BMP signaling regulates Id1 mediated neural stem cell quiescence in the adult zebrafish brain via a phylogenetically conserved enhancer module

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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 (SBMs) under both homeostatic and regenerative conditions. Transcriptome analysis of injured and uninjured telencephala 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.

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, 8, 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 [9, 10]. The cell bodies of RGCs extend two processes: a short one to the ventricular surface and a long one that crosses the brain parenchyma to reach the pial surface [9, 11]. Under homeostatic conditions, the majority of RGCs are quiescent (type 1) [9, 12, 13] and express typical neural stem cell markers such as glial acidic fibrillary protein (Gfap), brain lipid binding protein (Blbp) and the calcium-binding protein ß (S100ß) [9, 10, 14]. 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 direct conversion [15].

A stab wound injury inflicted upon the telencephalon of adult zebrafish leads to an increase in NSC proliferation from 48 hours to 13 days post-injury [16] and a concomitant sustained production of new neurons that migrate from the ventricular layer to the injury site to replace the damaged/lost neurons [17-20]. Remarkably, three weeks after the brain injury, the fish feed and breed normally, barely exhibiting histological traces of the traumatic damage [17-21]. 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 [15, 17, 19, 20]. Inflammatory signaling molecules cause expression of Gata3, a zinc finger transcription factor necessary for proliferation of RGCs, neurogenesis and migration of newborn neuroblasts [5, 22]. 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 [23-25]. *Id1* is mostly expressed in quiescent RGCs, and its expression is up regulated in the ventricular zone upon telencephalic injury. Forced expression of *id1* causes quiescence of neural stem cells, while *id1* knockdown increases the number of proliferating RGCs [25]. *Id1* reduces cycling NSCs both during constitutive neurogenesis and after the initial wave of induction of proliferation in reactive neurogenesis [25]. Our data argue for a role of *id1* in maintaining the balance between dividing and resting neural stem cells 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 [25]. These data raised the central question of how and by which specific signals *id1* expression is restricted to adult neural stem cells and is up regulated 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.

Materials and methods

Zebrafish strains and maintenance

All experiments were performed on 6-12 months old AB wildtype fish or on the transgenic reporter line described in this paper. Husbandry and experiments on animals were performed 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").

Identification and Cloning of putative cis-regulatory modules (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.genereg.net) to select sequences for functional analysis. We identified sequences conserved in all of the species *Oryzias latipes*, *Gasterosteus aculeatus* and *Tetraodon nigroviridis* using Ancora's default parameters within a 100 kb window around the *id1* locus. Putative CRMs chosen to test for regulatory potential were PCR-amplified from genomic DNA. Amplicons were subcloned into pCR™8/GW/TOPO® (Invitrogen) to create entry vectors for subsequent cloning into the Tol2-GFP-destination vector pT2KHGpzGATA2C1 as described by [26].

Mutation and deletion of different binding sites in the *id1* core sequence

Individual transcription factor binding sites were mutated by converting the core sequence of the binding site as defined by MatInspector to a stretch of thymidins. 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 supplementary data file table S1.

Injection of plasmids

For the generation of transgenic fish via a Tol2 based approach [27], 50 ng/µl plasmid DNA were 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 selected and the expression patterns documented. Identified F0 were out-crossed with wild-type fish to obtain stable progenies that express the transgene in the F1 generation. Each reporter construct was tested in at least 3 independent transgenic lines.

Stab wound and chemical treatment of adult zebrafish

The stab wound procedure was performed as described in [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 seven 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 two 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.

Preparation of adult zebrafish brains and *in situ* hybridization and immunohistochemistry

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

Quantitative Realtime PCR

Total RNA was isolated from adult brains 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. The relative levels of mRNA were calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences are listed in supplementary material Table S2.

RNA sequencing

RNA sequencing data were retrieved from a previous data set generated by [25]. The data analysis was carried out as described in [28] using the latest version of the zebrafish reference genome, assembly GRCz11. (https://www.ncbi.nlm.nih.gov/assembly/GCA_000002035.4/).

Results

Identification of a CRM mediating expression of *id1* in adult neural stem cells

To elucidate the mechanisms underlying *id1* expression in adult neural stem cells and its injury-induced up regulation, 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 (Fig.1A). The identified conserved non-coding sequences were inserted in front of a *gata2* minimal promoter [29] coupled to a GFP reporter cassette [26] 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 (Fig. 1B-K). All five CRMs mediated expression in 24-hour post fertilization (hpf) zebrafish embryos in somewhat overlapping but distinct and specific patterns (Fig. 1B-F). In the adult telencephalon (Fig. 1G-K), only *id1-CRM2* mediated specific GFP expression in the ventricular zone (Fig. 1J), while CRM5 (Fig. 1G), CRM4 (Fig. 1H), CRM3 (Fig. 1I) and CRM1 (Fig. 1K) drove ectopic GFP expression presumably at the tela choridea (white arrowhead, Fig. 1G), in neurons (yellow arrowheads, Fig. 1H and K) and in oligodendrocytes (yellow arrowheads, Fig. 1I). The ventricular GFP positive cells resembled RGCs in morphology and co-expressed the neural stem cell marker S100ß, but not the proliferation marker PCNA (Fig. 1L-O). GFP-positive cells were also excluded from the rostral migratory stream (RMS), a highly proliferative domain in the telencephalon composed of type 3 progenitors (Fig. 1J, white arrowhead). These data show that GFP-positive cells correspond to quiescent type 1 RGCs. Furthermore, GFP expression was up regulated upon stab injury of the telencephalon compared to non-injured control hemispheres (Fig. 1P, Q) similar to the endogenous *id1* gene and the previously described GFP tagged *id1* BAC (bacterial artificial chromosome) transgenic line, *TgBAC(id1:GFP)* [25]. 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.

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 (Fig. 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 3 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 (Fig. 2A and data not shown).

The deletion construct designed as *id1-CRM2-core* which contains a 119 bp long stretch of the *CRM2* sequence (Fig. 2A, chr11:19,127,209-19,127,328) drove expression in the ventricular zone of the adult telencephalon (Fig. 2B). Double labeling experiments of this transgenic line *Tg(id1-CRM2-core:GFP*) revealed that this shorter version of the *CRM2* drives expression in PCNA-negative, S100β-positive RGCs (Fig. 2B-E), as observed for the endogenous *id1* gene. Moreover, this short sequence responded to injury by increased expression of GFP (Fig. 2F, G). Thus, our deletion approach led to the identification of a 119 bp sequence that appears to harbor all relevant sequences to drive expression in neural stem cells 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 (supplementary Fig. 1) indicating that both embryonic and adult regulatory signals act through this *CRM*.

id1-CRM2-core is structurally and functionally conserved between fish and human

Sequence analysis of zebrafish *id1-CRM2-core* with the *MatInspector* software [30] showed that this regulatory module harbors transcription factor (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)* (Fig. 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 (Fig. 3B-E). The human sequence also mediated expression in the ventricular zone, in type 1 progenitors corresponding to quiescent RGCs (PCNA-negative, S100ß-positive), similar to the zebrafish sequence. Moreover, when a brain injury was inflictaed in one hemisphere of the telencephalon the *Hsid1-CRM2-core* carrying transgene also responded to the stab wound by increased expression at 5 days post-lesion (dpl) (Fig. 3F, G). 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.

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

The central region (chr11:19,127,219-19,127,297) of the *id1-CRM2-core*, situated between the *foxA2* and *egr1* binding sites and containing two *SBMs* (Fig. 3A and 4A), is similar to a previously identified *BMP response element* (*BRE*) of the mouse and human *id1* gene [31, 32]. Because *id1* is a direct target of BMP signaling pathway [33] and smads transduce BMP signal from the cytoplasm to the nucleus [34] (Fig. 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*) (Fig. 4B) or both *SBM1* and *SBM2* sequences were mutated (*id1-CRM2-mut-SBMs*) (Fig. 4C). GFP expression in the ventricular zone and in RGCs was abolished in transgenic lines carrying the mutant constructs (Fig. 4D-I) and no induction of GFP expression upon injury could be detected for both constructs (Fig. 4J-M and Supplementary Fig. 2). Only ectopic GFP expression in cells of neural morphology could be observed within the telencephala parenchyme (D) or periventricular zone (E, F, I). 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 BRE* [31, 32] that contains multiple *SBMs* would control expression in the telencephalon. A construct containing 2 tandem copies of the mouse *BRE* was shown to mediate GFP expression in all BMP signaling target tissues of zebrafish embryos [35, 36]. Surprisingly, however, it did not show any activity in the RGCs of the ventricular zone of the adult telencephalon (Fig. 4O), and was unaffected by brain injury (Figure. 4P,Q). In the transgenic line *Tg(BRE:GFP)*, GFP expression is restricted to blood vessels of the adult brain (Fig. 4O), in contrast to the expression driven by *id1-CRM2-core*, which is RGC-specific (Fig. 2B). This blood vessel expression was observed with two independent lines of the *BRE:GFP* described in [35] and [37]. These findings suggest that in the adult zebrafish brain, the BMP pathway alone is critical but not sufficient to drive *id1* expression in the ventricular RGCs.

BRE and additional transcription factors are necessary for full activity of *id1-CRM2* in neural stem cells

The observation that the two tandem copies of the mouse *BRE* [31] are not sufficient to drive reporter gene expression in RGCs (Fig. 4O) suggests that further sequences are required in addition to *SBMs* for activity of *id1-CRM2-core* in RGCs. Indeed, the *id1-CRM2-core* contains several other well-conserved binding sites of transcription factors surrounding the *SBMs* (Fig. 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 (Fig. 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 (Fig. 5B and E), we observed a reduction in GFP expression for constructs with mutated *pknox* or *cre* binding sites (Fig. 5C, D). Notably, we still observed a strong and specific response to stab injury of the telencephalon in these two mutant lines (Fig. 5F-J and Fig. 5K-O). This finding indicates that these sites together with *SBMs* are necessary for RGC-specific expression. However, since mutations of the individual *cre* (Fig. 5F-J) and *pknox* Fig. 5K-O) 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.

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 *BREs* for *CRM2* activity. To address this question, in a first step we analyzed our deep sequencing data set of the transcriptomes of injured and non-injured adult zebrafish telencephala [25]. We discovered an increase in the transcription levels of several key genes involved in canonical BMP signaling (Fig. 6A, B). Among those, we identified 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* (Fig. 6B). For genes which are significantly up regulated (Fig. 6B) and which display a restricted pattern of expression in the telencephalon under homeostatic conditions (*bambia*, *smad5* and *id3*), the results were further verified by *in situ* hybridization (ISH) on telencephalic cross sections at 5 days post lesion (dpl; Fig. 6 C-E). A distinct up regulation 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 (Fig. 6 C-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 1; [38, 39]). The fish were analyzed at 5 dpl. As visualized by *in situ* hybridization (Fig. 7A, B, E, F) and by quantitative polymerase chain reaction (qPCR) analysis (Fig. 7C, D, G, H), suppression of BMP signaling by exposure to DMH1 led to a significant loss of induction of the endogenous *id1* gene (Fig. 7B) and *id1-CRM2:GFP* reporter gene (Fig. 7F) 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 telencephala hemispheres upon DMH1 treatment (Fig. 7B, C, F, G).

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

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 reactive 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.

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 [40]. However, *id1* expression was not affected by inflammation [25]. Additionally, Notch signaling, previously shown to be involved in the control of neurogenesis in the zebrafish [41, 42], did not affect *id1* expression, either [25]. 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 up-regulation of BMP target genes are always restricted to the injured side of the telencephalon (e.g. [20, 25] 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 towards 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. Therefore, it is tempting to speculate that the lesion triggers either release of BMPs or their maturation.

BMPs as regulators of quiescence and proliferation of neural stem cells 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 [43-45]. However, in mice the situation is more complicated as several *id* genes act redundantly [45]. A single *id* gene appears to provide this function in the zebrafish: Other zebrafish *id* family members are not expressed in the RGCs of the telencephalon [24].

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 down-regulate 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 [41, 46]. 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 neural stem cells [47, 48].

***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, [49]). 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 [31, 32]. The two zebrafish *SBM1* and S*BM2* located in the central region perfectly match the smad binding site consensus GGCGCC ([50, 51]) and that of the *smad binding element (SBE)*, AGAC ([52, 53]). These elements are critical for BMP-induced *id1* expression in the mouse C2C12 myoblast cell line ([31]). Transgenes harboring tandem copies of this central *BRE* served as reliable reporters of canonical BMP signaling activity in mice and zebrafish embryos [35, 36, 54, 55].

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. A 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 [56]. In mouse osteoblasts, a cAMP response element was shown to enhance the response of *id1* to BMP signals [57], showing that this interaction is not only restricted to neural stem cells in the zebrafish but is also employed during bone formation in mammals.

The *pknox* binding site in *id1-CRM2* partially overlaps with the *BRE* sequences (CGCC, CAGC) identified in mouse *id1* to be necessary for strong responsiveness to BMP signaling [31]. 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 co-factors.

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 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 ([49]). It is thus expected to contain other elements, some of which may not reveal themselves by conserved sequence homology [49].

Conclusion

Here, we have identified a RGC cis-regulatory module of *id1*, which mediates the input from the BMP signaling pathway into the adult neural stem cells 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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Role of authors

SR and US: designed the experiments and supervised the work. GZ, MF, LL, ND and TB: conducted the experiments; VG: performed the RNA sequencing data analysis; SR and ND: analyzed the data; SR and US wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest

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Figure legends

Figure 1: **Homology-based search for cis-regulatory modules (CRMs) driving ventricular expression in the adult zebrafish telencephalon and mediating induced expression upon stab wound.**
(A) Schematic representation of the *id1* locus. Possible CRMs are represented by yellow rectangles. (B-K) GFP expression in stable zebrafish lines generated by injection of the *gata2:GFP* reporter construct fused to *id1-CRM1 to 5: Tg (id1-CRM5:GFP*), CRM5, panels B, G), *Tg (id1-CRM4:GFP)* (CRM4, panels C, H), *Tg (id1-CRM3:GFP)* (CRM3, panels D, I), *Tg (id1-CRM2:GFP)* (CRM2, panels E, J) and *Tg (id1-CRM1:GFP)* (CRM1, panels F, K). (B-F): GFP expression in 24 hpf transgenic embryos. (G-Q): GFP expression in transverse sections of the telencephalon of adult zebrafish. Only in the *Tg(id1-CRM2:GFP)* transgenic line (J), GFP is expressed in the ventricular zone (white arrows) and excluded from the rostral migratory stream (RMS, white arrow head). The white arrowhead in (G) indicates the tela choridea. The yellow arrowheads in (H, K) and (I) show possible neurons and oligodendrocyte cells, respectively. (L-O) Immunohistochemistry on transverse sections of the telencephalon of the *Tg*(*id1-CRM2:GFP)* line with antibodies against GFP (L, green), PCNA (M, red) and S100ß (N, blue; O, merged). The yellow arrows indicate PCNA positive cells (L-O). *Tg(id1-CRM2:GFP)* is exclusively expressed in RGCs of the ventricular zone. (P) Expression of the transgene is up regulated on the injured side of the telencephalon (white star in P and Q)). Anterior-posterior positions of sections or the area of magnification are indicated in the upper right hand corner of each image. Scale bar = 200 µm (G, H, I, J, K, P, Q); 20 µm (L, M, N, O).

Figure 2: Deletion mapping of *id1-CRM2* identified a 119 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. Table on the right: + indicates specific GFP expression in embryos or adult telencephalon. (B) Immunohistochemistry of telencephalic transverse sections with antibodies against GFP (B, F), PCNA (C, G) and S100ß (D) (merged panels: E). (F, G) Upon stab wound injury reporter construct expression is up regulated (F, G). The injured side is labeled with a white star. White arrows show the proliferating (PCNA-positive) type 2 RGCs. Note that these cells are GFP negative. Section levels and areas of magnification are indicated in the upper right hand corner of the panels. Scale bar = 20 µm (B, C, D, E); 200 µm (F, G).

Figure 3: Conservation of zebrafish *id1-CRM2* core sequences and function across evolution.
(A) Sequence comparison of *id1-CRM2-core* with human and mouse orthologous sequences. Conserved nucleotides are indicated with a star. Conserved motifs are outlined by yellow boxes comprising putative DNA recognition sequences for the transcription factors FoxA2, Smad (SBM1 and 2), CREB, Pknox and EGR1. Nucleotide sequences in green, red or blue correspond to previously identified sequences in the human *id1* orthologue: *Smad binding element (SBE)*, a Smad 1/5 binding site and a binding site for an unknown binding protein, respectively. Immunohistochemistry of zebrafish telencephalic transverse sections with antibodies against GFP (B, F), PCNA (C, G) and S100ß (D). (E) Merged panels. (B) Expression of GFP in RGCs at the telencephalic ventricular zone driven by the human *id1* regulatory sequences in the zebrafish adult telencephalon. (F, G) Expression of a human *Tg(Hsid1-CRM2)* driven reporter construct is up regulated upon stab wound injury. The injured telencephalic side is labeled with a

white star. White arrows in B, C, D and E show the proliferating (PCNA positive) type 2 RGCs. Note that these cells are GFP-negative. Anterior-posterior positions of sections or area of magnification are indicated in the upper right hand corner of each image. Scale bar = 20 μm (B, C, D, E); 200 μm (F, G).

Figure 4: The conserved BMP response element in the *id1-CRM2* is crucial

(A-C) Scheme showing mutated *id1-CRM2* reporter constructs: (A) *id1-CRM2* wt construct with putative TF binding sites boxed in grey, (B) *id1-CRM2-Δ74* construct which contains a deletion of the 74 bp stretch of the most conserved sequence in *id1-CRM2*, (C) *id1-CRM2-mut-SBMs* construct with mutation in the 2 SBMs (1 and 2) of *id1-CRM2*

(D) Deletion of the 74 bp stretch of in the *id1-CRM2* abolished GFP expression in the ventricular zone. (E to I) Mutations in both *Smad binding motifs* (*SBM1 and 2*) abolished both GFP expression in the RGCs and induction of GFP expression upon injury (J to M). (F to I) magnification of white-boxed region in E. Note in E the ectopic GFP expression in cells looking like neurons. (O to Q) The *BRE* does not drive RGC expression of GFP and is not inducible by telencephalic injury. Immunohistochemistry of telencephalic cross-sections with GFP (green), PCNA (red) and S100ß (blue). Anteroposterior positions of transverse sections are indicated in the upper right hand corner of each picture. Scale bar = 20 μm (F, G, H, I, J, K, L, M); 200 μm (D, E, N, O, P, Q).

Figure 5: Cre and pknox binding sites in close vicinity to *SBM1* and *-2* are necessary for full activity of *id1-CRM2*.

(A-E) Immunohistochemistry with GFP antibody on telencephalic cross-sections of the *id1-CRM2* transgenic line containing a mutation in *foxA2* (B), *cre* (C), *pknox* (D) and *egr1* (E). Mutation in *cre* (C) and *pknox* (D) binding sites led to a reduction of GFP expression in the ventricular zone. (F-J) Expression of the *id1-CRM2-mut-CRE* construct is inducible by brain injury (white star, injured hemisphere). (K-O) Expression of the *id1-CRM2-mut-pknox* construct is induced upon brain injury (white star, injured hemisphere). (G-J and E-H: magnification of the framed region in F and K, respectively). Anterior-posterior positions of transvers sections are indicated in the upper right corner of each picture. Scale bar = 200 μm (A, B, C, D, E, F, K); 25 μm (G, H, I, J, L, M, N, O).

Figure 6: Genes involved in canonical BMP signaling are induced in response to telencephalon injury.

(A) Schematic diagram of the BMP signaling pathway. (B) RNAseq analysis of injured telencephala in comparison to uninjured brains reveals an up-regulation of BMP signal transducers (*smad5*), regulators (*bambia*) or downstream target genes (*id3, id1*) mRNA. (C to E) *In situ* hybridization on sections of the adult zebrafish telencephalon 5 days post lesion. (C) *bambia,* (D) *smad5,* (E) *id3*. Arrowheads indicate up regulation of gene expression in left telencephalic hemisphere upon stabbed injury. Scale bar = 200 μm (C, D, E).

Figure 7: Inhibition of BMP signaling with DMH1 reduces injury induced *id1* and *id1-CRM2:GFP transgene* expression.

ISH against *id1* (A, B) and *gfp* mRNA (E, F) showed reduction in induction of *id1* (B) and *gfp* transgene upon BMP signaling inhibition by DMH1 treatment. (C, D, G, H) qPCR experiments confirmed reduction in *id1* and *gfp* expression in DMH1 treated fish. Arrowheads indicate up regulation of gene expression in left telencephalic hemisphere upon stabbed injury. The data represent the mean±s.d. of three independent experiments. **, $.01 \leq p \leq .001$. Scale bar = 200 μm (A, B, E, F). n = 3 animals for (C, D, G, H).

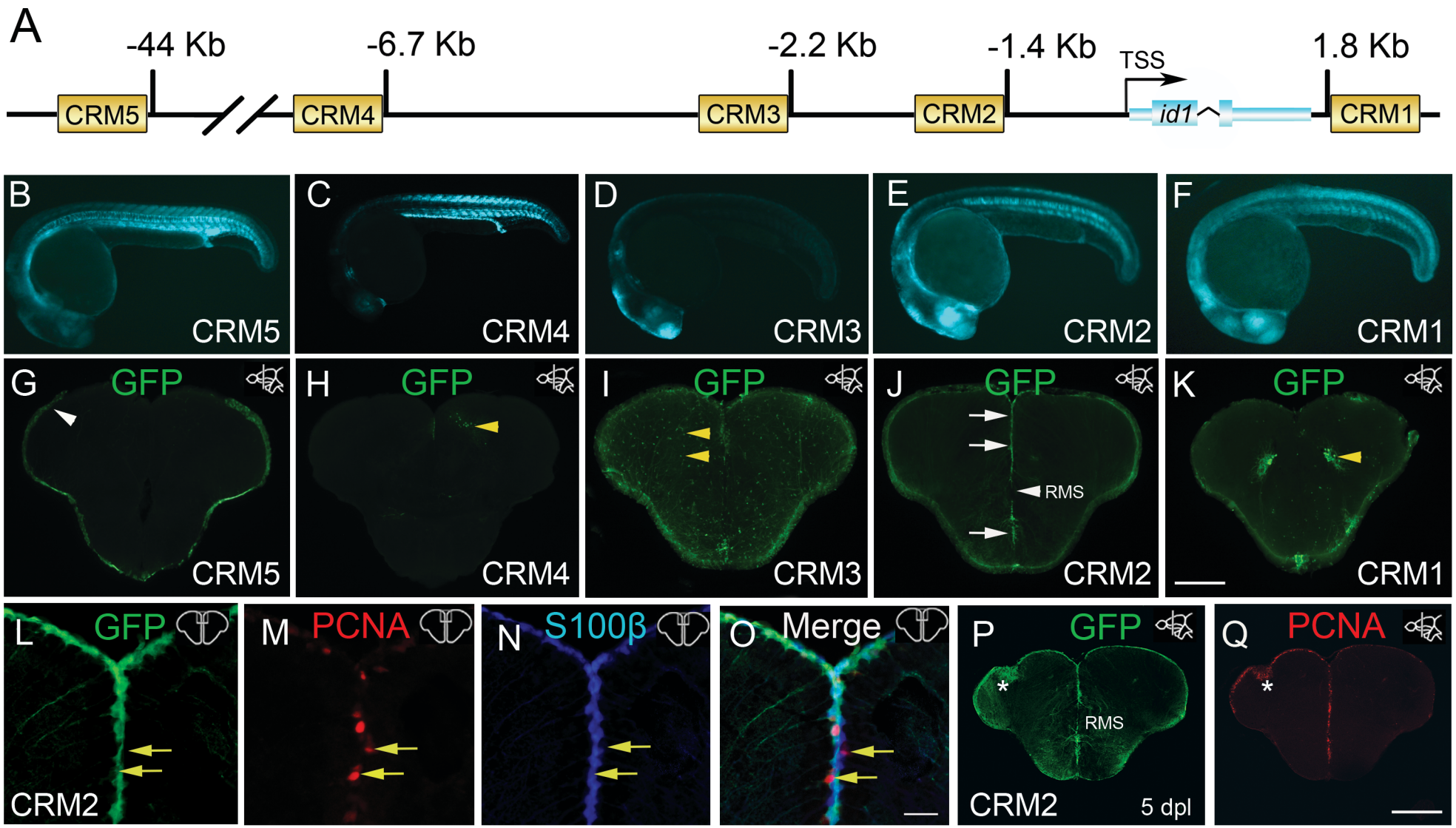

Figure 1. Zhang and Ferg et al

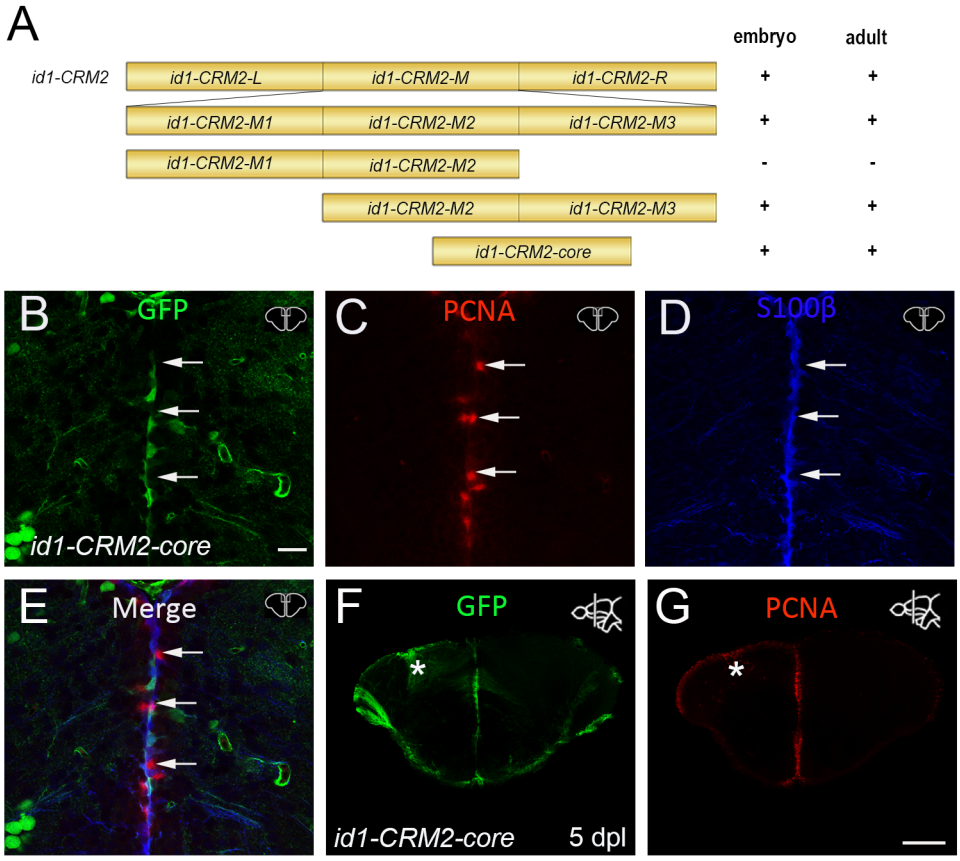

Figure 2. Zhang and Ferg et al

A

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                FoxA2                          SBM1          CRE
Danio    -GTCCAAACAAACCGAAGCAGACTGCCGGGCGCCAGCGCCGTGACGTCACCCATTCATTC
Mus      CGTCTGAACAAG-CGGCTCAGACCGTTAGACGCCAGGCCCGTGACGTCACCCATTCA-TA
Homo     CGTCTGAACCAG-CGGCTCAGACCGTTAGACGCCAGGCCTGTGACGTCACCCATTCA-TA
          ***  *** *  **   ***** *   * ******  * ***************** *

                        pknox     SBM2     EGR1
Danio    GAGCCTTGAC-GCCTGTCAGCCTGGCGCCGAGCGGGCGGTCCCTATAGCAACAACAAACAA-
Mus      AAACCCGGACGGGCTGTCAGGCTGGCGCCGCGCGGGCGGTCGCCATGGAGACGGCAAACTGG
Homo     AAACCCGGACGGACTGTCAGGCTGGCGCCGCGCGGGCGGTCGCCATGGAGACGACAAACTGG
          * **  *** * ******* ********* ********** * ** *  **  *****
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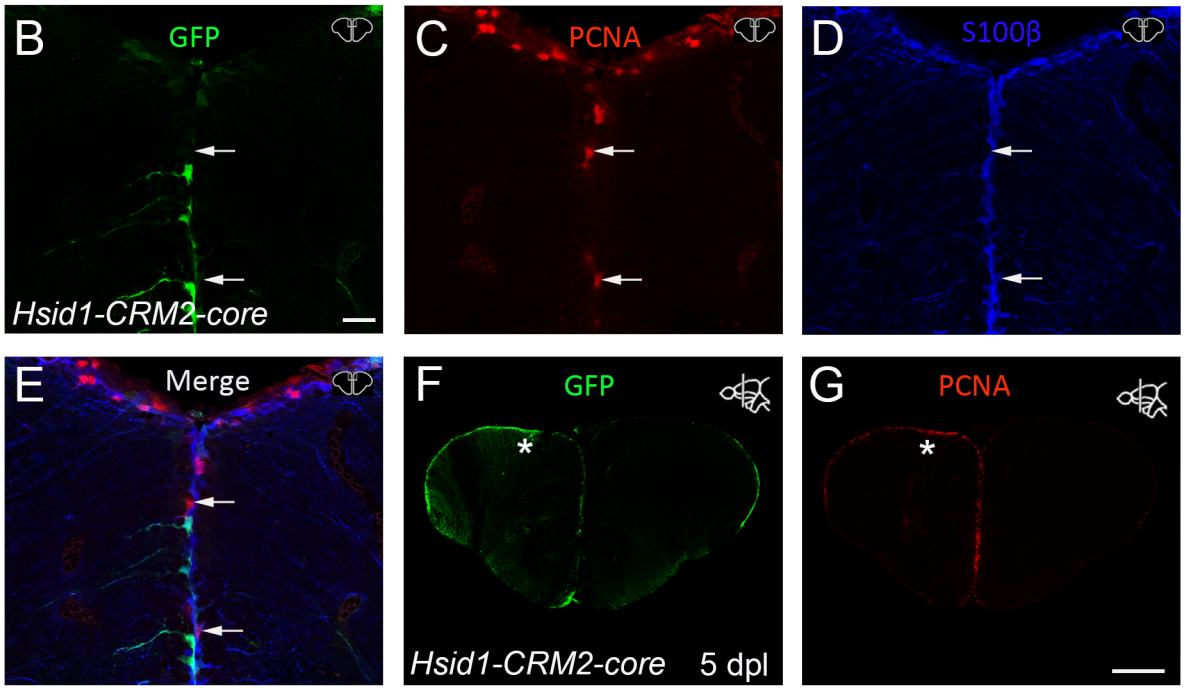


Figure 3. Zhang and Ferg et al

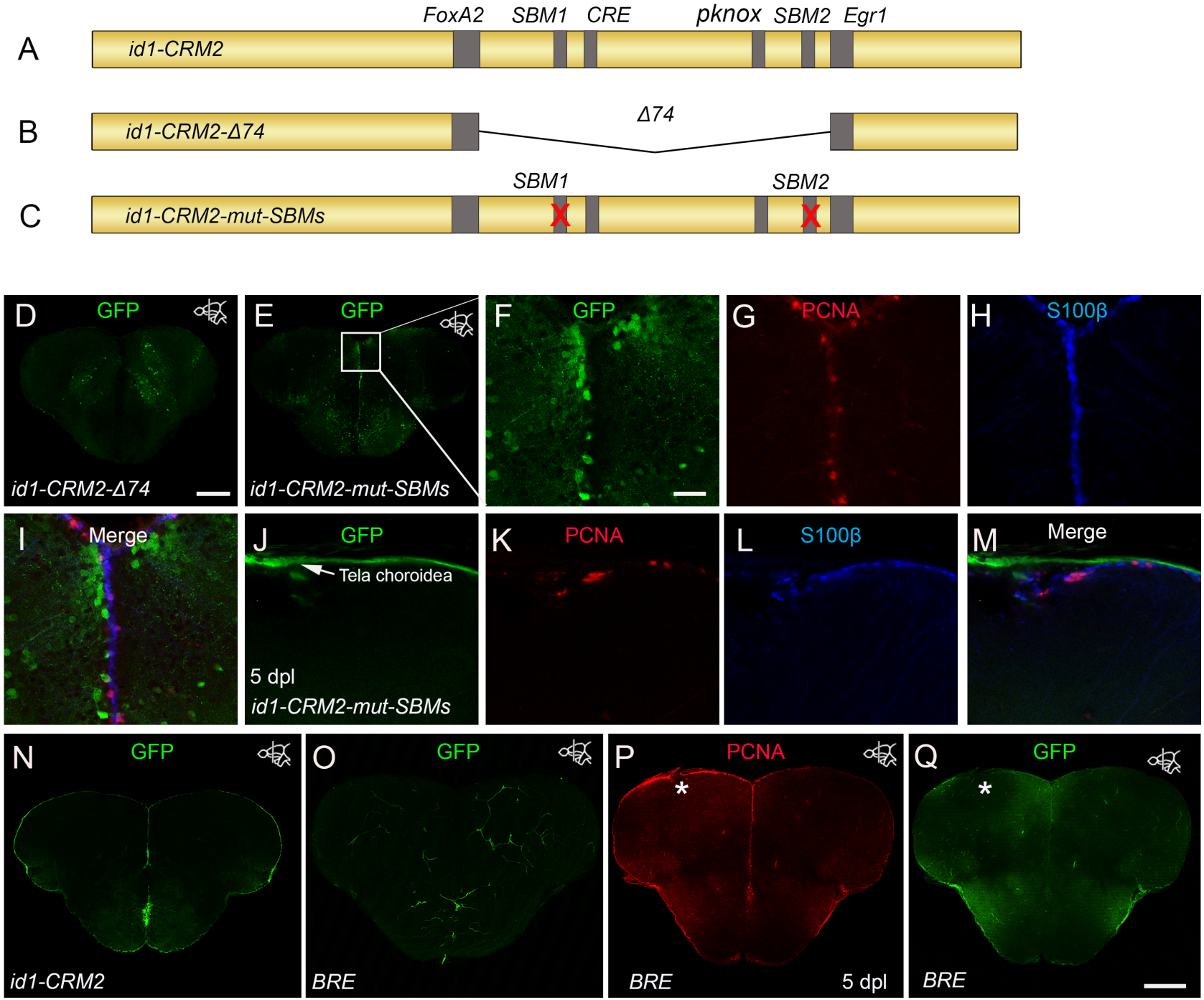


Figure 4. Zhang and Ferg et al

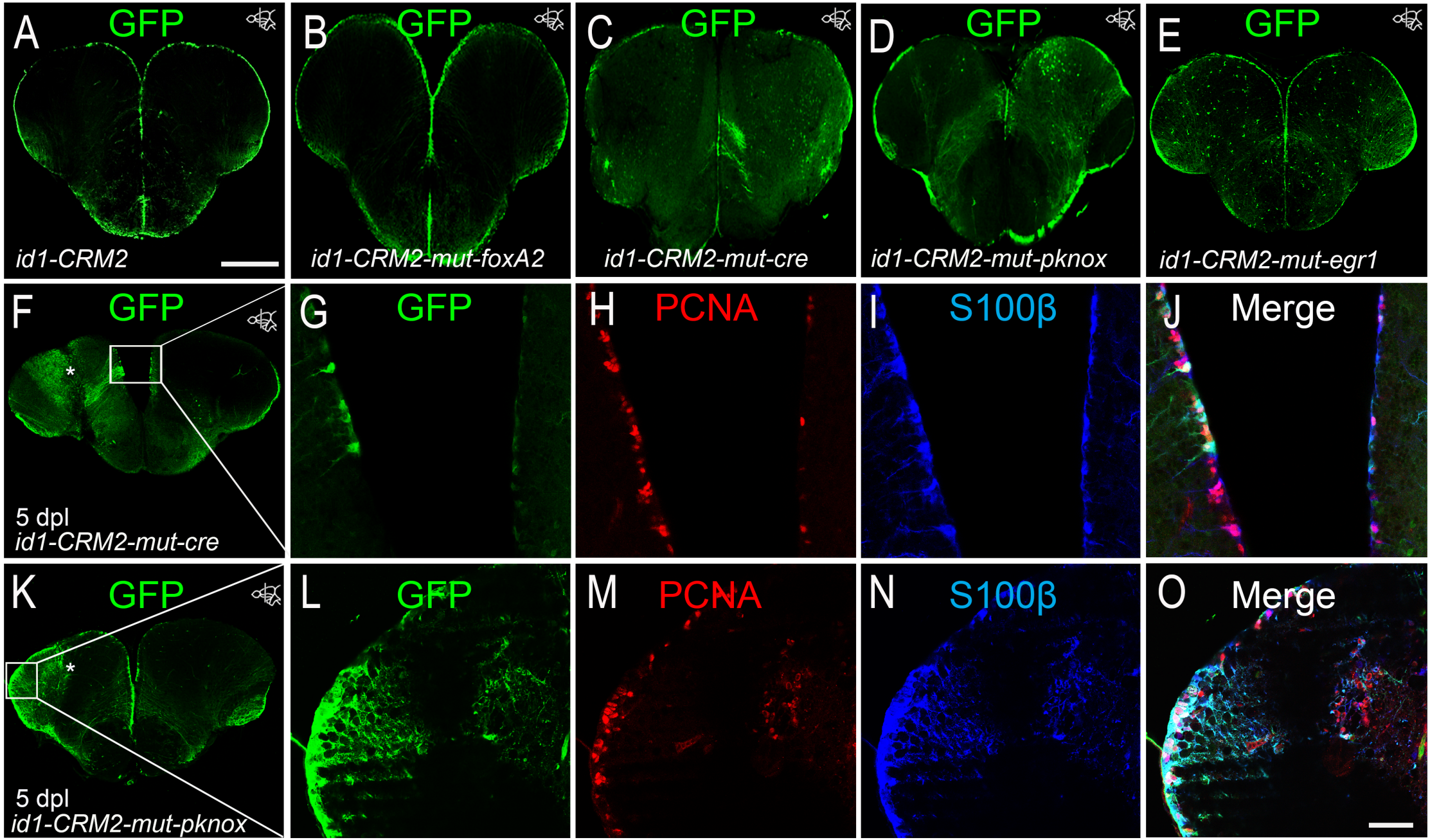
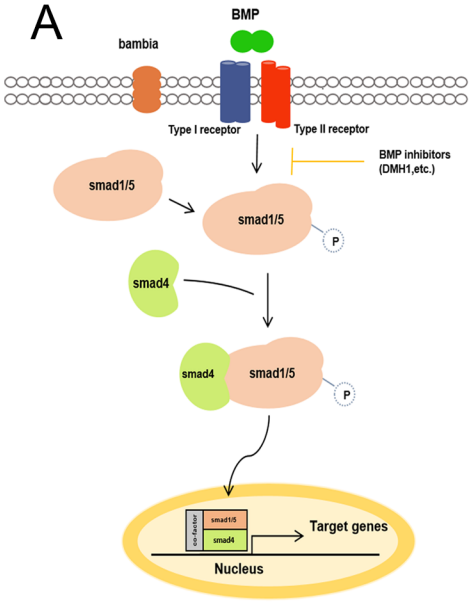


Figure 5. Zhang and Ferg et al

A



B

Name	Ensembl Gene ID	Log2 (Fold Change)	Adjusted p-Value
bambia	ENSDARG00000055381	0.568724373	0.024064413
lzts2a	ENSDARG00000074597	0.722445583	2.75523E-08
smad5	ENSDARG00000037238	0.601838324	4.89998E-03
smad1	ENSDARG00000027199	0.357495052	0.002743393
id3	ENSDARG00000054823	0.389943903	2.22946E-05
id1	ENSDARG00000040764	0.352186235	0.00309219

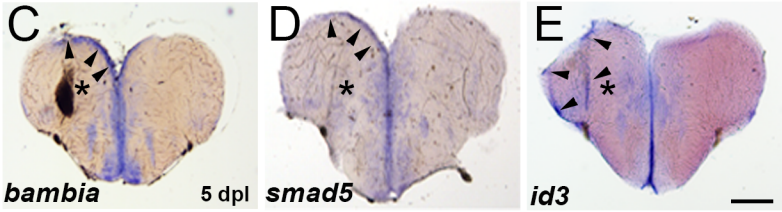
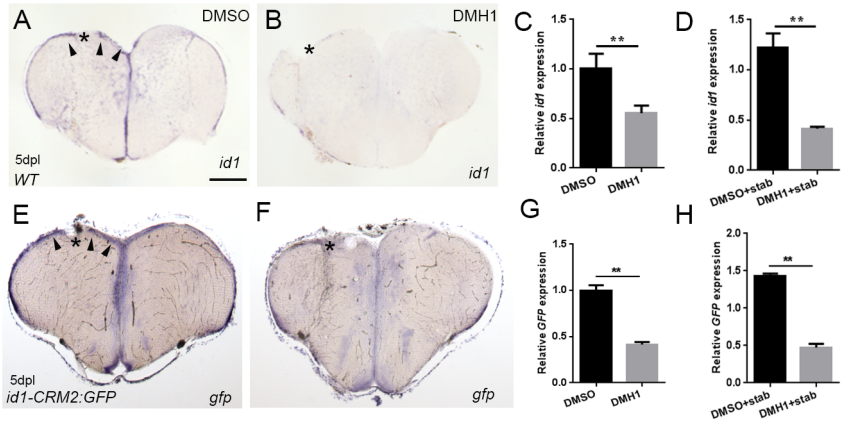


Figure 6. Zhang and Ferg et al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DMSO
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WT
id1
B
DMH1
id1
C
Relative id1 expression
1.5
1.0
0.5
0.0
**
DMSO
DMH1
D
Relative id1 expression
1.5
1.0
0.5
0.0
**
DMSO+stab
DMH1+stab
E
5dpl
id1-CRM2:GFP
gfp
F
gfp
G
Relative GFP expression
1.5
1.0
0.5
0.0
**
DMSO
DMH1
H
Relative GFP expression
2.0
1.5
1.0
0.5
0.0
**
DMSO+stab
DMH1+stab