in comparison to uninjured hemispheres reveals an upregulation in mRNAs
expression of BMP signal transducers (smad5), regulators (bambia) or downstream target genes (id3, id1). C–E, In situ hybridization on sections of
the adult zebrafish telencephalon 5 days postlesion. C, bambia, D, smad5, E, id3. Arrowheads indicate upregulation of gene expression in the left
telencephalic hemisphere upon stab injury. C‘-E‘, Quantification of C’bambia, D’, smad5, and E’, id3 expression upregulation 5 days postlesion.
Only upregulated areas were quantified along the control and injured ventricular zone from the dorsomedial to the dorsolateral region of the
telencephalon (scheme in the upper right-hand corner of (C’) shows the quantified area in blue). Significance is indicated by asterisks: ***P < .001.
P-values: bambia: 0.00008917 (C’), smad5: 0.003684 (D’), id3: 7.455 x 10⁻⁷ (E’). Scale bar = 200 μm (C, D, E)
DISCUSSION

Here, we identified the DNA module id1-CRM2 as a key regulator of id1 expression in the ventricular zone of the adult zebrafish telencephalon. Moreover, we show that a BRE is crucial for the activity of this CRM and that inhibition of BMP signaling reduces expression of the CRM driven reporter, both during constitutive and regenerative neurogenesis. This requirement of BMP signaling is correlated with an increase in mRNAs encoding BMP pathway components and BMP-controlled genes in the transcriptome of injured telencephala.

4.1 BMP as a regulator of id1 expression during constitutive and regenerative neurogenesis

A key question is which cues control id1 expression in RGCs during constitutive and regenerative neurogenesis. Inflammatory signals were previously implicated in the induction of regenerative neurogenesis in the zebrafish telencephalon. However, id1 expression was not affected by inflammation. Additionally, Notch signaling, previously shown to be involved in the control of neurogenesis in the zebrafish, did not affect id1 expression, either. Our systematic deletion and mutation analysis of the CRM2 module, as well as pharmacological inhibition of the BMP/Smad signaling pathway strongly suggest that BMP signals are crucial for id1 expression in the adult zebrafish telencephalon during both constitutive and regenerative neurogenesis. The observation that BMP signaling components (bmp1aa, smad1, smad5), as well as known BMP-controlled genes such as bambia, id3, and lzts2a are induced upon brain lesion in addition to the id1 gene suggests that injury increases the basal level of BMP signaling in the telencephalon.

Remarkably, injury-induced proliferation of NSCs and upregulation of BMP target genes are always restricted to the injured side of the telencephalon (eg, and this study). Given the close juxtaposition of left and right hemispheres in the medial parts of the telencephalon, this restriction of induction in gene expression and proliferation to the injured half is rather remarkable and implies a highly limited diffusion of the BMP signals toward the uninjured hemisphere. Rather than diffusing freely within the injured side, BMP signals may be sensed and relayed down to the cell bodies at the ventricular zone by the long processes of the RGCs. Bmp2, bmp4, and bmp7 mRNAs are expressed in the telencephalon (unpublished data), but the type of BMP signals involved in injury response as well as their origin are unclear. Although, we detected mRNA expression of these bmps in our transcriptome data, the change in response to injury was not significant. We did also not observe a reduction in the expression of BMP signaling inhibitors, such as smad6, smad7, noggin, and follistatin genes (data not shown). Therefore, it is tempting to speculate that the lesion triggers either release of BMPs or their maturation.
4.2 | BMPs as regulators of quiescence and proliferation of NSCs in reactive neurogenesis

In agreement with our data, previous work in mouse associated BMP signaling and id genes with maintenance of the adult NSCs of the hippocampus and lateral ventricle during constitutive neurogenesis.47-49 However, in mice the situation is more complicated as several id genes act redundantly.49 A single id gene appears to provide this function in the zebrafish.25

Here, we provide evidence that the mechanism of neural stem cell maintenance by id1 is conserved not only in constitutive adult neurogenesis, as observed in mouse, but also in the teleost-specific reactive neurogenesis, which is involved in injury repair. BMP signals appear to play a crucial role in elevating id1 expression in response to injury and in this way to eventually downregulate proliferation of RGCs, securing a pool of resting stem cells for future activation. In this context, it is important to stress that id1 expression reacts in a delayed fashion relative to the induction of proliferation.25 Another signaling system maintaining adult neural stem cell quiescence is the Notch pathway in both zebrafish and mice.45,50 It remains to be assessed whether the BMP/Id1 and Notch/Her4.1 pathways are redundant or parallel pathways with distinct functions in stem cell maintenance during constitutive and reactive neurogenesis. In mouse it was recently suggested that both Notch/Hes and Bmp/Id pathways interact to enhance quiescence of NSCs.51,52

4.3 | Id1-CRM2 is structurally and functionally highly conserved

Id1-CRM2 is a highly conserved CRM with homologous sequences in all-vertebrate species examined so far (this report.53). The 74 bp central region of id1-CRM2-core situated between the foxA2 and egr1 binding sites is very similar to a previously identified BRE of the mouse and human id1 regulatory sequence.34,35 The two zebrafish SBM1 and SBM2 located in the central region perfectly match the smad binding site consensus GGGGCC 54,55 and that of the smad binding element (SBE), AGAC 56,57. These elements are critical for BMP-induced Id1 expression in the mouse C2C12 myoblast cell line 34. Transgenes harboring tandem copies of this central BRE served as reliable reporters of canonical BMP signaling activity in mice and zebrafish embryos.39,40,58,59

However, this region was found to be necessary but not sufficient in id1-CRM2 to drive expression of id1 in RGCs of the adult zebrafish telencephalon. Moreover, the tandem copy of the BRE did not mediate expression in RGCs of the telencephalon. Conserved cre and a pknox site were found to be necessary in addition to the BRE core of id1-CRM2 for basal expression in the RGCs. The combination of smad binding sites with cre is a conserved feature of the id1-CRM2, which is shared with many other BMP target modules in the mammalian genome.60 In mouse osteoblasts, a cAMP response element was shown to enhance the response of id1 to BMP signals,43 showing that this interaction is not only restricted to NSCs in the zebrafish but is also employed during bone formation in mammals.

The pknox binding site in the id1-CRM2 partially overlaps with the BRE sequences (CGCC, CAGC) identified in mouse id1 to be necessary for strong responsiveness to BMP signaling.34 Therefore, it may be possible that these mutations impair the BRE. However, the mutations of the pknox and cre sites did not impair the BMP mediated response to injury. Our data suggest that their function is dispensable for BMP mediated induction of id1 reporter expression in response to injury, suggesting that basal and induced expression may involve different cofactors.

Taken together this regulatory region of the id1 gene appears to be structurally highly conserved between fish and mammals and serves as a regulatory interface that integrates multiple inputs. The high structural conservation is reflected by the fact that the human sequence can drive expression in RGCs of the adult zebrafish telencephalon. Remarkably, this conservation of function is not restricted to constitutive neurogenesis, but the human sequence also faithfully reproduces the response to injury. Thus, despite the vast difference in regenerative capacity, the underlying basic mechanism of reactive neurogenesis appears to be conserved between fish and mammals. This underscores the value of studies in the zebrafish as a model to develop therapies for injuries of the human brain. Clearly, id1-CRM2 is a highly versatile CRM that drives expression in the zebrafish embryo in various tissues including the notochord.53 It is thus expected to contain other elements, some of which may not reveal themselves by conserved sequence homology.53

5 | CONCLUSION

Here, we have identified an RGC CRM of id1, which mediates the input from the BMP signaling pathway into the adult NSCs during constitutive and regenerative neurogenesis in the zebrafish telencephalon. This CRM has a high potential to serve as an interface, which will permit to alter the balance between proliferation and maintenance of stem cells in experimental, as well as medical applications.

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CONFLICT OF INTEREST
The authors declared no potential conflicts of interest.
AUTHOR CONTRIBUTIONS
S.R.: designed the experiments and supervised the work, analyzed the data, and wrote the manuscript; U.S.: analyzed the data, and wrote the manuscript; G.Z., M.F.: conducted the experiments and analyzed the data; L.L., T.B.: conducted the experiments; V.G.: performed the RNA sequencing data analysis; M.T., N.D.: analyzed and quantified the data.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author.

ORCID
Sepand Rastegar https://orcid.org/0000-0003-4411-5646

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