Differential expression of *id* genes and their potential regulator *znf238* in zebrafish adult neural progenitor cells and neurons suggests distinct functions in adult neurogenesis

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A B S T R A C T

Teleost fish display a remarkable ability to generate new neurons and to repair brain lesions during adulthood. They are, therefore, a very popular model to investigate the molecular mechanisms of constitutive and induced neurogenesis in adult vertebrates. In this study, we investigated the expression patterns of inhibitor of DNA binding (*id*) genes and of their potential transcriptional repressor, *znf238*, in the whole brain of adult zebrafish. We show that while *id1* is exclusively expressed in ventricular cells in the whole brain, *id2a, id3* and *id4* genes are expressed in broader areas. Interestingly, *znf238* was also detected in these regions, its expression overlapping with *id2a, id3* and *id4* expression. Further detailed characterization of the *id*-expressing cells demonstrated that (a) *id1* is expressed in type 1 and type 2 neural progenitors as previously published, (b) *id2a* in type 1, 2 and 3 neural progenitors, (c) *id3* in type 3 neural progenitors and (d) *id4* in postmitotic neurons. Our data provide a detailed map of *id* and *znf238* expression in the brain of adult zebrafish, supplying a framework for studies of *id* genes function during adult neurogenesis and brain regeneration in the zebrafish.

1. Introduction

Adult neurogenesis is a conserved feature of the central nervous system across vertebrates. In mammals, the capacity to generate new neurons during adulthood is limited to two main regions of the telencephalon: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Braun and Jessberger, 2014; Grandel and Brand, 2013). In contrast, adult zebrafish exhibit widespread neurogenesis throughout the whole brain during their entire lifespan (Edelmann et al., 2013) and possess a high regenerative capacity for repairing lesions of the central nervous system (Diotel et al., 2013; Grandel and Brand, 2013; Lindsey and Tropepe, 2006; März et al., 2011; Zupanc, 2008; Zupanc et al., 2005). This strong neurogenic activity is enabled by the persistence of neural progenitors during adulthood (Adolf et al., 2006; Lindsey et al., 2012; März et al., 2010; Pellegrini et al., 2007; Schmidt et al., 2013). The ventricular telencephalic neural progenitors of the adult zebrafish can be classified into three distinct types regarding their morphology, division rate and marker expression (März et al., 2010). Type 1 progenitors correspond to quiescent radial glial cells that display a long radial process and express a wide variety of radial glial markers such as the calcium binding protein S100β, the steroid metabolizing enzyme aromatase B, the brain lipid binding protein (blbp) or the glial fibrillary acidic protein (gfap). Type 2 progenitors are actively dividing radial glial cells, and are consequently expressing proliferating cell nuclear antigen (PCNA) in addition to the markers found in quiescent stem cells. Finally, type 3 progenitors are fast dividing cells with absent or very low expression of radial glial cell markers, but which express the polysialylated neuronal cell adhesion molecule (PSA-NCAM). Type 3

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cells have been proposed to correspond to neuroblasts, the more committed precursors of differentiating neurons (März et al., 2010; Schmidt et al., 2013).

Although the neurogenic capacities of teleost fish are well documented, only a limited number of studies have investigated the expression and potential involvement of regulatory factors known to play key roles in mammalian neurogenesis. Among these factors, helix-loop-helix (HLH) and basic helix-loop-helix (bHLH) proteins are important regulators of transcription that play crucial roles in cell proliferation, differentiation and survival during early development and adult life (Lasorella et al., 2014; Norton, 2000; Patel et al., 2015). Their HLH regions mediate homo or heterodimerization, a step necessary for DNA binding through the basic domain. Inhibitors of DNA binding (Id) proteins belong to the HLH family and lack the basic DNA binding domain. They act as dominant negative regulators by binding to the HLH domains of bHLH factors, thereby preventing their binding to DNA (Lasorella et al., 2014; Ling et al., 2014; Norton, 2000; Perk et al., 2005). In mammals, four different Id proteins encoded by distinct genes (Id1-Id4) have been described (Norton, 2000). They are involved in multiple physiological and pathological processes, acting on cell growth, differentiation, senescence and survival (Lasorella et al., 2014; Ling et al., 2014; Sikder et al., 2003; Yokota, 2001). The different *Id* genes play key roles in embryonic and adult neurogenesis by regulating quiescence and self-renewal of neural stem cells as well as neuronal differentiation (Aloia et al., 2015; Bai et al., 2007; Havrda et al., 2008; Jen et al., 1997; Jung et al., 2010; Lyden et al., 1999; Nam and Benezra, 2009; Neuman et al., 1993; Ohtaka-Maruyama et al., 2007). Given that expression patterns of Id proteins strongly overlap in various tissues, redundancy in their function has been suggested (Langlands et al., 1997; Sun et al., 1991). Indeed, Id1 and Id3 functions in the mouse brain are partially redundant, as a single knock-out for either Id1 or Id3 has almost no effects on brain development, whereas double knock-out mice exhibit precocious neurogenesis (Bai et al., 2007).

Recently, the C_2H_2-type zinc finger protein Rp58, the product of the *Znf238* gene, has been shown to negatively regulate *Id* genes in the developing cerebral cortex of mouse. Rp58 contributes to the regulation of both neuronal and astroglial differentiation (Hirai et al., 2012). In addition, it modulates proliferation of neural progenitors, neuronal migration and neuronal differentiation during cortical development (Baubet et al., 2012; Hirai et al., 2012; Ohtaka-Maruyama et al., 2007; Xiang et al., 2012). It is currently not understood whether these effects are all mediated via Rp58 regulation of Id expression, or whether Rp58 also regulates other genes functioning in this context. Consistent with the latter hypothesis, Rp58 negatively regulates *Rnd2* (a RhoA-like GTPase), which plays an important role in the control of neural migration during cerebral corticogenesis in mouse (Heng et al., 2015).

In zebrafish, five *id* genes have been identified (*id1*, *id2a*, *id2b*, *id3* and *id4*; www.ensembl.org) and were shown to have distinct but partially overlapping expression patterns during embryogenesis (Armant et al., 2013; Chong et al., 2005; Dickmeis et al., 2002; Sawai and Campos-Ortega, 1997; Thisse et al., 2001). In the adult zebrafish telencephalon, *id1* was recently shown to control homeostatic and regenerative neurogenesis by promoting stem cell quiescence (Rodriguez Viales et al., 2015). Expression and function of the other four *id* genes in the zebrafish central nervous system during adulthood are only poorly documented, which raises the question whether they also contribute to the regulation of adult neurogenesis. Notably, some aspects of Id1 function may have been masked by potential redundancy with these other Id members. Additionally, the zebrafish genome also contains one homologue of *znf238*, the expression and function of which has not yet been studied.

Given the redundancies of *Id* genes function in other model organisms (Bai et al., 2007), we decided to investigate their expression in the whole zebrafish adult brain by *in situ* hybridization. Together with the expression of their potential negative regulator Rp58 encoded by the *znf238* gene, we thereby aimed to create a map of potential Id activity. We placed particular emphasis on the expression patterns in the different telencephalic progenitor cell types. With this, we provide a framework for studies of *id* genes function during adult neurogenesis and brain regeneration in the zebrafish.

2. Results

2.1. Id and znf238 genes expression in the brain of adult zebrafish

In mammals, *id* genes and their negative regulator *znf238* are expressed in neurogenic niches. To examine if the zebrafish homologues of these genes are expressed in similar regions and also show enriched expression in the additional neurogenic areas of the teleost brain, we first mapped expression of *id1-4* and of *znf238* by *in situ* hybridization. Representative results of the *in situ* hybridization stainings are shown in Fig. 1 along with schemes indicating the section levels and the anatomic subdivisions visible in the sections. To facilitate comparison of the expression patterns, Table 1 additionally summarizes expression levels of the examined genes in all expressing brain nuclei and regions.

As shown in Fig. 1 and Table 1, *id2b* transcripts were barely or not detected in our *in situ* hybridization experiments: we observed only a very weak ubiquitous expression in 1 out of 5 brains investigated (Fig. 1 and data not shown). Therefore, we excluded *id2b* from further analysis.

At the olfactory bulb/telencephalon junction, we observed that *id1* expression is restricted to the ventricular cells of the dorsal telencephalic area (D) (Fig. 1, A1) as previously described (Rodriguez Viales et al., 2015). Also *id2a* expression is detected along this part of the ventricular layer, and in addition in numerous parenchymal cells (Fig. 1, B1). *Id3* is barely detected along the ventricular layer (Fig. 1, D1), and *id4* staining is observed in a dorsal and medial periventricular stripe of the dorsal telencephalic area (Fig. 1, E1). On the other hand, the *znf238* gene is strongly expressed in a large periventricular stripe in the dorsal telencephalic area and its expression pattern partially overlaps with those of *id2a*, *id3* and *id4*, namely in the periventricular layer (Fig. 1, F1).

More caudally in the telencephalon, *id1* staining is detected in the ventricular cells of the ventral and dorsal nuclei of the ventral telencephalon (Vv and Vd) as well as in the medial (Dm) and lateral (Dl) zone of the dorsal telencephalon (Fig. 1, A2 and A3), as previously reported (Rodriguez Viales et al., 2015). Similarly, *id2a* staining is also observed in the ventricular cells of the Vv, Vd, Dm and Dl, but also in the Dp (posterior zone of the dorsal telencephalic area) and in the brain parenchyma (Fig. 1, B2 and B3). *id3* transcripts are strongly expressed along the ventricle of the subpallium, mainly in the Vv (Fig. 1, D2 and D3), and *id4* is barely detectable in the ventricular layer (Vv, Vd, Dm and Dl), but is strongly expressed in parenchymal cells of the Vv (Fig. 1, E2 and E3). Interestingly, at this section level, *znf238* is mainly (and strongly) expressed in a large periventricular stripe in the Dm, Dl, Dm and Dp (Fig. 1, F2 and F3). In addition, numerous parenchymal cells deep in the Dl parenchyma express *znf238*.

In the anterior part of the preoptic area (PPa), numerous *id1* and *id3* positive cells are detected along the diencephalic ventricular layer (Fig. 1, A3 and D3). This is also the case for *id2a*, but its expression is not restricted to the ventricular zone, and it is also observed in the parenchyma, in a pattern similar to that of *id4*

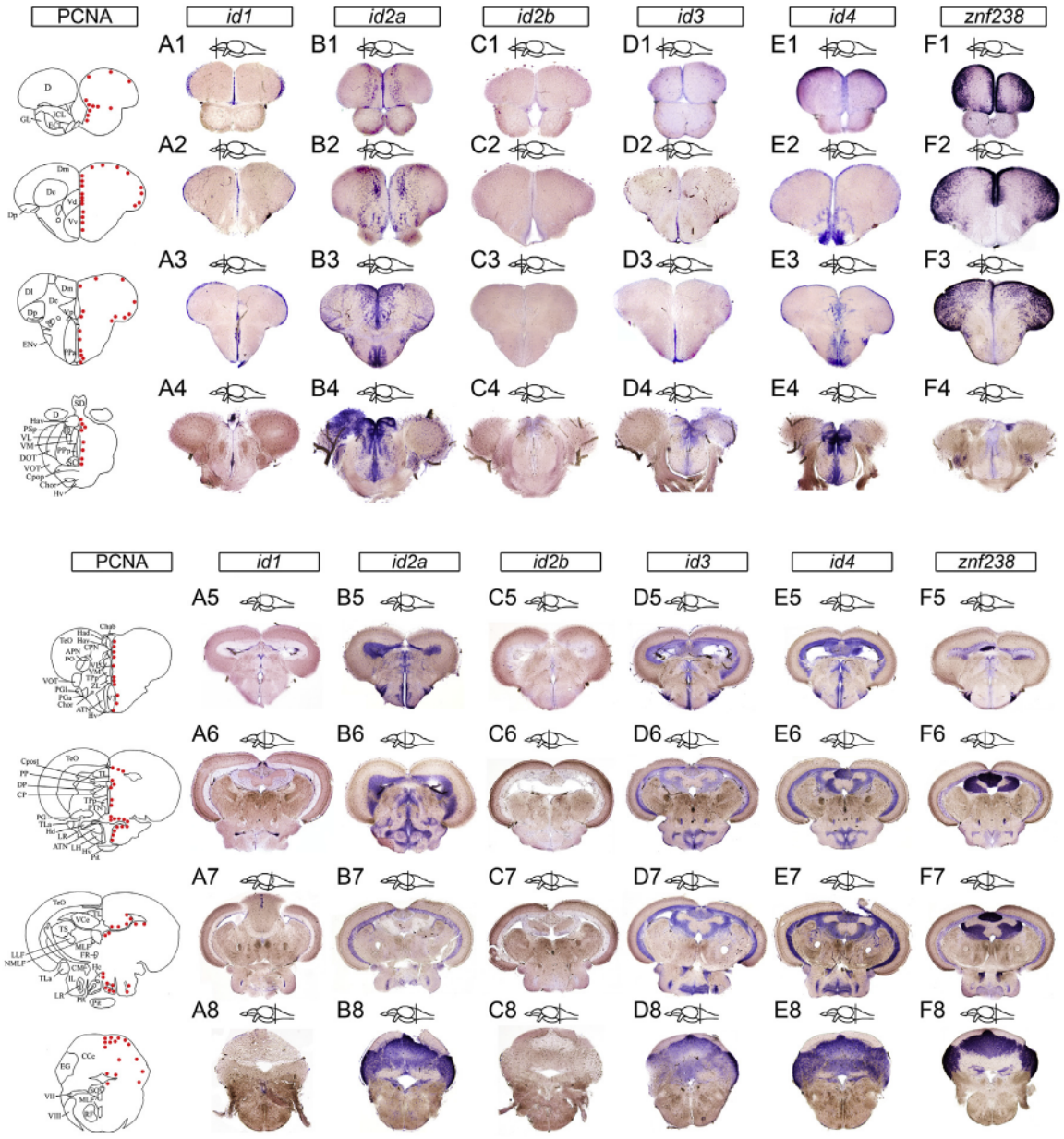


Fig. 1. *Id* genes and *znf238* expression in the brain of adult zebrafish. *Id1*, *id2a*, *id2b*, *id3*, *id4* and *znf238 in situ* hybridization on transverse sections through the olfactory bulb/telencephalon junction (A1-F1), the telencephalon (A2-F2), the anterior (A3-F3) and posterior part (A4-F4) of the preoptic area, the anterior (A5-F5), the mediobasal (A6-F6) and the caudal (A7-F7) hypothalamus and through the medulla oblongata (A8-F8). A1 to F4: *id1-id4* and *znf238* expression in the anterior part of the brain, from the olfactory bulb/telencephalon junction to the end of the preoptic area. *id1* as well as *id3* is expressed along the brain ventricle. However, *id3* is mainly detected in the subpallium. In contrast, *id2a* and *id4* are more widely expressed and also found in the brain parenchyma. *Id2b* transcripts are hardly detected. *znf238* is strongly expressed in the pallium, but the ventricular layer does not appear to be stained. A5 to F8: *id1-id4* expression in the posterior part of the brain, from the hypothalamus to the medulla oblongata. *Id1* staining is detected along the brain ventricles notably at the level of the hypothalamus and optic tectum. In contrast *id2a*, *id3* and *id4* transcripts are widely expressed in ventricular and periventricular layers of the neurogenic regions and also in the brain parenchyma. Their expression appears more or less ubiquitous. Similarly to the anterior part of the brain, *id2b* transcripts are hardly detectable. *znf238* is strongly expressed in the valvula of the cerebellum and the cerebellum, and shows weak expression in the hypothalamus. Red dots in the zebrafish section schemes correspond to proliferative areas, which are indicated based on PCNA expression. Bars: 200 μm (A1 to F4); 280 μm (A8 to F8); 300 μm (A5 to F7).

(Fig. 1, B3 and E3). Only a weak *znf238* staining is observed in the PPa (Fig. 1, F3).

In the posterior part of the preoptic area (PPp), *id1* expression is only detected along the ventricular layer (Fig. 1, A4), while *id2a, id3* and *id4* are widely expressed in the entire PPp but also in the ventromedial thalamic nucleus (VM) and the habenula (Hav) (Fig. 1, B4, D4 and E4). *Znf238* is also widely, if only weakly, expressed in the PPp, VM and Hav (Fig. 1, F4).

More caudally, *id1* expression is consistently detected along the ventricular layer of the periglomerular gray zone of the optic tectum (TeO) and of the anterior hypothalamus (Hv), while *id2a*, *id3* and *id4* appear to be widely expressed with a similar distribution in the Hv, zona limitans (ZL), the periventricular nucleus of the posterior tuberculum (TPp), the VM, the thalamus (A), the periventricular gray zone of the TeO and the anterior preglomerular nucleus (PGa) (Fig. 1, A5, B5, D5 and E5). *Znf238* displays a similar pattern, but is only weakly expressed, except in the PGa (Fig. 1, F5).

In more posterior sections, *id1* is also detected again in the ventricular layer, along the diencephalic ventricle of the mediobasal hypothalamus, notably in cells surrounding the lateral recess

Table 1
id and *znf238* expressions in brain nuclei and regions of the adult zebrafish brain 0: no expression; +/−: weak expression; +: low expression; ++: moderate expression; +++: strong expression; X: no data.

Brain regions(according to Wullimann et al., 1996)	id1	id2a	id2b	id3	id4	znf238
A, anterior thalamic nucleus	+/−	++	0	+	++	+/−
APN, accessory pretectal nucleus	0	0	0	0	0	0
ATN, anterior tuberal nucleus	+	+	0	+	+	0
CCe, corpus cerebelli	+/−	+++	0	+/−	+++	+++
Chab, habenular commissure	0	0	0	0	0	0
Chor, horizontal commissure	0	0	0	0	0	0
CM, corpus mamillare	0	+	0	++	++	+++
CP, central posterior thalamic nucleus	0	+/−	0	0	0	0
CPN, central pretectal nucleus	0	0	0	0	0	0
Cpop, postoptic commissure	0	0	0	0	0	0
Cpost, posterior commissure	0	0	0	0	0	0
D, dorsal telencephalic area	+	++	0	+/−	++	+++
Dc, central zone of dorsal telencephalic area	0	+/−	0	0	0	+
Dl, lateral zone of dorsal telencephalic area	+	0	+/−	+/−	+	+++
Dm, medial zone of dorsal telencephalic area	+	++	0	+/−	+	+++
DOT, dorsomedial optic tract	0	+/−	0	0	+/−	+/−
Dp, posterior zone of dorsal telencephalic area	++	++	0	+/−	+	+++
DP, dorsal posterior thalamic nucleus	0	+	0	0	0	0
ECL, external cellular layer of olfactory bulb	0	0	0	0	0	0
EG, eminentia granularis	0	+++	0	+	+++	+++
ENv, entopendoncular nucleus, ventral part	0	++	0	0	++	+
FR, fasciculus retroflexus	0	+/−	0	0	0	0
GL, glomerular layer of olfactory bulb	0	++	0	0	0	0
Had, dorsal habenular nucleus	X	X	X	X	X	X
Hav, ventral habenular nucleus	0	+++	+/−	++	+++	0
Hc, caudal zone of periventricular hypothalamus	+	+	0	+++	+++	+
Hd, dorsal zone of periventricular hypothalamus	++	+++	0	+++	+++	+
Hv, ventral zone of periventricular hypothalamus	+	+++	0	+++	+++	+/−
ICL, internal cellular layer of olfactory bulb	0	0	0	0	0	0
IL, inferior lobe	0	+/−	0	+/−	+/−	+/−
LH, lateral hypothalamic nucleus	0	+++	0	++	+++	+/−
LLF: lateral longitudinal fascicle	0	+/−	0	+/−	++	0
LR, lateral recess of diencephalic nucleus	X	X	X	X	X	X
MLF, medial longitudinal fascicle	0	0	0	0	0	0
NMLF, nucleus of medial longitudinal fascicle	0	0	0	0	0	0
PG, preglomerular nucleus	0	0	0	0	0	0
PGa, anterior preglomerular nucleus	0	+++	0	+++	+++	+++
PGl, lateral preglomerular nucleus	0	+++	0	+++	+++	+++
Pit, pituitary	X	X	X	X	X	X
PO, posterior pretectal nucleus	0	+/−	0	0	0	0
PP, periventricular pretectal nucleus	0	++	0	++	+++	+/−
PPa, parvocellular preoptic nucleus, anterior part	++	+++	0	++	+++	+/−
PPp, parvocellular preoptic nucleus, posterior part	++	+++	0	+/−	+++	+/−
PR, posterior recess of diencephalic ventricle	X	X	X	X	X	X
PSp, parvocellular superficial pretectal nucleus	0	+/−	0	0	0	+/−
PTN, posterior tuberal nucleus	0	++	0	++	++	+/−
R, rostrolateral nucleus	0	+/−	0	+/−	+/−	0
RF, reticular formation	0	+/−	0	0	0	0
SC, suprachiasmatic nucleus	+/−	+++	0	+/−	+++	+/−
SD, saccus dorsalis	+/−	X	X	X	X	X
SO, secondary octaval population	+/−	0	0	0	0	0
TeO, tectum opticum	+	+++	0	+++	+++	+/−
TL, torus longitudinalis	0	+	0	++	++	+/−
TLa, torus lateralis	0	+/−	0	+/−	+/−	+/−
TPp, periventricular nucleus of posterior tuberculum	0	++	0	0	0	0
TS, torus semicircularis	0	+	0	0	0	0
V, ventral telencephalic area	+	++	0	+	++	++
V3, third ventricle	X	X	X	X	X	X
VII, sensory root of the facial nerve	0	0	0	0	0	
VIII, octaval nerve	0	0	0	0	0	0
VCe, valvula cerebelli	0	0	0	+++	+++	+++
Vd, dorsal nucleus of ventral telencephalic area	+	+	0	+	++	0
VL, ventrolateral thalamic nucleus	0	++	+/−	0	+++	+/−
VM, ventromedial thalamic nucleus	0	++	+/−	0	+++	+/−
VOT, ventrolateral optic tract	0	0	0	0	0	0
Vp, postcommissural nucleus of ventral telencephalic area	+	+++	0	0	++	+/−
Vv, ventral nucleus of dorsal telencephalic area	+	++	0	++	+++	0
ZL, zona limitans.	+/−	+	0	+/−	+/−	0

(LR) (Fig. 1, A6). The other *id* genes, except *id2b* that is not detected, exhibit an overall similar expression pattern, showing a broad expression in the Hv around the LR and also in the periglomerular grey zone of the TeO (Fig. 1, B6, D6 and E6). *Znf238* displays a similar pattern of expression, but the staining is weaker in these regions than that of the *id* genes (Fig. 1, F6).

In addition, *id1* is consistently expressed in the ventricular cells lining the LR and posterior recess (PR) of the hypothalamus and in the ventricular zone of the periglomerular gray zone of the TeO. A weak *id1* expression is also observed in the cerebellum (Fig. 1, A7). Again, *id2a*, *id3* and *id4* exhibit a similar expression pattern such as in the caudal hypothalamus and the valvula of the cerebellum (VCe). In these regions, *znf238* pattern is similar to those of *id2a*, *id3* and *id4* (Fig. 1, B7, D7, E7 and F7).

Finally, *id1* is detected along the rhombencephalic ventricle while *id2a*, *id3*, *id4* and *znf238* are also detected in the periventricular layer of the rhombencephalic ventricle as well as in the cerebellum (CCe) (Fig. 1, A8-F8).

In summary, all *id* genes with the exception of *id2b* were found to be specifically expressed in the zebrafish adult brain, as was their potential negative regulator *znf238*. *id1* is detected only in the ventricular cells across the whole brain and in few cells of the cerebellum (Fig. 1, A1-A8). The other *id* genes show a different type of distribution: In the anterior part of the brain (from the olfactory bulb/telencephalon junction to the PPa), *id2a*, *id3* and *id4* exhibit specific patterns of expression with only a few overlapping areas such as the ventricular zone of the Vv/Vd or the PPa. In contrast, from the PPp to the cerebellum, *id2a*, *id3*, *id4* and *znf238* display almost identical expression patterns, while *id1* remains restricted to the ventricular cells. Interestingly, and potentially consistent with a negative cross-regulation of expression between *znf238* and the *ids*, their expression levels sometimes show a negative correlation. Thus, *id* genes are weakly detected in the D, Dl and Dm, while *znf238* is highly expressed in these regions. In contrast, *id* genes are strongly detected in the Vd, Vv, PPa, PPp and TeO whereas *znf238* is weakly expressed in these regions. However, we also noticed regions that display high levels of both *znf238* and *id* expression, such as the PGa and PGl, suggesting that here the negative cross regulation may be overridden by other factors (Table 1). Strikingly, all four *id* genes present in the brain as well as *znf238* are expressed in well-known proliferative/neurogenic regions, such as the ventricular and periventricular layers of the telencephalic ventricle and in the hypothalamus where the lateral recess of the diencephalic ventricle starts to open (Grandel et al., 2006; Lindsey and Tropepe, 2006; Pellegrini et al., 2007) (Fig. 1, schemes in the left column with PCNA-positive cells indicated by red dots).

2.2. Characterization of id-expressing cells in the telencephalon

Given that the telencephalon contains the homologues of the mammalian adult neurogenic regions, making this the preferred subdivision of the brain targeted in zebrafish neurogenic and neural regeneration studies, we decided to examine which cell types express *id* genes in the telencephalon, by performing co-stainings with markers for the different subtypes of neural progenitors. Along the ventricular zone of the adult zebrafish telencephalon, three main types of neural progenitor cells have been defined by characteristic marker combinations: type 1 cells, corresponding to quiescent radial glial cells (S100β-positive and PCNA-negative); type 2 cells, corresponding to proliferative radial glial cells (S100β and PCNA-positive); and type 3 cells which are believed to be actively dividing neuroblasts (S100β-negative and PCNA-positive) and constitute non radial glial progenitors (März et al., 2010). These latest progenitors are mainly found in the rostral migratory stream like region (RMS). Thus, we characterized *id*-expressing cells by performing fluorescent *in situ* hybridization against each of the four selected *id* genes, followed by immunohistochemistry against S100β/PCNA or the panneuronal marker HuC/D (Figs. 2–4).

To examine *id1* expression in detail we made use of the transgenic line *Tg(id1:EGFP)* (Rodriguez Viales et al., 2015). This reporter line has been shown to closely mimic endogenous *id1* mRNA expression. Consistent with previous findings (Rodriguez Viales et al., 2015), transgene expression driven by *id1* regulatory regions was detected in radial glial cells (Fig. 2A–L). In the Dm, we observed *Tg(id1:EGFP)* expression in type 1 cells (Fig. 2 E–H, arrows), while expression in type 2 progenitors was only low or not detected (Fig. 2 E–H, arrowheads). In the RMS-like region, which is composed of type 3 progenitors, we did not detect expression of *Tg(id1:EGFP)* (Fig. 2I–L, asterisks).

Id2a mRNAs were widely expressed in the brain, notably along the ventricular layer and in the brain parenchyma of the Dl, Dm, Vd and Vv (Figs. 1 and 2). In the Dm, *id2a* transcripts were detected in type 1 (Fig. 2Q–T, arrows) and type 2 progenitors (Fig. 2Q–T, arrowheads). In the RMS, we also observed that type 3 progenitors strongly express *id2a* (Fig. 2U–X, asterisks).

Although *id3* staining was only detected in the subpallium with colorimetric ISH (Fig. 1 D2), we also detected a weak *id3* staining in the pallium (Dm and Dl) with fluorescent labelling (Fig. 3A–H), probably because the fluorescent ISH is more sensitive than the colorimetric one used in Fig. 1. In the medial ventricular zone of the pallium (Fig. 3E–H), type 1 (arrow) and 2 progenitors (arrowhead) exhibit a very weak *id3* staining (Fig. 3H, see high squares boxes 1 and 2). In the RMS (lower box in Fig. 3D), a strong *id3* staining is observed at the level of type 3 cells (Fig. 3I–L, asterisks).

Finally, with the exception of the parenchymal cells which widely expressed *id4*, we did not observe any obvious *id4* expression in type 1 (arrows) and type 2 cells (arrowheads) in the Dm, as well as in type 3 cells (asterisks) in the RMS (Fig. 3M–X).

To further characterize *id*-expressing cells in the telencephalon, we also investigated *id* genes expression in HuC/D-positive neurons because of the known role of *ids* in neuronal differentiation/specification (Tzeng, 2003). Numerous *id2a*, *id3* and *id4*-expressing cells in the parenchyma correspond to HuC/D-positive neurons (Fig. 4D–L, arrows), while *id1* was never co-expressed with this neuronal marker (Fig. 4A–C). This suggests that, in contrast to *id1*, *id2-4* might play a role in neuronal differentiation.

Taken together, our data show that a) *id1* expression is mainly restricted to type 1 cells, b) *id2a* is detected in type 1, 2 and 3 cells as well as in neurons, c) *id3* transcripts are strongly expressed in type 3 progenitors and weakly in radial glial cells and neurons, and finally 4) *id4* is mainly detected in neurons The overlapping expression in some progenitors (*id1* and *id2a* in type 1 and 2 cells; *id2a* and *id3* in type 3 cells) may be consistent with some redundancy of Id function in these cell types.

2.3. znf238 gene expression in id-expressing cells and neurons in the adult zebrafish brain

Rp58, the product of *znf238*, was shown to negatively regulate *Id* expression in mammals, and we observed that the expression levels of *id* genes and *znf238* show opposite tendencies in some areas of the adult zebrafish brain (Fig. 1 and Table 1). Therefore, we examined a) whether the same cell types shown to express *id* genes also express *znf238*, and b) whether *znf238* is indeed co-expressed with *id* genes in these cells. *znf238* is not expressed in the ventricular S100β-positive radial glial cells (Fig. 5A–I), and we also do not observe *znf238* in the RMS region (data not shown). Thus, *znf238* is not expressed in any of the three neural progenitor cell types. In contrast, numerous HuC/D-positive neurons strongly express *znf238* (Fig. 5J–L). In line with these observations, we show that *znf238* is co-expressed within many *id2a*, *id3* and *id4*-positive cells localized in the brain parenchyma, but not along the ventricular surface, again indicating that it is only expressed in neurons.

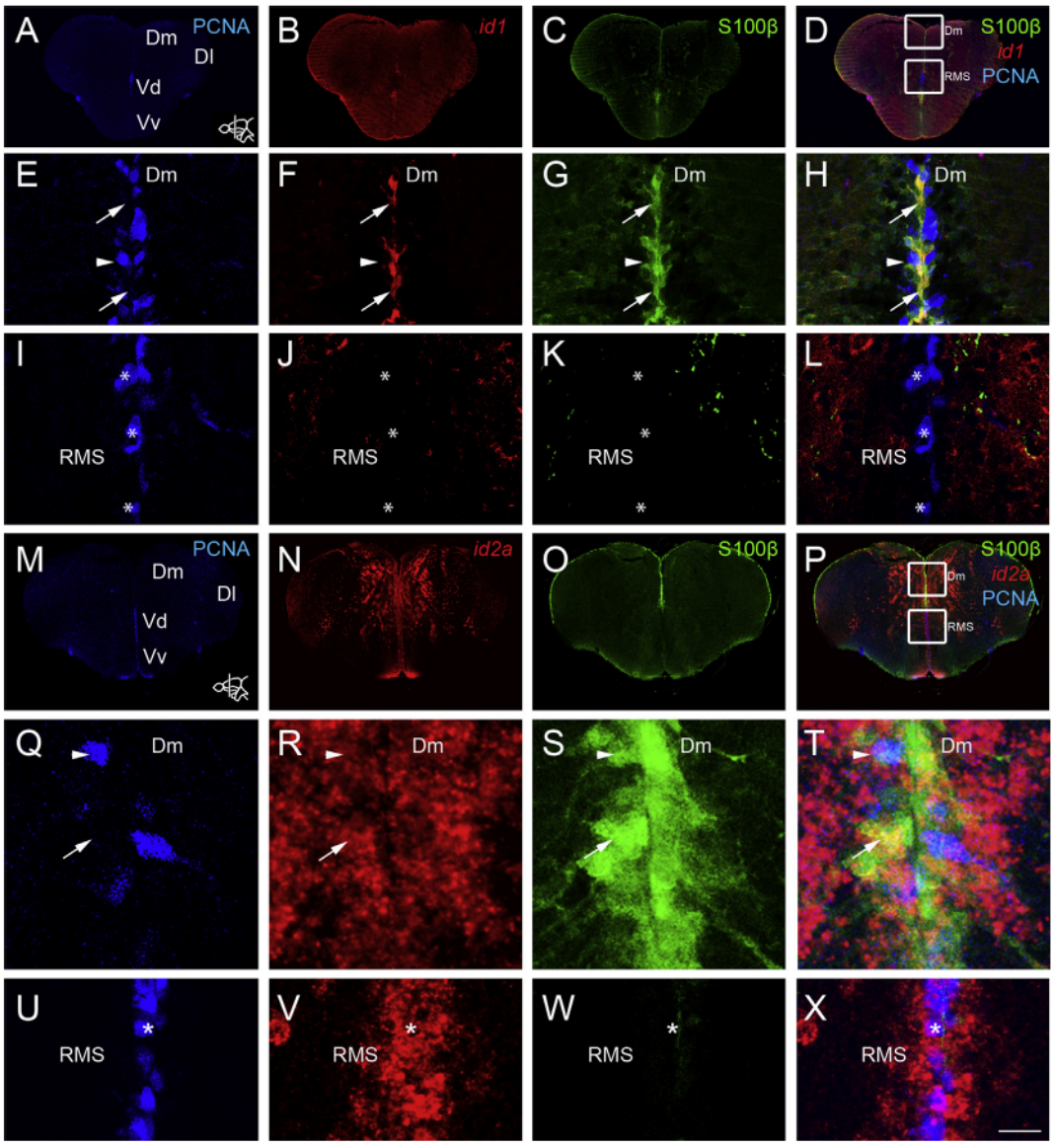


Fig. 2. *id1* and *id2a* expression in neural progenitors. A to L: PCNA (blue) and S100β (green) immunohistochemistry in the telencephalon of transgenic *Tg(id1:EGFP)* fish (GFP artificially put in red). *Id1* transgene is expressed in type 1 (S100β-positive and PCNA-negative) and in type 2 (S100β and PCNA-positive) radial glial cells. E to H: high magnification views of the upper white box in D corresponding to the Dm region. *Id1* transgene is strongly expressed in type 1 cells (arrows) and weakly or not by type 2 cells (arrowhead). I to L: high magnification views of the lower white box in D corresponding to the rostral migratory stream like region (RMS) region. Type 3 progenitors (S100β-negative and PCNA-positive) of the RMS do not express *id1* (asterisks). M to X: *id2a in situ* hybridization (red) followed by PCNA (blue) and S100β (green) immunohistochemistry in the telencephalon. *Id2a* mRNA appears to be widely expressed in the telencephalon, in the parenchyma and the ventricular layer. Q to T: high magnification views of the upper white box in P corresponding to the Dm region. The arrows show an example of a type 1 progenitors (S100β and PCNA-negative) expressing *id2a*, while the arrowhead shows a type 2 progenitor (S100β-positive and PCNA-positive) exhibiting *id2a* expression. U to X: high magnification views of the lower white box in P corresponding to the RMS region. The asterisk shows an example of a type 3 progenitor (S100 β -negative and PCNA-positive) of the rostral migratory stream like region (RMS), expressing *id2a* transcripts. Scale bar: 8 μm (Q to T); 12 μm (I to L); 20 μm (U to X); 25 μm (E to H); 250 μm (A to D); 310 μm (M to P).

Consistently, we do not observe co-expression of *id1* and *znf238* in the ventricular zone (Fig. 6).

3. Discussion

Id proteins play key roles in embryonic and adult neurogenesis, as shown in mammals, birds and more recently in adult zebrafish for Id1 (Bai et al., 2007; Jen et al., 1997; Liu et al., 2013; Lyden et al., 1999; Nam and Benezra, 2009; Riechmann and Sablitzky, 1995; Rodriguez Viales et al., 2015; Sugimori et al., 2007). In this study, we have (a) characterized the expression of *id* and *znf238* genes in the whole brain of zebrafish, (b) highlighted their overlapping distribution and their expression in neurogenic niches. Detailed expression analysis in neural progenitors of the telencephalon revealed patterns of *id* expression that are consistent with a potential redundancy of Id function in these cells (Fig. 7).

3.1. Id genes expression in the whole brain

Our data show that *id1*, *id2a*, *id3* and *id4* are widely expressed within the entire brain of adult zebrafish, while *id2b* is barely detectable, in agreement with RNAseq data (Armant et al., 2013; Diotel et al., 2015). Thus, we present new data showing that *id1*-expressing cells are not limited to the telencephalon (Rodriguez

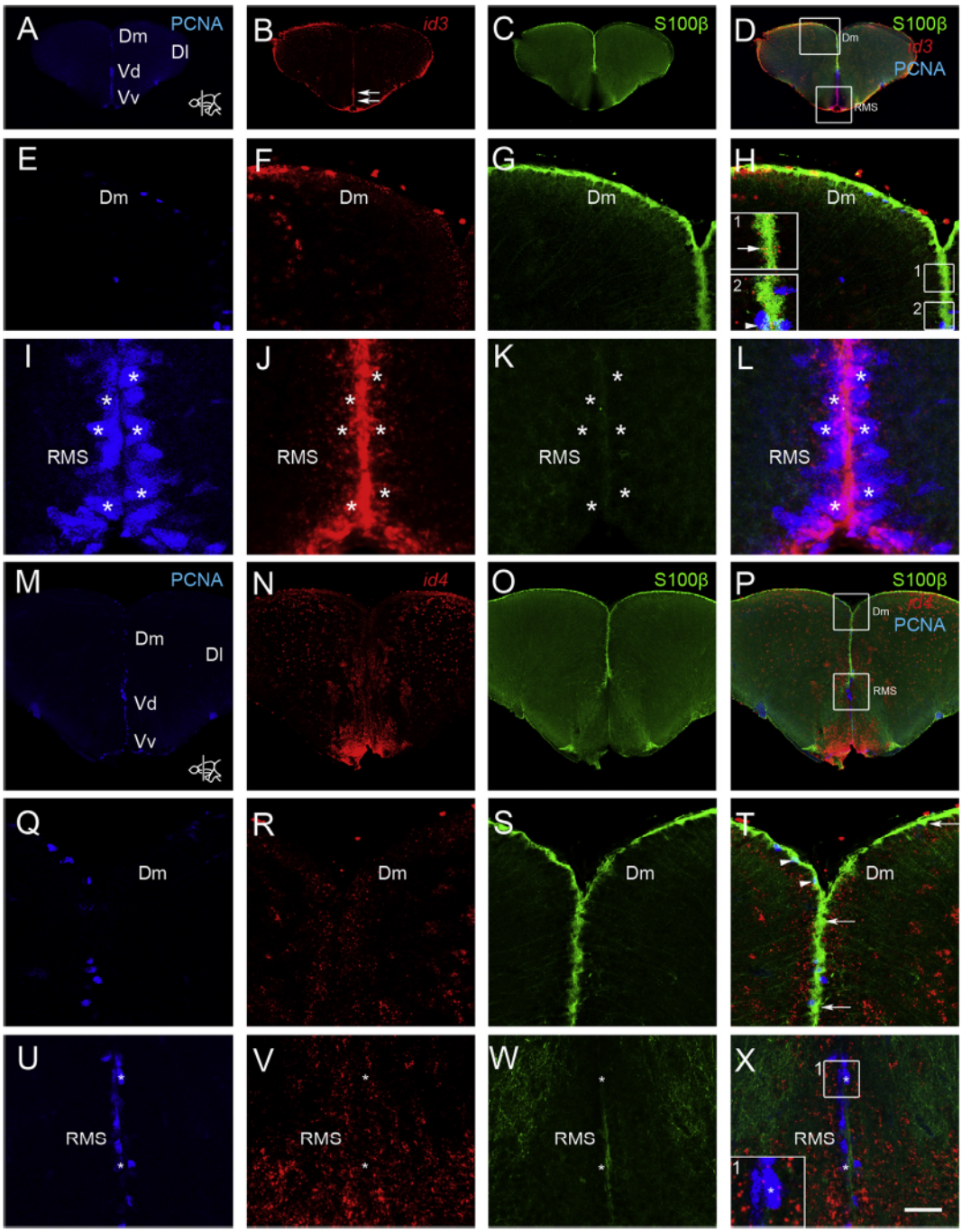


Fig. 3. Strong *id3* expression in type 3 progenitors and weak *id4* expression in neural progenitors. A to L: *id3 in situ* hybridization (red) followed by PCNA (blue) and S100β (green) immunohistochemistry in the telencephalon. *Id3* appears to be mainly and strongly expressed in the ventricular layer of the subpallium. E to H: high magnification views of the upper white box in D corresponding to the Dm region. A weak *id3* expression is detected in non-proliferative (arrow) and proliferative (arrowhead) S100β-positive radial glial cells (type 1 and type 2 cells, respectively). I to L: high magnification views of the lower white box in D corresponding to the rostral migratory stream like region (RMS) region. The asterisks show a strong *id3* expression in type 3 progenitors (S100β-negative and PCNA-positive) of the RMS. M to X: *id4 in situ* hybridization (red) followed by PCNA (blue) and S100β (green) immunohistochemistry in the telencephalon. *id4* expression appears to be widely expressed in the parenchyma but not in the ventricular layer. Q to T: high magnification views of the upper white box in D corresponding to the Dm region. *id4* transcripts are mainly absent from non-proliferative (arrow) and proliferative (arrowhead) S100β-positive radial glial cells (type 1 and type 2 cells, respectively). U to X: high magnification views of the lower white box in P corresponding to the RMS region. In the RMS, *id4* expression is barely detected in type 3 progenitor (S100β-negative and PCNA-positive) as shown with asterisks. Scale bar: 15 μm (I to L); 20 μm (U to X); 35 μm (Q to T); 40 μm (E to H); 130 μm (M to P); 250 μm (A to D).

Viales et al., 2015), but can also be found in the ventricular zone of the diencephalon (i.e. preoptic area and hypothalamus), the mesencephalon (periglomerular gray zone of the optic tectum), and in cells lining the rhombencephalic ventricle. Interestingly, expression always remains restricted to the ventricular cells. Given the well-established role of Id1 in mammalian neurogenesis (Bai et al., 2007; Lyden et al., 1999; Nam and Benezra, 2009; Tzeng and de Vellis, 1998) and its recently described function in the control of stem cell proliferation in the zebrafish telencephalon (Rodriguez Viales et al., 2015), these data suggest a potential

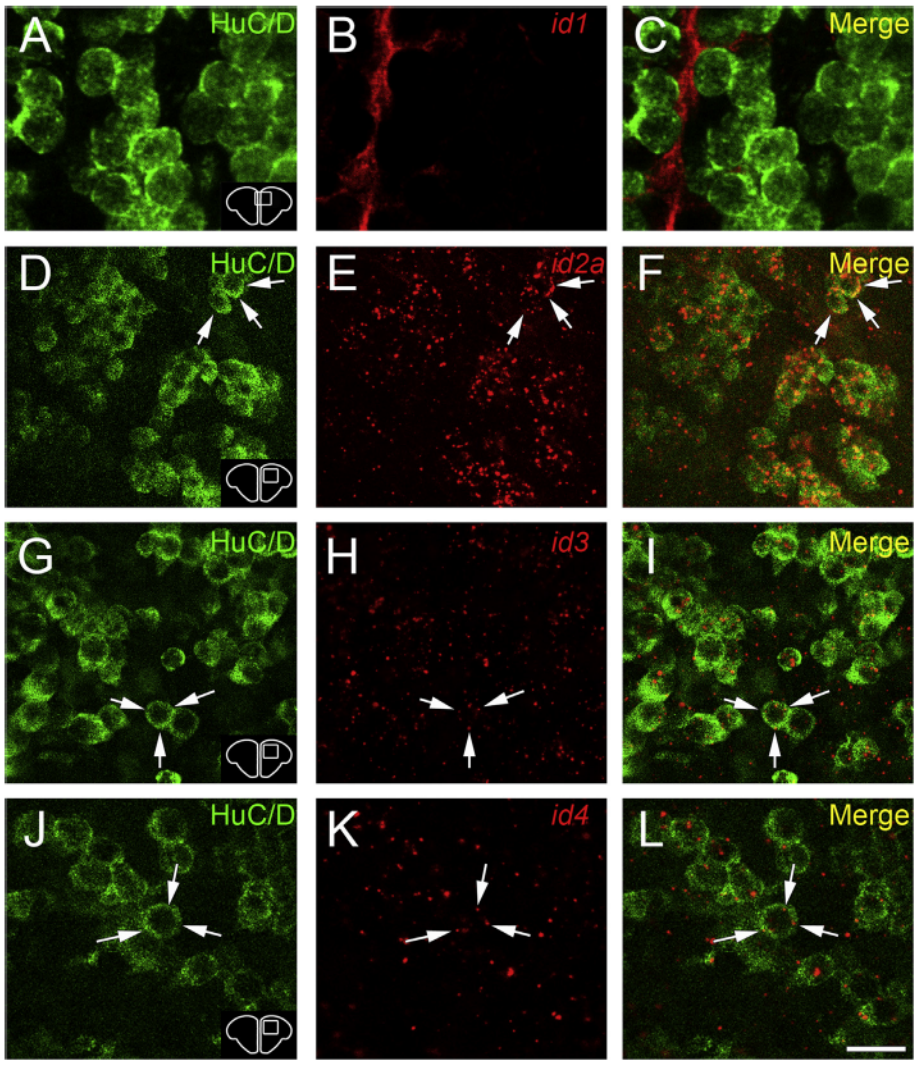


Fig. 4. *id2a, Id3* and *Id4* expression in HuC/D-positive neurons. A to L: Anti-GFP immunohistochemical staining on *Tg(id1:EGFP)* fish (GFP artificially put in red) and *id2a, id3* and *id4 in situ* hybridization on WT fish (red) followed by HuC/D immunohistochemistry (green). The arrows show *id2a, id3* and *id4* mRNAs detection in HuC/D-positive neurons. The *id1* transgene is not detected in neurons. Scale bar: 7 μm (A to C); 16 μm (D to I); 11 μm (J to L).

involvement also in midbrain and hindbrain neurogenesis. Importantly, in contrast to *id1*, the other *id* transcripts appear not to be restricted to the ventricular zone. Also, they exhibit distinct and specific gene expression patterns in the anterior part of the brain (i.e., from the olfactory bulb/telencephalon junction to the PPa), while they show a strong overlapping expression in the mes-, met and rhombencephalon. However, also some regions of the telencephalon express all *id* genes including *id1*, notably the Dp, which has been suggested to be the homologue of the mammalian hippocampus.

In rodents, *Id* genes are also widely expressed in the brain. *Id1* is mainly detected in the dentate gyrus of the hippocampus and in the SVZ of the lateral ventricles, while *Id2-4* are also detected in the olfactory areas, the isocortex, the thalamus, the hypothalamus and the cerebellum (Kitajima et al., 2006; Lau et al., 2008; Lee et al., 2015; Nam and Benezra, 2009; Ng et al., 2009; Tzeng and de Vellis, 1998). It consequently appears that *id* expression in the brain is relatively well conserved between fish and rodents.

3.2. *Id gene expression in neural progenitors*

Focussing on the well-characterized telencephalon, we show that (a) *id1* is expressed in type 1 and 2 progenitors (as previously described (Rodriguez Viales et al., 2015)), (b) *id2a* is detected in type 1, 2 and 3 cells as well as in neurons across the parenchyma of the entire telencephalon, (c) *id3* is mainly observed in type 3 progenitors and neurons, while (d) *id4* is mainly detected in post-mitotic neurons of the pallium (Figs. 2–4 and Fig. 7). With these data, we provide the first comprehensive description of *id* gene expression in adult neural progenitors in zebrafish. Our results suggest that other *id* genes in addition to *id1* could be involved in the activity of neural progenitors, and that some redundancy in Id function and/or compensatory mechanisms could be present in neural stem cells, as described in mammals and chick for Id1 and Id3 (Bai et al., 2007). This could apply particularly to the pairs of *id1/id2a* and *id2a/id3*, which are strongly expressed in type 1 and type 3 progenitors, respectively. Interestingly, in the rodent brain, Id1 and Id3 appear to be preferentially expressed in progenitor cells while Id2 and Id4 persist in post-mitotic neurons (Hirai et al., 2012; Jen et al., 1997; Neuman et al., 1993; Riechmann and Sablitzky, 1995; Tzeng and de Vellis, 1998; Yokota, 2001), similar to what we observe in fish. In addition, in the mouse SVZ, Id1 has been shown to be strongly expressed in a rare population of astrocytes displaying stem cell properties (Nam and Benezra, 2009) that could be the equivalent of type 1 cells in zebrafish. Furthermore, *id2a* expression in neural progenitors of the telencephalon strongly

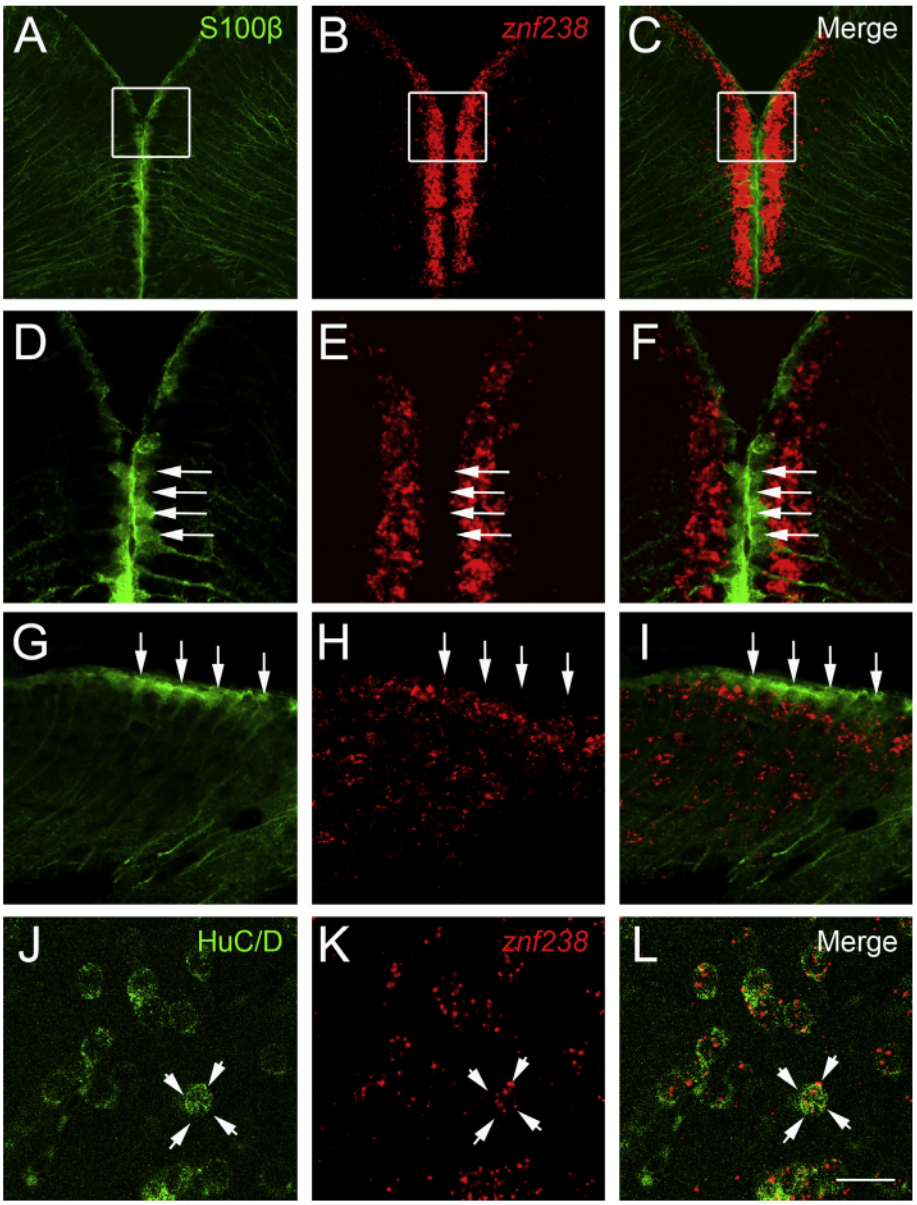


Fig. 5. *znf238* is not expressed in radial glial cells but in neurons. A to I: *znf238 in situ* hybridization (red) followed by S100β (green) immunohistochemistry in the telencephalon. D to F: high magnification views of the corresponding white boxes in A to C. The arrows show that the S100β-positive radial glial cells do not express (or only barely for few of them) *znf238* mRNA in the dorsomedian telencephalon. J to L: *znf238 in situ* hybridization (red) followed by HuC/D (green) immunohistochemistry in the telencephalon. *Znf238* transcripts were detected in HuC/D-positive neurons (arrows) Scale bar: 12 μm (J to L); 20 μm (D to I); 70 μm (A to C).

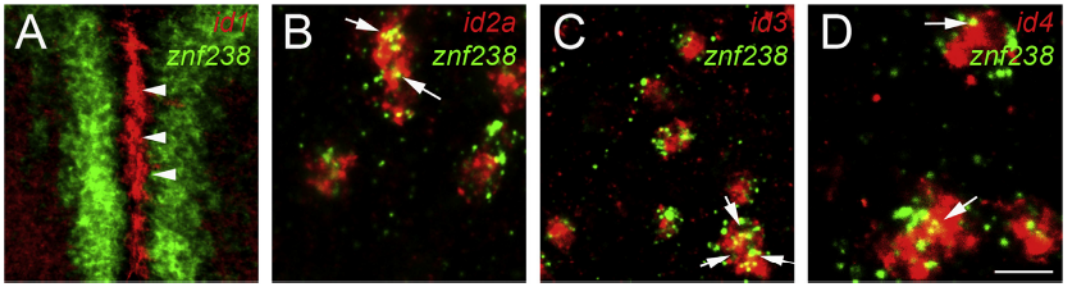
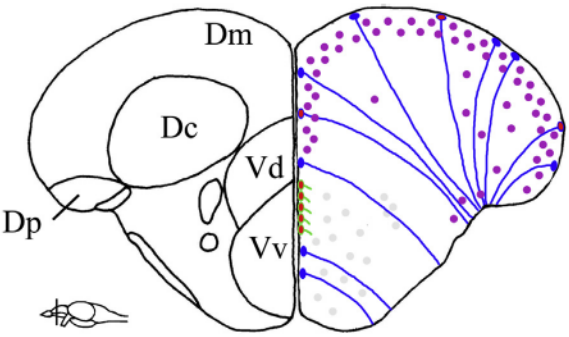


Fig. 6. *id2a*, *id3* and *id4* are expressed in *znf238*-positive cells, but not *id1*. A to D: Double fluorescent *in situ* hybridization in the pallium showing *id1*, *id2a*, *id3* and *id4* transcripts in red and *znf238* mRNA in green. *Id1* transcripts are not detected in *znf238* expressing cells (arrowheads), while *id2a*, *id3* and *id4* transcripts are expressed in *znf238* positive cells (arrows). Scale bar: 6 μm (D); 12 μm (B to C); 35 μm (A).



	Type 1	Type 2	Type 3 (RMS)	Neuron (pallium)	Neuron (subpallium)
id1	+++	+	0	0	0
id2a	+++	+++	+++	+++	+++
id3	+/-	+/-	+++	++	++
id4	+/-	+/-	+/-	+++	+++
znf238	0	0	0	+++	0

Fig. 7. Summary of *id1, id2a, id3, id4* and *znf238* expression in progenitor cells and neurons of the adult telencephalon. The different cell types are schematized on cross-sections at the anterior level of the telencephalon (as indicated on the lateral view scheme). Type 1 and type 2 progenitors correspond to quiescent and actively dividing radial glial cells, respectively. They express different levels of *id* genes but do not express *znf238*. Type 3 progenitors correspond to actively dividing neuroblasts. They mainly express *id2a* and *id3* but do not express *id1* and *znf238*. Pallial neurons (purple) express *znf238* and *id2a, id3 and id4* genes but do not express *id1*. Subpallial neurons (grey) do not express *id1* and *znf238*. This figure highlights the potential roles of *id* and *znf238* genes in neurogenesis and also suggests the potential redundancy of Id function in progentior cells. 0: no expression; +/−: weak expression; +: low expression; ++: moderate expression; +++: strong expression. Dc, central zone of dorsal telencephalic area; Dm, medial zone of dorsal telencephalic area; Dp, posterior zone of dorsal telencephalic area; Vd, dorsal nucleus of ventral telencephalic area; Vv, ventral nucleus of dorsal telencephalic area.

suggests its involvement in telencephalic neurogenesis. Interestingly, *id2a* was shown to regulate retinoblast proliferation and differentiation during zebrafish retinogenesis by modulation of the Notch pathway activity (Uribe and Gross, 2010; Uribe et al., 2012), and it is tempting to speculate that it might have analogous functions in the regulation of neural progenitors. Evidence from mammals indicates that Id2 is required for dopaminergic neuronal specification during adult olfactory neurogenesis (Havrda et al., 2008), and that it functions as a pro-proliferative gene for neural progenitor cells (Paolella et al., 2011).

Taken together, these data strongly suggest key roles of Id proteins in neurogenic activity in the brain of adult zebrafish, as well as potential redundancy (Fig. 7).

3.3. *Znf238 expression in the brain: correlation with id distribution*

In rodents, *Znf238* expression is detected in the neocortex, the hippocampus, parts of the amygdala and in granular cells of the cerebellum (Ohtaka-Maruyama et al., 2007). In *Rp58 knock-out mice*, Id members are ectopically expressed in the ventricular and subventricular zone (Hirai et al., 2012). This shows that Rp58 is an important factor driving the correct expression of *id* members and controlling neural stem cell proliferation in the developing cortex of mammals (Hirai et al., 2012).

In zebrafish, our investigations show that the *znf238* is also widely expressed in the brain, both in the telencephalon as recently described (Diotel et al., 2015) and also in the remaining parts of the brain. *Znf238* is co-expressed with *id2a*, *id3* and *id4* in numerous parenchymal pallial cells (Figs. 6 and 7). In contrast, no co-expression was detected with *id1* nor with S100β, which both label radial glia cells (type 1 and/or type2). Interestingly, in the adult telencephalon, the ventricular layer of the pallium is surrounded by a broad *znf238*-positive periventricular stripe. Given that *id1* and *znf238* are not co-expressed, *znf238* could participate in the restriction of *id1* expression to the ventricular cells of the pallium, inhibiting its expression outside of the ventricular zone. In addition, some regions strongly expressing *znf238* appear to barely express *id* genes, such as the dorsal telencephalic area (Fig. 1, row A1-F1) and the dorsolateral domain of the telencephalon (Fig. 1, rows A2-F2 and A3-F3). In contrast, regions that do not express *znf38*, or show very weak expression, generally strongly express *id* genes, notably the preoptic area (PPa and PPp) and the habenula (Fig. 1, rows A3-F3 and A4-F4), the optic tectum and also the ventricular and periventricular regions of the hypothalamus (Fig. 1, A5-F7). These data argue in favour of a potential regulation of *id* genes by *znf238*. However, in other regions, such as in the valvula of the cerebellum (VCe) and the cerebellum, strong expression of both *znf238* and *id2a*, *id3* and *id4* is observed, showing that there is no strict mutually exclusive expression of the two gene groups, and indicating that other, unknown regulators may influence their expression patterns.

3.4. *Conclusions*

To conclude, our work provides a detailed expression of *id* and *znf238* genes in the adult zebrafish brain. It suggests important roles for *id and znf238* genes in the control of neural progenitor proliferation and neuronal cell-specification in the whole brain including the telencephalon, the preoptic area, the hypothalamus and the cerebellum. Our work is a first step towards more comprehensive studies concerning the role of Id proteins in adult neurogenesis in zebrafish and their potential regulation by *znf238* and other factors.

4. Experimental procedure

4.1. Zebrafish strains and maintenance

Fish were maintained under standard conditions of photoperiod (14-h light/10-h dark) and temperature (28.5 °C) in recirculation systems (Schwarz Ltd Germany, Müller and Pfleger Ltd Germany), and fed with commercial food as previously described (Westerfield, 2007). Experiments were performed on 3–6 month old adult zebrafish of the AB wildtype strain or of the *Tg(id1:EGFP)* reporter line (Rodriguez Viales et al., 2015). Experiments on animals were performed in accordance with the German animal protection standards and were approved by the Government of Baden-Wurttemberg, Regierungspräsidium Karlsruhe, Germany (Aktenzeichen 35-9185.81/G-272/12 'Adulte Neurogenese').

4.2. Dissection and fixation

Fish were anesthetized in 0.02% tricaine methanesulfonate (MS-222, Sigma, pH7) before being sacrificed in ice water (Westerfield, 2007) and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) overnight at 4 °C. On the next day, brains were stepwise dehydrated in a methanol/PBS concentration series before being stored at −20 °C in 100% methanol (Adolf et al., 2006; Schmidt et al., 2014).

4.3. In situ hybridization

In situ hybridization (ISH) was performed on vibratome brain slices (50 μm thickness) as previously described (Adolf et al., 2006; Schmidt et al., 2014). We used digoxigenin (DIG) labelled anti-sense probes for chromogenic *in situ* hybridization and DIG or dinitrophenol-11-UTP (DNP-11-UTP) labelled probes for fluorescent *in situ* hybridization. Probe synthesis and ISH on whole adult brains were performed as previously described (Adolf et al., 2006; Diotel et al., 2015; Lam et al., 2009; Rodriguez Viales et al., 2015). Briefly, after rehydration, brains were washed in 0.1% Tween, PBS buffer (PTw; pH 7.4) and subsequently incubated in PTw containing proteinase K (10 μg/ml) at room temperature (20 °C) for 30 min. Next, brains were post-fixed in 4% PFA for 30 min, washed in PTw and prehybridized for 3 h before overnight incubation at 65 °C in hybridization buffer containing the DIG labelled probes. After several washing steps, brains were briefly incubated in blocking buffer, embedded in 2% agarose and sectioned using a Leica vibrating blade microtome VT1000 S at 50 μm thickness. After a 1 h blocking step at room temperature, sections were incubated overnight at 4 °C with Anti-Digoxigenin-AP Fab fragments (1:4000, Roche, Cat# 11093274910, RRID: AB_514497). Finally, sections were washed in PTw and stained with NBT/BCIP solution.

Fluorescent *in situ* hybridization was performed using tyramide amplification according to the manufacturer's instructions (TSA Plus Cyanine 3 System, Perkin Elmer, Boston, MA). Briefly, brains were treated with 3% H_2O_2 in PBS before sectioning for inactivating endogenous peroxidase. After blocking for 1 h, the sections were incubated with anti-dinitrophenol-HRP (1:200, Perkin Elmer) and/or anti-digoxigenin-POD (poly) antibody (1:1000, Roche) and stained with tyramide Cy3 solution (1:100) in 0.002% H_2O_2 in PBS-Tween 0.1% as previously described (Yang et al., 2010).

Each ISH was repeated at least three times, and no specific staining was observed with sense probes (data not shown).

4.4. Immunohistochemistry

Immunohistochemistry was performed on free-floating 50 μm thickness transverse vibratome sections as previously described by Adolf and colleagues (Adolf et al., 2006), using the following primary antibodies: chicken anti-GFP (1:1000, Aves Labs, Cat# GFP-1020, RRID: AB_10000240), rabbit anti-GFP (1:1000, Torrey Pines Biolabs), mouse anti-PCNA (1:500, DAKO, Cat# M0879, clone PC10; RRID: AB_2160651), rabbit anti-S100 (1:400, DAKO, Cat# Z0311, RRID: AB_10013383) and mouse anti-HuC/D (1:300, Invitrogen, Cat# A21271, Clone 16A11, RRID: AB_221448). The secondary Alexa fluor antibodies used for fluorescent staining were goat anti-chicken, anti-mouse and anti-rabbit from the Alexa series (Alexa 488, Alexa 546, Alexa 680; 1:1000, Invitrogen). The sections were mounted on slides with Aqua-Poly/Mount (Polysciences).

4.5. Microscopy

For bright field microscopy, pictures were acquired using a Leica compound microscope (DM5000B). For fluorescent microscopy, pictures were acquired using a laser scanning confocal microscope Leica TCS2 SP5 and processed using Leica software. Pictures were adjusted for brightness and contrast in Adobe Photoshop CS5.

4.6. Nomenclature and abbreviations

The nomenclature and the schemes of brain sections correspond to those provided in the zebrafish brain atlas (Wullimann et al., 1996).

A, anterior thalamic nucleus; APN, accessory pretectal nucleus; ATN, anterior tuberal nucleus; CCe, corpus cerebelli; Chab, habenular commissure; Chor, horizontal commissure; CM, corpus mamillare; CP, central posterior thalamic nucleus; CPN, central pretectal nucleus; Cpop, postoptic commissure; Cpost, posterior commissure; D, dorsal telencephalic area; Dc, central zone of dorsal telencephalic area; Dl, lateral zone of dorsal telencephalic area; Dm, medial zone of dorsal telencephalic area; DOT, dorsomedial optic tract; Dp, posterior zone of dorsal telencephalic area; DP, dorsal posterior thalamic nucleus; ECL, external cellular layer of olfactory bulb; EG, eminentia granularis; ENv, entopendoncular nucleus, ventral part; FR, fasciculus retroflexus; GL, glomerular layer of olfactory bulb; Had, dorsal habenular nucleus; Hav, ventral habenular nucleus; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; ICL, internal cellular layer of olfactory bulb; IL, inferior lobe; LH, lateral hypothalamic nucleus; LLF: lateral longitudinal fascicle; LR, lateral recess of diencephalic nucleus; MLF, medial longitudinal fascicle; NMLF, nucleus of medial longitudinal fascicle; PG, preglomerular nucleus; PGa, anterior preglomerular nucleus; PGl, lateral preglomerular nucleus; Pit, pituitary; PO, posterior pretectal nucleus; PP, periventricular pretectal nucleus; PPa, parvocellular preoptic nucleus, anterior part; PPp, parvocellular preoptic nucleus, posterior part; PR, posterior recess of diencephalic ventricle; PSp, parvocellular superficial pretectal nucleus; PTN, posterior tuberal nucleus; R, rostrolateral nucleus; RF, reticular formation; SC, suprachiasmatic nucleus; SD, saccus dorsalis; SO, secondary octaval population; TeO, tectum opticum; TL, torus longitudinalis; TLa, torus lateralis; TPp, periventricular nucleus of posterior tuberculum; TS, torus semicircularis; V, ventral telencephalic area; V3, third ventricle; VII, sensory root of the facial nerve; VIII, octaval nerve; VCe, valvula cerebelli; Vd, dorsal nucleus of ventral telencephalic area; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VOT, ventrolateral optic tract; Vp, postcommissural nucleus of ventral telencephalic area; Vv, ventral nucleus of dorsal telencephalic area; ZL, zona limitans.

Conflict of interest

The authors declare no conflict of interest.

Role of authors

ND, SR and US designed the experiments and supervised the work. ND and TB conducted the ISH experiments. ND and SR analyzed the ISH data and wrote the manuscript.

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