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Six *cadm/SynCAM* Genes Are Expressed in the Nervous System of Developing Zebrafish

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Abstract

The Cadm (cell adhesion molecule) family of cell adhesion molecules (also known as IGSF4, SynCAM, Necl and TSLC) has been implicated in a multitude of physiological and pathological processes, such as spermatogenesis, synapse formation and lung cancer. The precise mechanisms by which these adhesion molecules mediate these diverse functions remain unknown. To investigate mechanisms of action of these molecules during development, we have identified zebrafish orthologs of *Cadm* family members and have examined their expression patterns during development and in the adult. Zebrafish possess six *cadm* genes. Sequence comparisons and phylogenetic analysis suggest that four of the zebrafish *cadm* genes represent duplicates of two tetrapod *Cadm* genes, whereas the other two *cadm* genes are single orthologs of tetrapod *Cadm* genes. All six zebrafish *cadms* are expressed throughout the nervous system both during development and in the adult. The spatial and temporal patterns of expression suggest multiple roles for Cadms during nervous system development.

Keywords

Cadm; SynCAM; IGSF4; Necl; zebrafish; central nervous system

INTRODUCTION

The Cadms (cell adhesion molecules) are a family of type I transmembrane proteins that have been described in several pathological and physiological processes such as the progression of lung and other cancers (reviewed in Murakami, 2005), mast cell adhesion (Ito et al., 2003, 2007b; Furuno et al., 2005; Ito and Oonuma, 2006), spermatogenesis (Wakayama et al., 2001; Wakayama et al., 2003; Fujita et al., 2006; van der Weyden et al., 2006; Yamada et al., 2006), epithelium development and homeostasis (Ito et al., 2007b), and central nervous system development (Biederer et al., 2002; Sara et al., 2005; Spiegel et al., 2007).

Cadms have been independently identified in various model systems, thus these genes and the proteins they encode have acquired several different names, such as *Necl* (Nectin-like molecules), *Igsf4* (Ig-like spermatogenic factor), *TSLC* (tumor suppressor in lung cancer), and *SynCAM* (synaptic cell adhesion molecule; Table 1). Recently, the Human Genome Organization (HUGO) Gene Nomenclature Committee renamed the genes *CADM* (Table 1).

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So far, four *Cadm* genes have been identified in tetrapod vertebrates, and the proteins they encode show a strict conservation of their structural organization. Cadm proteins are composed of three extracellular Ig-like loop domains, a trans-membrane domain, and a highly conserved short cytoplasmic tail containing two known protein–protein interaction domains, namely a juxtamembrane protein 4.1 binding motif and a C-terminus type II PDZ-binding domain (Fig. 1A; Biederer, 2006). Interestingly, genes with this protein structure are only found in vertebrates and appear to be an innovation of this phylum (Biederer, 2006).

The extracellular Ig-like domains of Cadm proteins can mediate both hetero- and homophilic interactions that are Ca^{2+} and Mg^{2+}-independent. To date, in addition to various combinations of Cadm to Cadm interactions (Takai et al., 2003; Maurel et al., 2007), two types of heterophilic binding partners have been identified, the nectins and class I-restricted T-cell-associated molecules (CRTAM). Furthermore, the intracellular tails of the Cadms have been shown to interact with several scaffolding molecules in various *in vitro* systems. These include CASK (Biederer et al., 2002), syntenin (Biederer et al., 2002; Meyer et al., 2004), GRIP (Meyer et al., 2004), MPP3 (ortholog of the *Drosophila* tumor suppressor gene Dlg; Fukuhara et al., 2003), and DAL-1 (Yageta et al., 2002). However, very little is known about how these interactions relate to the functions of the Cadms during development.

Extensive analyses have revealed that *Cadm1* is expressed during early development in rodents (Fujita et al., 2005; Ohta et al., 2005). It has been found in most epithelia and neuroepithelia, such as hair follicles, lung, liver, gut, tongue, olfactory epithelium, dorsal root ganglia, various regions of the central nervous system, being particularly enriched in the marginal zone of the cortex, the external granule layer of the cerebellum, the habenular nucleus, and the thalamus (Fujita et al., 2005; Ohta et al., 2005). Additionally, *Cadm1* has been shown to be highly expressed in the testis during germ cell development.

Of interest, inactivation of the *Cadm1* gene in mice produces infertile males (oligo-astheno-teratozoospermia; Fujita et al., 2006). Infertility is probably caused by a delay in the maturation of sperm cells, leading to an increase in apoptosis. The deficit in sperm cell maturation is due to an alteration of the adhesion between spermatogenic cells and the Sertoli cells (Wakayama et al., 2003; Fujita et al., 2006; van der Weyden et al., 2006; Yamada et al., 2006).

Given the widespread expression of *Cadm1*, it is interesting to note that no other deficits have been detected in *Cadm1* knockout mice (Fujita et al., 2006), revealing probable compensation mechanisms in other tissues. Nonetheless, *in vitro* studies have determined several functions for *Cadm1* in the nervous system. *Cadm1* is expressed during the period of synaptogenesis and localizes to pre- and postsynaptic sites in the rodent brain. Its overexpression in cultured neurons increases spontaneous synaptic activity (Biederer et al., 2002; Sara et al., 2005). Moreover, the expression of recombinant Cadm1 in non-neuronal cells cocultured with neurons induces the formation of functional presynaptic terminals onto the non-neuronal cells. Cadm1 may therefore act as a synaptogenic molecule during nervous system development (Biederer et al., 2002; Sara et al., 2005).

In addition to promoting neuron to neuron interactions, Cadm1 also promotes a homophilic adhesion link between neurons and mast cells. This interaction grants Cadm1 the ability to modulate the immune system by enhancing mast cell response to nerve activation (Ito et al., 2003; Furuno et al., 2005; Ito and Oonuma, 2006; Ito et al., 2007a). Another function of *Cadm1* in the immune system includes immunosurveillance. Loss of *Cadm1* may provide an escape mechanism from detection by natural killer (NK) cells and cytotoxic T cells, which express CRTAM (Murakami, 2005). Also, cell adhesion through Cadms appears necessary to promote tumor suppression. For instance, absence of *CADM1* expression and mutated

forms of *CADM1* in carcinoma have been found to enhance malignancy, through destabilization of cell–cell contacts (Fuchs and Colonna, 2006).

In contrast to *Cadm1*, *Cadm3* and *Cadm4* expression seems to be mostly restricted to neurons and glial cells (astrocytes, oligodendrocytes, and Schwann cells; Maurel et al., 2007; Spiegel et al., 2007). Recently, it has been proposed that a heterophilic interaction between Cadm3 and Cadm4 mediates Schwann cell adhesion to peripheral axons. Perturbation with dominant-negative forms of either Cadm3 or Cadm4 or knockdown of their expression blocks the myelination process (Maurel et al., 2007; Spiegel et al., 2007). Expression patterns and function of *Cadm2* have not been reported to date.

As a prerequisite to better understanding the function and mechanism of all the *Cadm* genes during development, we have isolated orthologs of the tetrapod *Cadm* genes from the zebrafish (*Danio rerio*). We have characterized their pattern of expression during development and in the adult.

RESULTS AND DISCUSSION

Isolation and Characterization of the *cadm* Genes From Zebrafish

In tetrapods, four distinct *Cadm* gene family members have been identified to date (Biederer, 2006). By searching the zebrafish genome database (http://www.ensemble.org/Danio_rerio) using mammalian family members as a template, we have identified six orthologs in zebrafish. In accordance with the HUGO Gene Nomenclature Committee and the nomenclature guidelines proposed by the Zebrafish Model Organism Database (www.zfin.org), we will call these genes *cadm* (Table 1).

By using a combination of reverse transcriptase-polymerase chain reaction (RT-PCR) and 5′–rapid amplification of cDNA ends (RACE) -PCR, we have identified the coding sequences for the six *cadm* genes. This finding indicates that all six genes are expressed in zebrafish. The coding sequences show the same protein domain organization that characterizes the tetrapod *Cadm* family: a signal peptide followed by three immunoglobulin domains in the extracellular portions of the proteins, a transmembrane domain and a short cytoplasmic tail that encompass a juxtamembrane 4.1B binding domain and a C-terminal PDZ type II binding domain (Fig. 1A,C).

Alignment of the protein sequences of the six *cadm* genes (Fig. 1B,C) and comparison with tetrapod species (Fig. 2B; Supplementary Figure S1A–D, which can be viewed at http://www.interscience.wiley.com/jpages/1058-8388/suppmat) revealed that two genes are very similar to mouse *Cadm1*, whereas two other genes are very similar to mouse *Cadm2*. Given that the ancestor of teleost fish underwent a genome duplication before branching off from the tetrapod lineage during evolution (Postlethwait et al., 2004), we hypothesized that the genes similar to mouse Cadm1 and 2 are co-orthologs. We have named these *cadm1a* and *cadm1b*, and *cadm2a* and *cadm2b*. The respective co-ortholog protein sequences share higher homology between each other (80.6% and 74.1% identity for *cadm1* and *2* genes, respectively, Fig. 1B) than with any other *cadm* genes (42.2% average identity in pairwise comparison).

Analysis of conserved synteny between phyla can provide evidence of orthology and is necessary to determine whether genes are duplicates arising from a genome duplication (Postlethwait et al., 2004). Radiation hybrid mapping determined that *cadm1a*, *1b*, *2a*, *2b*, *3*, and *4* map to linkage groups 21, 16, 2, 15, 10, and 15, respectively. Because some of these locations differ from the assigned chromosomal locations in the latest zebrafish genome assembly (Zv7), we have compared the scaffolds containing the zebrafish *cadm* genes with

the chromosomal region surrounding the murine *Cadm* genes. This analysis demonstrates that the neighboring genes of the zebrafish co-orthologs of *Cadm1* are also found flanking the murine gene. In fact, three orthologous genes are found in all three chromosomal regions: *jam3*, *igsf9b*, and *grit* (Supplementary Figure S2). This confirms that *cadm1a* and *1b* are derived from a duplication of the ancestral chromosomal region (Postlethwait et al., 2004).

Similar analyses revealed conserved syntenies between zebrafish and murine *Cadm3* and *4* genes (not shown). However, comparison of syntenic regions for *cadm2a* and *2b* was not possible due to the paucity of neighboring genes. Further supporting the conclusion that *cadm1a*, *1b*, *2a*, and *2b* arose from the teleost genome duplication, all *cadm* genes share the same gene structures as the respective mouse ortholog (Fig. 3). No duplicates have been found to date for *cadm3* and *4*. Because no duplicates have been found for these genes in other bony fish, we conclude that, subsequent to the genome duplication within the teleost lineage, these gene duplicates were probably lost.

Alignment of the six zebrafish Cadm protein sequences (Fig. 1B,C) reveals that the Cadm2 protein sequences are more closely related to Cadm3 (49.5% average identity) than to Cadm1 and 4 (45.9% and 35.2% average identity, respectively). Cadm4 is a more distant member of the family, with an average amino acid identity of 37.1%. The phylogenetic comparison between, human, mouse, toad, and zebrafish sequences confirms this relationship of zebrafish Cadm proteins (Fig. 2A).

When compared with their murine orthologs, the zebrafish Cadm proteins show on average 55% identity (Supplementary Fig. 1A–D). The strongest divergence is seen in the N-terminus of the proteins, encoding the signal peptides. In contrast, the C-terminal portions of the Cadms, including the intracellular protein–protein interaction domains are highly conserved across phyla (Fig. 2B). This striking conservation reflects constraints upon evolutionary mechanisms, suggesting an important function for both the 4.1B and PDZ type II binding domains. The Cadm1 PDZ binding domain is relevant to synaptogenesis as demonstrated by *in vitro* experiments (Biederer et al., 2002), while the 4.1B binding domain has been shown to-mediate interactions with proteins important for actin cytoskeleton stabilization (Yageta et al., 2002).

We have also compared the genomic organization of mouse and zebrafish *Cadm* genes. We focused our attention on their coding sequences, excluding 5′- and 3′-untranslated regions of the genes. Thus, the number of exons might be under-represented. Nevertheless, there are striking similarities between the mouse and zebrafish gene structures (Fig. 3). Almost all of the exons are of the same size and encode the same protein region, suggesting that, despite the large evolutionary distance that separates these species, the genes are remarkably well conserved. Here, we highlight the few differences.

When comparing mouse and zebrafish genes, we see three cases of missing introns, resulting in extended exons. Loss of introns appears to have happened both in mouse and in zebrafish, such that zebrafish *cadm1a* and *1b* exons 1 and 2 correspond exactly to mouse *Cadm1* exon 1, and mouse *Cadm4* exons 3 and 4 appear to have been contracted into zebrafish exon 3. This loss of an intron from zebrafish *cadm4* gene is substantiated by the medaka *cadm4* gene (EnsEmbl ENSORGL00000004868), which possesses the same structure as its zebrafish ortholog. Unusually, the lengths of the first two exons of the *cadm3* gene do not correspond to the lengths of the murine counterparts. We have confirmed the sequence of zebrafish *cadm3* by identifying expressed sequence tags that cover at least the first three exons of the gene (for example, EST CN507252).

Another interesting point is that the murine *Cadm* genes show an increased number of alternatively spliced exons when compared with the zebrafish genes (hatched boxes in Fig. 3). Both *Cadm2* and *Cadm3* show additional exons 9 and 2, respectively, which are alternatively spliced for exons 3 and 8. The zebrafish *cadm1* genes contain two exons (9 and 10) that can be alternatively spliced, and potentially produce four different isoforms, just as in mouse and human (Biederer, 2006). Using zebrafish adult brain cDNA, we were able to recover three of these isoforms for *cadm1a*, revealing an identical splice pattern between mouse and zebrafish. However, we have identified only one isoform for the *cadm1b* locus. Additionally, a third alternatively spliced exon in *Cadm1* has been identified in the mouse and human genomes (Biederer, 2006). It is probable that additional splice variants also exist in zebrafish and that they are differentially regulated during development and in various tissues. This finding would explain the different number of isoforms we recovered for the *cadm1* loci by screening adult central nervous system cDNA.

In conclusion, despite their relatively large evolutionary distance, the *cadm* genes are remarkably conserved between zebrafish and mammals, suggesting that their functions are probably also conserved. In addition, the maintenance of the duplicated *cadm1* and *cadm2* loci in zebrafish indicates that multiple functions of the mammalian orthologs may have been partitioned to the co-orthologs. On the other hand, it is possible that the co-orthologs may carry out the same function but in different tissues or at different times during development, as has been seen for other co-orthologs in teleost fish (Postlethwait et al., 2004).

Expression Patterns of *cadm* Genes

We assayed the temporal and spatial distribution of *cadm* gene expression during zebrafish development by whole-mount RNA in situ hybridization (ISH), from approximately 10 hours postfertilization (hpf) through 72 hpf. We are confident that the probes to all six genes are specific and do not cross-react, given their distinct expression patterns and the fact that ISH using sense probes did not show any staining.

No expression was seen at 10 hpf, but by 15 hpf, all of the *cadm* genes showed evidence of expression, in particular in the developing head (not shown). At 24 hpf, development has progressed to the point that most major organ systems have begun to form, and expression of all six *cadms* was seen throughout the central nervous system, including the eye and spinal cord (Fig. 4). While expression for all *cadm* genes was consistently stronger in the head than in the trunk and tail, their expression patterns in the spinal cord were dynamic and divergent between the *cadm* family members. Expression was evident in different domains of the spinal cord at 24 hpf (Fig. 4, discussed below, see Fig. 7). Furthermore, the spinal cord expression of *cadm* genes was evident as a wave of expression during development. For example, the expression of *cadm2a* was decreased in the rostral spinal cord by 48 hpf, becoming undetectable throughout the spinal cord by 72 hpf (Fig. 4C, insets). Also, the *cadm1* genes were present in a rostrocaudal gradient at 24 hpf, with no expression seen above the yolk tube and caudal to this (Fig. 4A,B, dashed lines). These patterns are confirmed for all *cadm* genes by ISH on sections at 24 and 48 hpf (not shown). Because many neurons in the spinal cord develop in a rostrocaudal gradient (Lewis and Eisen, 2003), it is probable that the *cadm* genes are important for a specific event during neuronal maturation.

The general pattern of strong expression throughout the brain persisted to 72 hpf. However, some expression was also seen outside of the nervous system; all 6 *cadms* were expressed in the precartilage of the pectoral fin buds at 48 hpf (arrows in Fig. 5A–F), the pancreas, gut, and developing swim bladder (not shown). All *cadm* genes were expressed at low levels in the adult testis, with *cadm3* showing the strongest expression (not shown), suggesting that,

as in mammals (Fujita et al., 2006), these cell adhesion molecules may play a role in the maturation of sperm. Due to the predominant expression of the *cadm* genes in the nervous system during development, we have focused our attention on the brain, visual system and spinal cord of the zebrafish.

cadm Expression in Developing and Adult Brain

The *cadms* were broadly expressed throughout the brain both during development and in the adult (Fig. 4, Fig 5). We have summarized the brain expression patterns in Table 2 and Table 3, and detailed descriptions of the expression patterns are in the Supplementary Materials. The tables and descriptions are based on analyses of both horizontal and coronal sections of the whole brain, the majority of which are not shown here.

In general, the *cadm1* and *2* genes showed weaker and more punctate expression than *cadm3* and *4* (Fig. 5). This may suggest that the four *cadm1* and *2* genes are expressed in subsets of cells within a structure. This expression may be in distinct cells within each structure, however, more detailed analyses will be required to determine whether this is true. Conversely, *cadm3* and *4* appeared to be expressed in more cell types and more brain regions throughout the nervous system than *cadm1a*, *1b*, *2a*, and *2b* (Table 2, Table 3). Whereas as the co-orthologs were sparsely expressed in cells at 48 hpf, the same structures solidly expressed *cadm3* and *4* (for example Fig. 5K,L). This finding may reflect the expression of *cadm3* and *4* in both neurons and glial cells, as has been demonstrated in the mouse (Kakunaga et al., 2005;Gruber-Olipitz et al., 2006;Maurel et al., 2007;Spiegel et al., 2007).

One of the areas with the most striking expression at 48 hpf was the *medulla oblongata* (MO). Expression varied considerably for each *cadm* gene through the rostrocaudal length of the MO. While *cadm1a* showed weak punctate expression medially in the anterior portion of the MO, it was expressed predominantly in the periphery of the posterior MO (Fig. 5M, arrow). In contrast, *cadm1b* was hardly expressed in the anterior MO, showing a scattered punctate staining toward the middle, becoming concentrated dorsally toward the posterior end of the structure (Fig. 5N). *Cadm2a* showed strong staining mostly in the ventral region of the whole MO in a series of stripes (arrowheads in Fig. 5O), whereas *cadm2b* was only weakly expressed in the ventral region of the MO (Fig. 5P, asterisks). Both *cadm3* and *4* were expressed strongly throughout the MO (Fig. 5Q,R).

It was interesting to note that the co-orthologs for the *cadm* genes *1* and *2* demonstrated overlapping, yet more restricted expression patterns both at 48 hpf and in adult. For example, *cadm1a* was expressed throughout the dorsal thalamus and the posterior tuberculum at 48 hpf (arrows in Fig. 5G), while *cadm1b* was only expressed in the ventral part of the posterior tuberculum (arrow in Fig. 5N). Analogously, *cadm2a* was expressed throughout both the granular zone of the optic tectum (OT) and lateral division of the *valvula cerebelli* (VC) in adult zebrafish (arrows in Fig. 5U), whereas *cadm2b* was only present in the OT (arrows in Fig. 5V). The restriction of expression of one of the co-orthologs, rather than a distinct expression pattern, may suggest a partitioning of function between the two co-orthologs.

On close examination, it was apparent that, in many areas, the *cadm1* and *2* genes showed inverse expression patterns in the developing and adult brain. This is especially clear in Table 2 and Table 3, which reveal that inverse staining was seen for the olfactory epithelium, *griseum tectale*, VC, cerebellar plate, and the trigeminal ganglion at 48 hpf. In the adult, the olfactory bulb, the anterior thalamic nuclei, both the magnocellular and periventricular pretectal nuclei, the *torus longitudinalis* (TL), the valvula, the caudal lobe of the cerebellum and the octaval nucleus all showed inverse staining for *cadm1* and *2*. Clear

examples are the TL and VC. Both *cadm1* genes were expressed in the TL (arrows in Fig. 5S,T), while *cadm2a* and *2b* were absent (Fig. 5U,V). In contrast, both *cadm1a* and *1b* were not detected in the VC, whereas *cadm2a* and *2b* were present (arrows in Fig. 6S–V). This inverse expression pattern suggests that transcription for these genes may be coregulated in an exclusionary manner.

cadm Expression in the Developing and Adult Visual System

We examined in detail the *cadm* gene expression patterns in retina at 48 hpf, 5 days postfertilization (dpf) and adult eyes. Lamination of the retina occurs with a central to peripheral and inner to outer gradient of maturation and is generally complete by 48 hpf (Schmitt and Dowling, 1994). At this age of development, most *cadm* genes were expressed in a punctate, scattered manner. For instance, *cadm1b* and *2b* were expressed throughout the retina, lens, and the cornea in this manner (Fig. 6B and D, respectively). A similar scattered expression was seen for *cadm1a*, *2a*, and *4* in the inner nuclear layer (INL), outer nuclear layer (ONL), and the marginal zone (Fig. 6A, C, and F, respectively). Because the neuroepithelial cells within these regions are still actively dividing during this period of rapid growth, these expression patterns might represent mitotically active cells. Expression in mitotically active cells would then suggest a novel function for these *cadms* during early neuronal differentiation.

The ganglion cell layer (GCL) displayed the most varied expression pattern of all the layers of the retina at 48 hpf. *cadm1a* expression appeared to follow the wave of differentiation of this layer: high expression of *cadm1a* was seen in the periphery of the layer where the neuroblasts are still mitotically active at this stage, while being down-regulated in the more mature GCL (Fig. 6A, arrows). Conversely, *cadm4* expression in the GCL was higher in more mature neurons (Fig. 6F, arrows), while *cadm2a* was expressed in the entire GCL independently of the level of maturation of the ganglion cells (Fig. 6C). In contrast, and as seen in the brain, *cadm3* was expressed in the vast majority of cells in the retina (Fig. 6E).

Visual function is observed as early as 3 dpf and is fully functional by 5 dpf (Schmitt and Dowling, 1994). At this time, *cadm* genes were expressed in partially overlapping domains within most retinal layers. In the GCL, *cadm1a*, *2a*, and *3* were expressed in most of the neurons (Fig. 6G,I,K), while *cadm1b*, *2b*, and *4* (Fig. 6H, J and L) were seen in subsets of cells. In the INL, *cadm1a*, *1b*, *2a*, *2b*, and *4* were expressed in restricted populations of cells (Fig. 6G–J,L). These expression patterns suggest that all *cadm* genes are expressed in amacrine cells, bipolar cells, and Müller glia. In addition, it appears that *cadm1a*, *2b*, *3*, and *4* are expressed in horizontal cells at the outer limit of the INL (Fig. 6G,J–L) and *cadm3* and *4* in the ONL (Fig. 6K,L). Notably, the periphery of the ONL also expressed *cadm1a*, *2b*, and *4*, and might represent dividing retinal precursor cells (Fig. 6G,J,L, arrowheads).

All *cadm* genes remain expressed in the adult eyes with partially overlapping domains of expression, generally mirroring the expression at 5 dpf. However, *cadm1a* and *1b* were down-regulated in the GCL in adult eyes (Fig. 6M,N). *Cadm2a*, *2b*, *3*, and *4* were detected in the GCL, albeit at different levels (Fig. 6O–R). *Cadm2a* and *2b* were expressed in a small subset of the GCL cells (Fig. 6O,P), whereas *cadm3* and *4* were present in most of the ganglion cells (Fig. 6Q,R). All six *cadm* genes were expressed in the INL: *cadm1a*, *1b*, *2b*, and *4* were restricted to the medial domain of the INL, suggesting they are expressed in bipolar, but not amacrine or horizontal cells. In contrast, *cadm2a* and *3* presented a broader expression in the INL, including the amacrine cell domain; *cadm3* was expressed at a higher level in the amacrine cells than in the other regions of the INL. The ONL presented a sparse expression of *cadm1a*, *1b*, *2a*, and *4* in both inner and outer segments, suggesting they are present in a small subpopulation of rods and cones; *cadm3* was detected in virtually all cells

of the ONL. In contrast, *cadm2b* was restricted to the outer segment and, therefore, expressed in the rod population of the ONL.

In summary, *cadm* genes are highly regulated in the course of the development of the retina. They show partially overlapping domains of expression within the retina, but can be restricted to distinct cell populations composing each layer. These dynamic expression patterns suggest that the various *cadms* may perform distinct functions at discrete times during differentiation of retinal cells.

cadm Expression in the Developing Spinal Cord

We examined the expression of the six *cadm* genes in the spinal cord at 48 hpf, because the wave of expression of these genes was in the widest variety of cell types at this time. We present both dorsal views of RNA ISH in whole-mount embryos (Fig. 7A–F) and in sections (Fig. 7G–L). This strategy allows appreciation of the spatial distribution of expression along both rostrocaudal and dorsoventral axes. We used the following spatial subdivisions along these axes to assign expression to particular cell types. The dorsal domain is defined by the sensory Rohon-Beard neuron, whereas the intermediate domain is composed of various types of interneurons. Finally, the ventral domain comprises floor plate cells and motoneurons. The morphology, localization, and axon projections of zebrafish spinal cord neurons have been well described (Hale et al., 2001; Lewis and Eisen, 2003).

Diffuse expression was seen for all *cadms* in the various domains of the spinal cord; however, several expression patterns were suggestive of strong expression in distinct populations of spinal neurons (Fig. 4, Fig. 7). *cadm1a* was mostly detected in the intermediate domain of the spinal cord, probably in a subpopulation of interneurons. It was more strongly detected in the ventral part of this domain in the caudal spinal cord, suggesting that it is expressed first in a ventral population of interneurons, before expanding to a more dorsal domain. *cadm1b* was expressed in the intermediate domain of the spinal cord and, in particular, in its dorsal aspect, suggesting that *cadm1b* is expressed in dorsal interneurons.

cadm2a was expressed throughout the dorsal and intermediate region of the spinal cord and was particularly strong in cells at the midline in the dorsal spinal cord, suggesting expression in sensory Rohon-Beard neurons (Fig. 7C, arrowheads). In contrast, *cadm2b* was weakly expressed and excluded from the dorsal and ventral domain, suggesting that it may be exclusively present in interneurons.

cadm3 and *4* were widely expressed in the spinal cord, including the proliferating ventricular zone. *cadm3* showed strong staining in dorsal neurons, including Rohon-Beard neurons (Fig. 7, arrowhead in E and arrow in K). In contrast, *cadm4* was more highly expressed in the ventral domain of the spinal cord, suggestive of floor plate cells and motoneurons (arrow Fig. 7L). Additionally, *cadm3* was present in other neuronal tissues in the trunk and tail, the localization suggesting expression in dorsal root and sympathetic ganglia (Fig. 7K, arrowhead). *cadm4* was also detected in the developing neuromasts of the lateral line system (Fig. 5R).

As Cadm1 is a potential mediator of synaptogenesis (Biederer et al., 2002) and the expression of all six *cadm* genes is found in discrete neuronal subtypes of the developing spinal cord at a time when neuronal circuits are becoming functional (16–48 hpf; Drapeau et al., 2002), it is reasonable to hypothesize that these molecules could mediate synaptogenesis or determine synaptic specificity for the early circuits in spinal cord during development. With this expression data in hand, it may now be possible to perform a functional analysis of this family of cell adhesion molecules in vivo.

Concluding Remarks

The zebrafish *cadm* genes show strong similarities with their tetrapod orthologs in terms of genetic structure and protein organization. Their expression is highly regulated during development of the central nervous system, and they show partially overlapping domains of expression within each structure. The maintenance of two duplicated *cadm* loci in zebrafish with similar expression patterns indicates the probable acquisition of novel functions or the partition of functions during the course of evolution. The dynamic expression patterns during development suggest that these cell adhesion molecules may play multiple roles during neuronal differentiation, including steps in neuronal cell fate decisions and synaptogenesis.

EXPERIMENTAL PROCEDURES

Cloning the *cadm* Genes

To clone the zebrafish *cadm* genes, we searched the zebrafish genome assembly Zv6 from the Sanger Institute (http://www.ensemble.org/Danio_rerio) by tBLASTn (http://www.ncbi.nlm.nih.gov/BLAST) comparison with the mouse Cadm1 to Cadm4 protein sequences (NP_997558.2, NP_848836, NP_444429 and NP_694752 respectively). This search revealed six *cadm* genes in zebrafish, with a duplication of the *cadm1* and *cadm2* loci. The best hits on the zebrafish genome were then compared with the GenBank database to recover published sequences. Full-length cDNA for one *cadm2* (*Igsf4d*, renamed in this study *cadm2a*) and *cadm4* were found in the Genbank references NM_200664.1 (IMAGE clone 5603809) and BC085419 (IMAGE clone 7227860), respectively. Partial cloning of *cadm1a* (third Ig domain, transmembrane domain and cytoplasmic tail of the protein), based on a partial sequence of RA175 (AB183400) was performed by RT-PCR of zebrafish adult brain cDNA (forward primer: ATGCTAGCAAGGAGAGATAT reverse primer TCAGGACTCAGATATAGTAT). The database search with the mouse *Cadm1* did not allow recovery of the highly divergent 5′ fragment of zebrafish *cadm1a*. Comparison of the medaka *cadm1* sequence (EnsEmbl ENSORLG00000005159) by BLASTn with the zebrafish genome revealed a 5′ fragment (without the signal peptide sequence), which was cloned by RT-PCR (forward primer, CAGAATCTCATATCGGACAACGTC and reverse primer, GCCTGAAAGTCCTTGACTGC). We used the FirstChoice RLM-RACE Kit (Ambion) to recover the signal peptide of *cadm1a* (outer primer: TCCTCGCGGGACTCTATGAT and inner primer: CGAGCATCTTTCAGGGGTCT). Zebrafish *cadm1b* is derived from partial zebrafish cDNA sequences (XM_ 001337106, XM_685581 and BU710444) and cloned by RT-PCR of 72 hpf whole zebrafish embryo cDNA. These published sequences did not contain the signal peptide of *cadm1b*, which was cloned by 5′-RACE PCR (outer primer, CTCGCGGGACTCTAAGATTG and inner primer, CGGTGGATCCGTGTAGAGTT). *Cadm2b* and *cadm3* were deduced from XM_001342480 and XM_695311 (*cadm2b*) and NM_001045246 (*cadm3*) and cloned by RT-PCR from zebrafish adult brain cDNA (forward primer, TGCACGCAACAAATATCCTC and reverse primer, CTAAATGAAGTACTCTTTCTTTTCC for *cadm2b* and forward primer, CTATTGGCTGTAGCGTGCTG and reverse primer, GAAGCGTGTGAAGGAAGAGG for *cadm3*).

Protein Alignment and Phylogenetic Analysis

Deduced protein sequences for the zebrafish *cadm* genes based on the cloned cDNA sequences have been aligned with ClustalW. This alignment, using only the shortest isoforms, was used to generate a phylogenetic tree with the MEGA package, based on Poisson-corrected neighbor-joining amino-acid distances and considering gap pairwise deletion. Protein accession number (GenBank for human and mouse and EnsEmbl for

Xenopus) used for the analysis were, NP_055148.3 (*Homo-sapiens*; Hs_CADM1), NP_694854.2 (Hs_CADM2), NP_067012.1 (Hs_CADM3), NP_660339.1 (Hs_CADM3), NP_997559.1 (*Mus musculus*, Mm_Cadm1), NP_848836.1 (Mm_Cadm2), NP_444429.1 (Mm_Cadm3), NP_694752.1 (Mm_Cadm4), ENSXETP00000033613 (*Xenopus tropicalis*, Xt_cadm1), ENSXETG00000026489 (Xt_cadm2), ENSXETP00000041942 (Xt_cadm3), and ENSXETP00000033087 (Xt_cadm4).

Mapping and Conserved Synteny

The zebrafish *cadm* genes were mapped on the LN54 radiation hybrid mapping panel (Hukriede et al., 1999) as described (Postlethwait, 2000). The chromosomal position of each gene was determined through the use of the mapping program of Dr. I. Dawid (http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi). Two sets of primers were used to confirm the localization on the LN54 panel (primer sequences available on demand). For comparative mapping with murine *Cadm* genes, zebrafish *cadm* genes were considered orthologs when the human and zebrafish genes were best hits on the reciprocal BLASTp searches on the other genome. The mouse *Cadm* sequences used for this analysis are as indicated in Protein Alignment and Phylogenetic Analysis.

In Situ Hybridization

Zebrafish embryos, larvae and adults (AB/Tübingen strain) were raised at 28.5°C according to standard protocols (Westerfield, 2000). Sense and anti-sense probes were in vitro transcribed and digoxigenin tagged according to the manufacturer's protocol (Roche) from linearized pCRII-TOPO (Invitrogen) *cadm1a* plasmid or from PCR fragments of *cadm1b*, *2a*, *2b*, *3*, and *4* linked to a T7 or T3 promoter. *cadm1a* probes encompass the *cadm1a* cDNA from nt 623 to 1384 of the CDS; *cadm1b*, from nt 381 to 856, *cadm2a*, from nt 331 to 1154; *cadm2b*, from nt 351 to 1048; *cadm3*, from nt 239 to 992; *cadm4*, from nt 278 to 992. ISH on frozen sections was performed according to the protocol described by Jensen et al. (2001) with the following changes. Labeled probes were diluted 1:200 in hybridization buffer and heated to 68°C for 30 min, 200 µl of diluted probe was used per slide. Hybridization was carried out at 68°C. Posthybridization washes were increased to 3 washes in 50% form-amide, 1× standard saline citrate (SSC), 0.1% Tween-20 at 68°C for 30 min and three washes in MABT (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween-20) for 30 min at room temperature. Anti-digoxigenin conjugated to AP antibody (Roche) was used at a 1:1500 dilution. Whole-mount ISH was carried out according to the protocol as seen at http://courses.mbl.edu/zebrafish/faculty/houart_wilson/pdf/in_situ_hybridization.pdf. BM purple AP Substrate (Roche) was used for coloration. Images of sections and whole-mount embryos were taken on a Zeiss Axioplan microscope or a Leica MZ6 stereomicroscope using a Nikon Coolpix 990 or 4500 digital camera. The images were arranged for presentation purposes using Adobe Photoshop. Annotations of the anatomy of the embryonic and adult brain are done according to Wulliman et al. (1996) and Muller and Wulliman (2005).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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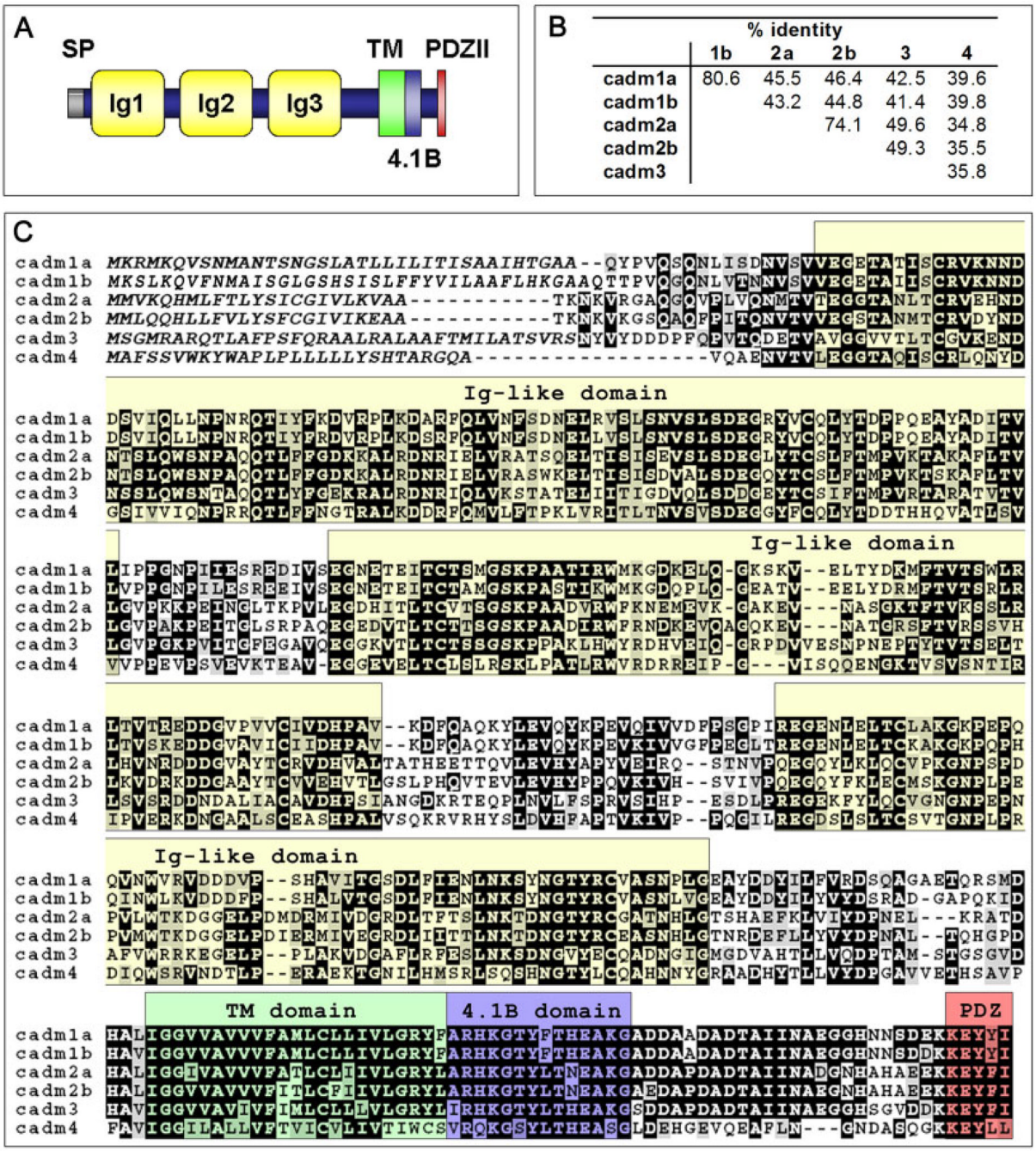

Fig. 1.

Structure and sequence of zebrafish Cadm proteins. **A**: Schematic representation of Cadm protein structure. All Cadms contain a signal peptide (SP), three Ig-like domains (Ig), a transmembrane (TM) domain, and a cytoplasmic tail with a 4.1B binding domain (4.1B) and the PDZ type II binding domain (PDZII). **B**: Amino acid identity as a percentage in pairwise alignments for the six Cadm protein sequences. **C**: Protein alignment of the zebrafish Cadm protein sequences. Conserved positions with an identical amino acid (black) and conserved subtitutions (gray) have been shadowed. Putative signal peptide for each cadm is in italic; each structural domain is indicated above the aligned sequences and color shaded as in A.

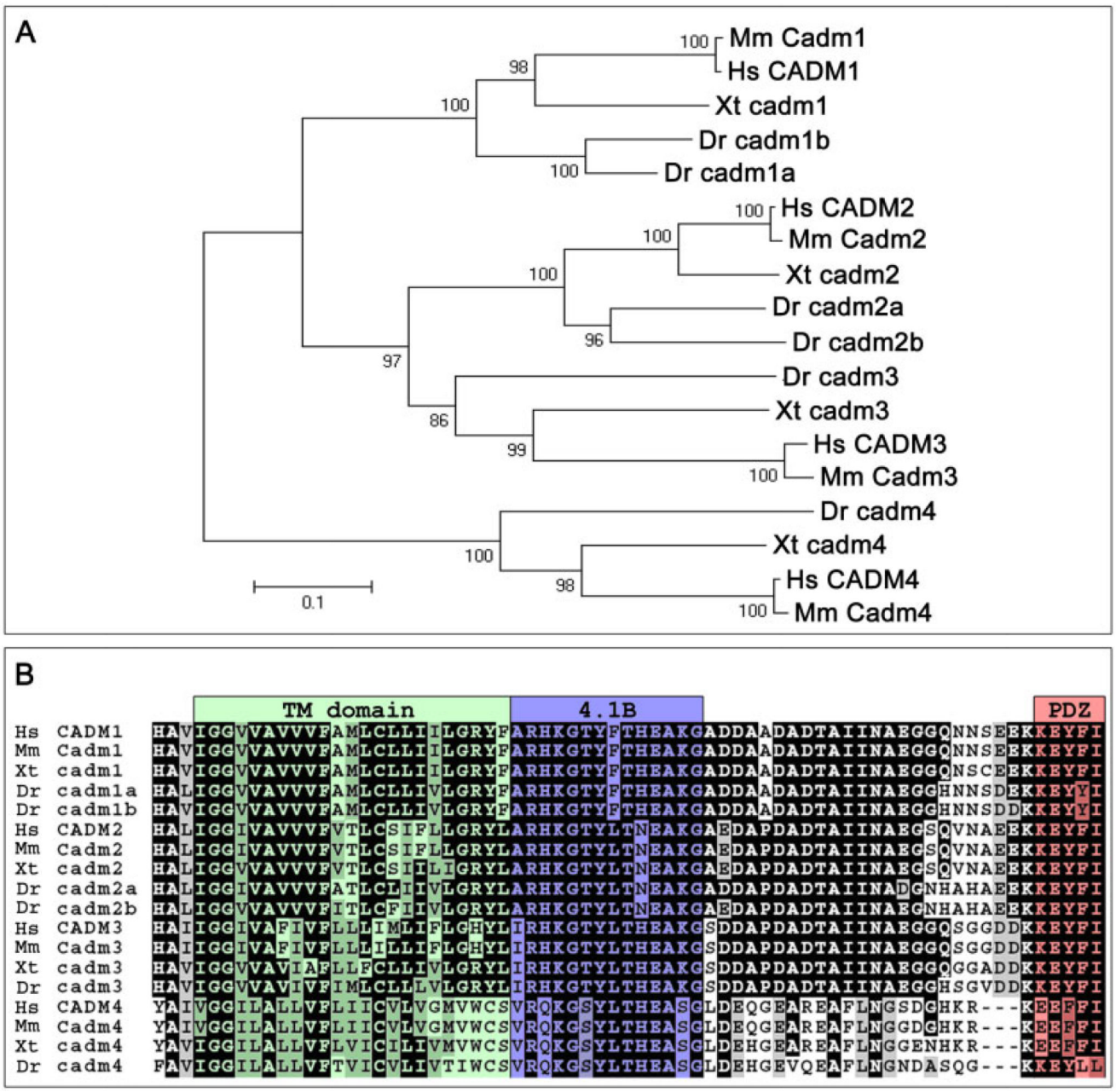

Fig. 2.

Phylogeny of the Cadm proteins. **A**: Relationships between the Cadm protein sequences of various vertebrates (Hs, *Homo sapiens*; Mm, *Mus musculus*; Xt, *Xenopus tropicalis*; Dr, *Danio rerio*) are shown in a phylogenetic tree. Amino acid sequences were aligned by Clustal-X. Sequences were trimmed to include unambiguously aligned regions, and phylogenic analysis used the Poisson-corrected neighbor-joining method. The branch lengths (numbers) are the percentage of bootstrap values for 1,000 replicas. Scale bar = 0.1 substitutions per site. **B**: Alignment of the transmembrane domain and cytoplasmic tail of the Cadm proteins highlights the high level of conservation of the structural domains among vertebrates. Conserved positions with an identical amino acid (black) and conserved subtitutions (gray) have been shadowed; the color shading of domains is as in Figure 1A.

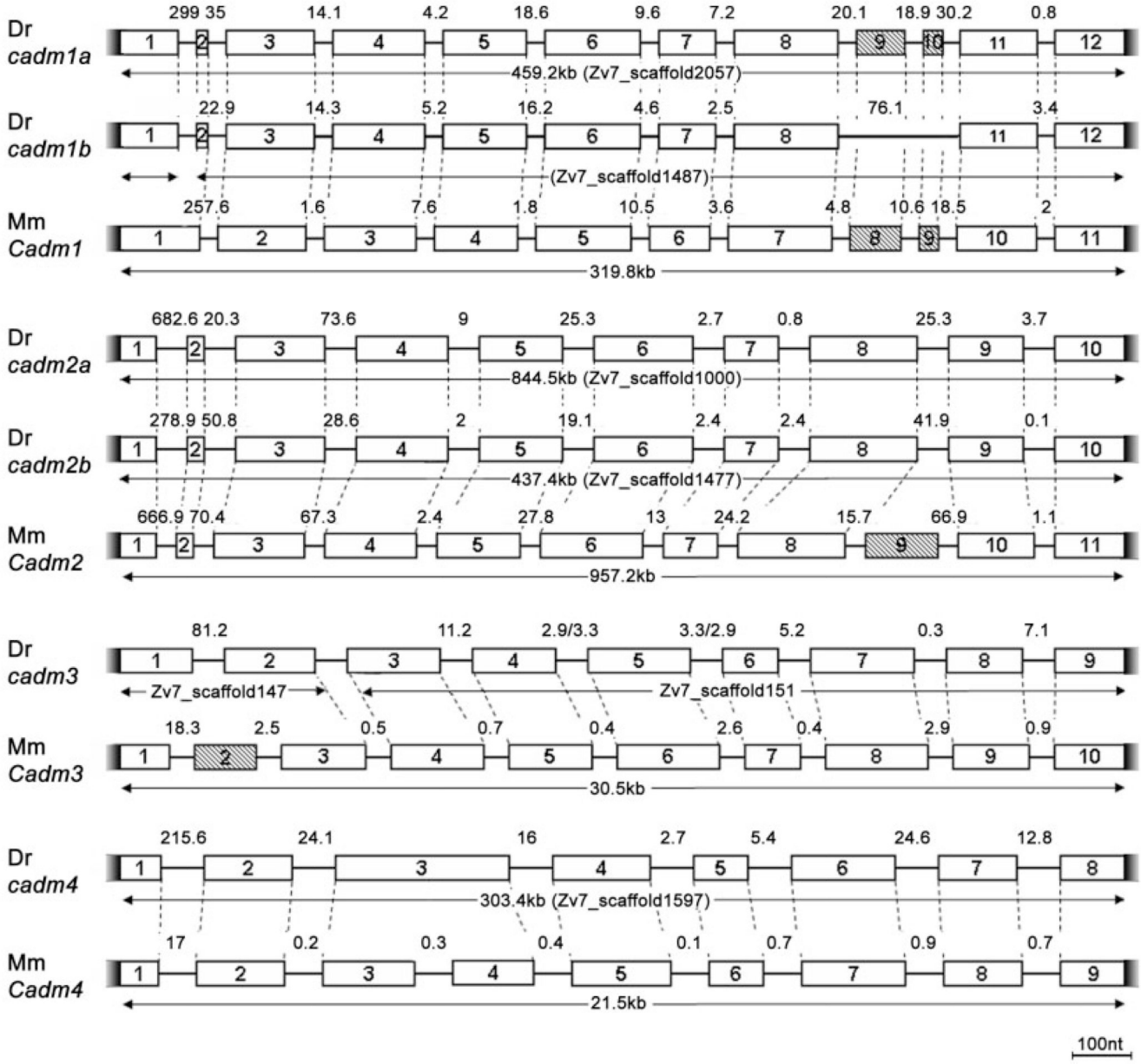

Fig. 3.

Genomic analysis of zebrafish *cadm* genes. Comparison of the zebrafish (Dr) and mouse (Mm) loci. Exons are represented by numbered boxes. Shaded boxes show exons subjected to alternative splicing. Exon–intron boundaries that correspond between zebrafish and mouse genes are represented by dashed lines. All corresponding exons between the two species are of identical length, unless indicated otherwise in the text. The scale bar (100 nt) is for exons; the sizes of introns are indicated in kilobases. Notice that zebrafish *cadm1b* and *3* are both aligned on two different scaffolds in Zv7 assembled zebrafish genome. The first exon of *cadm1b* is found on Zv7_scaffold1472, whereas the other exons on Zv7_scaffold1487, suggesting a large first intron. Similarly, the first two exons of *cadm3* are found in Zv7_scaffold147, while the rest of the gene is on Zv7_scaffold151.

Fig. 4.

Expression of *cadms* at 24 hours post-fertilization (hpf). In situ hybridization performed on 24 hpf whole-mount zebrafish embryos reveals that the six *cadm* genes are expressed throughout the central nervous system, in particular in the developing brain, the visual system and the spinal cord. **A–C**: Expression for *cadm1a* and *1b* is in a rostrocaudal gradient (A,B), being undetectable caudal to the dashed lines. *cadm2a* expression decreases rostrally at 48 hpf and is lost by 72 hpf (insets in C).

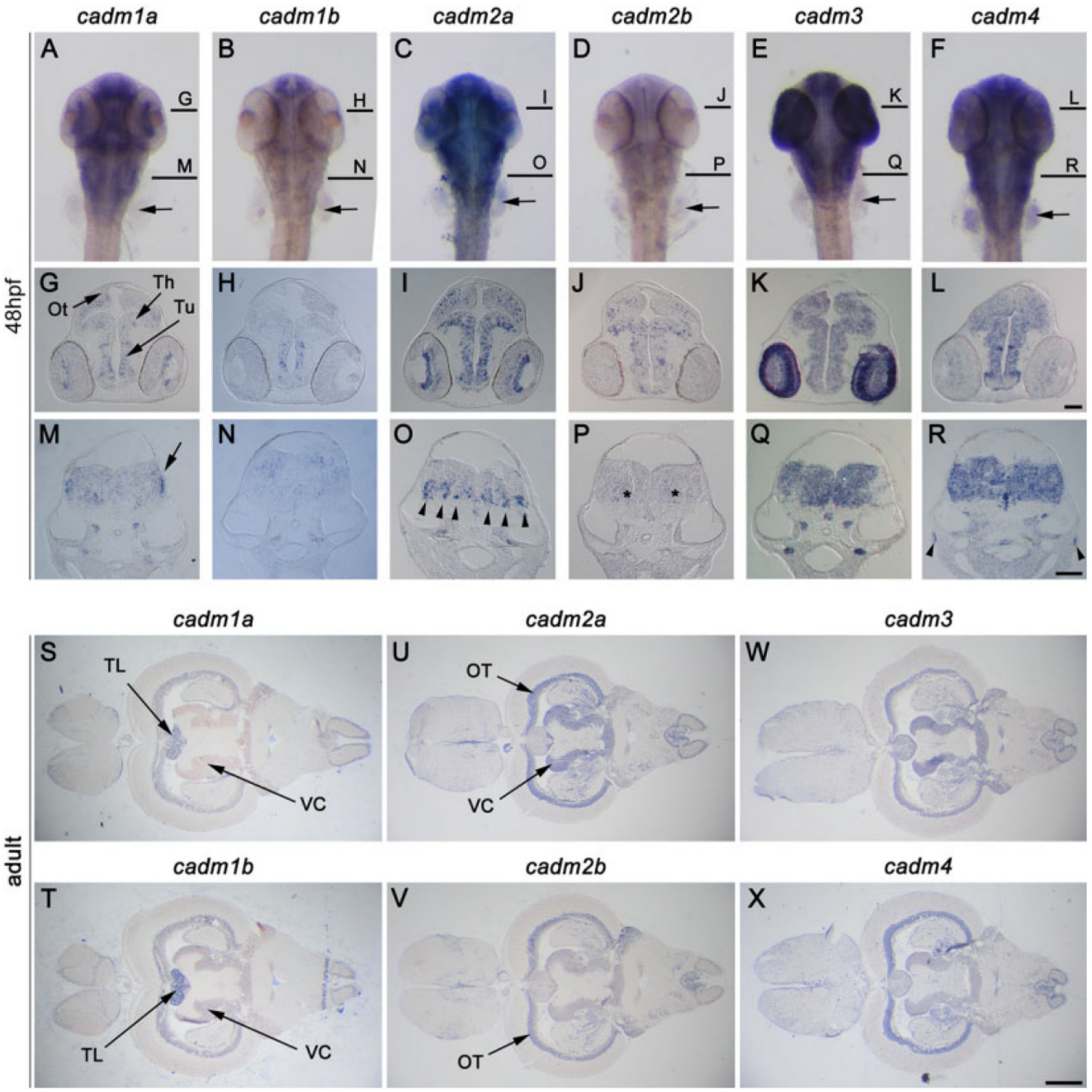

Fig. 5.

Expression of *cadms* in the brain. **A–R**: In situ hybridization (ISH) staining in 48 hours postfertilization (hpf) zebrafish embryos; a dorsal view of whole-mount zebrafish (A–F), and cross-sections at the midbrain (G–L), and hindbrain (M–R) are presented for each *cadm* gene. Lines in A–F represent levels of the sections in G–L and M–R. **S–X**: *cadm* expression visualized in horizontal sections of adult brain. The *cadm* genes are expressed broadly in the developing and adult brain and show partially overlapping domains of expression. *cadm2a* is strongly expressed in the ventral *medulla oblongata* (MO) (arrowheads in O), while *cadm2b* is more diffuse in this region (asterisks in P). The posterior part of the MO shows strong staining for *cadm1a* (arrow in M). Ot, developing optic tectum; OT, optic tectum granular

layer L3; Th, thalamus; TL, *torus longitudinalis*; Tu, tuberculum; VC, *valvula cerebellis*. Orientations are anterior at the top in A–F; dorsal at the top in G–R; anterior to the left in S–X. Scale bars = 50 μm in G–L, 50 μm in M–R, 0.5 mm in S–X.

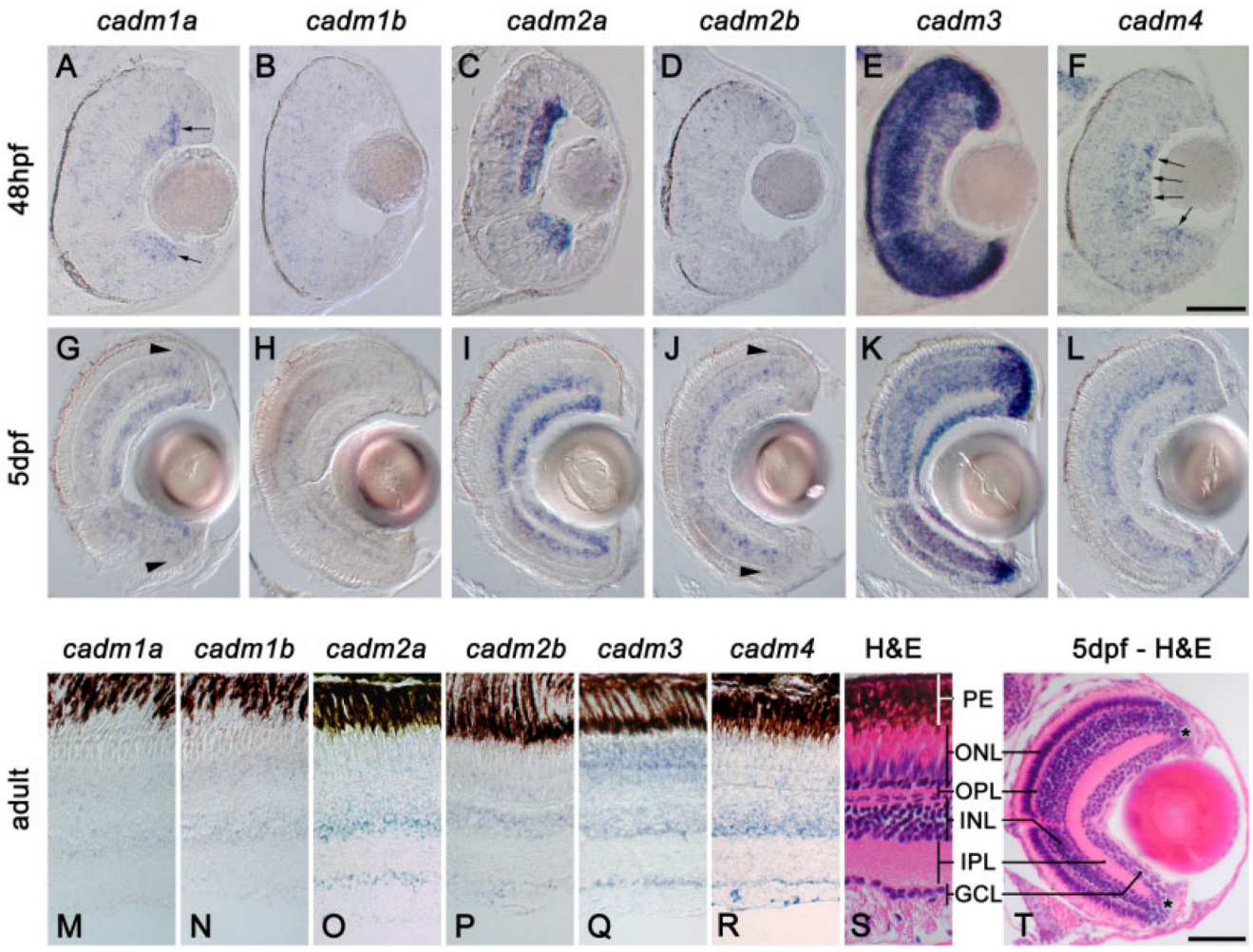

Fig. 6.

Expression of *cadms* in the visual system. In situ hybridization staining in sections of retina at 48 hpf (A–F), 5 days postfertilization (dpf; G–L), and adult zebrafish (M–R). S and T show hematoxylin and eosin staining (H&E) of sections of adult and 5 dpf, respectively; the different layers of the retina are indicated: PE, pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Asterisk in T denotes the location of the marginal zone. *cadm1a* is expressed in the dividing ganglion cells (arrows in A), while *cadm4* is more strongly expressed in the mature cells of the GCL (arrows in F). Arrowheads in G and J reveal precursors at the margin of the outer nuclear layer (ONL). Scale bars = 50 μm in A–F, 50 μm in G–L,T.

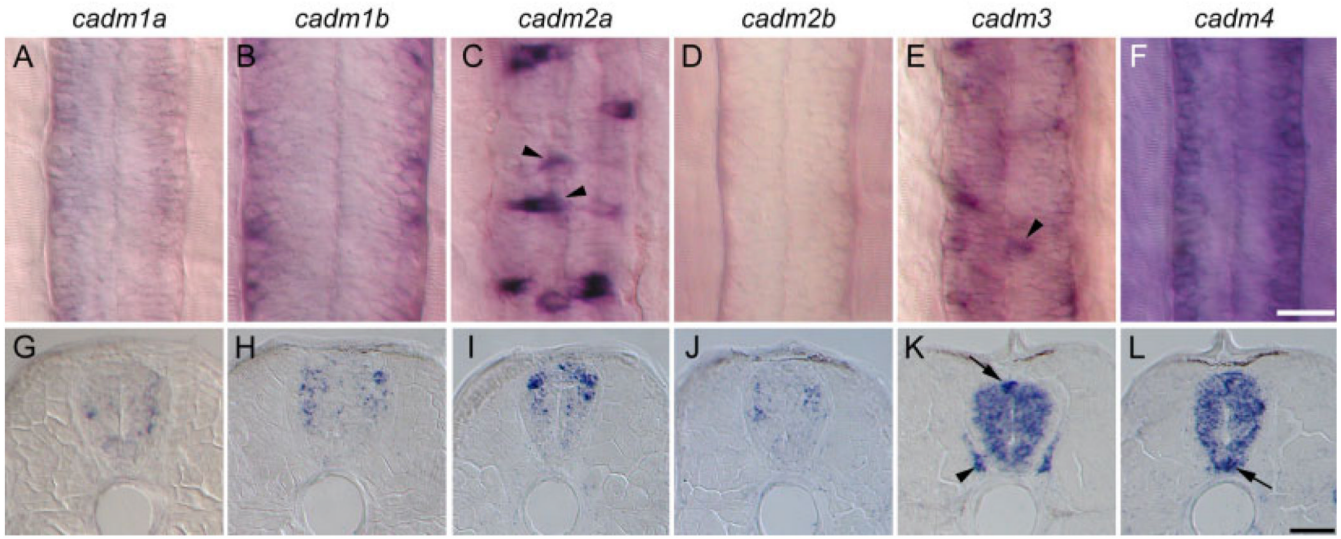

Fig. 7.

Expression of *cadms* in the developing spinal cord. **A–L**: The *cadm* expression revealed by in situ hybridization in whole-mount (dorsal view, A–F) and cross-sections (G–L) of 48 hpf embryos. Due to the strong rostrocaudal gradient of *cadm1b* expression, panels B and H show an anterior localized region of the spinal cord (somites 3–5), immediately behind the hindbrain, whereas all other images are from a region dorsal to the anus (somites 12–15). Staining for *cadm2a* and *3* is in dorsal cells at the midline of the spinal cord, suggestive of sensory Rohon-Beard neurons (arrowheads in C and E, arrow in K). Expression for *cadm3* is also seen in the dorsal root ganglion (arrowhead in K). *cadm4* is strongly expressed in the ventral domain of the spinal cord indicative of floor plate or motoneurons. Scale bars = 20 μm in A–F, 20 μm in G–L.

TABLE 1

Nomenclature of the *Cadm* Gene Family [a]

Human gene name	*CADM1*	*CADM2*	*CADM3*	*CADM4*
Mouse gene name	*Cadm1*	*Cadm2*	*Cadm3*	*Cadm4*
Zebrafish gene name	*cadm1*	*cadm2*	*cadm3*	*cadm4*
Alternate gene and protein names	Necl2	Necl3	Necl1	Necl4
	SynCAM1	SynCAM2	SynCAM3	SynCAM4
	IGSF4A	IGSF4D	IGSF4B	IGSF4C
	Tslc1		Tsll1	
	sgIGSF			
	RA175			

[a]The human and mouse gene names were recently changed from IGSF4 to CADM by the HUGO gene nomenclature committee. We have adopted this nomenclature. Previous names given to these genes and proteins are shown at the bottom. GenBank ID numbers for the novel zebrafish *cadm* genes and isoforms are: *cadm1a*, EU182349, EU182350, EU182351; *cadm1b*, EU182352; *cadm2b*, EU182353; *cadm3*, EU182354.

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TABLE 2

Summary of *cadm* Expression in Brain at 48 Hours Postfertilization [a]

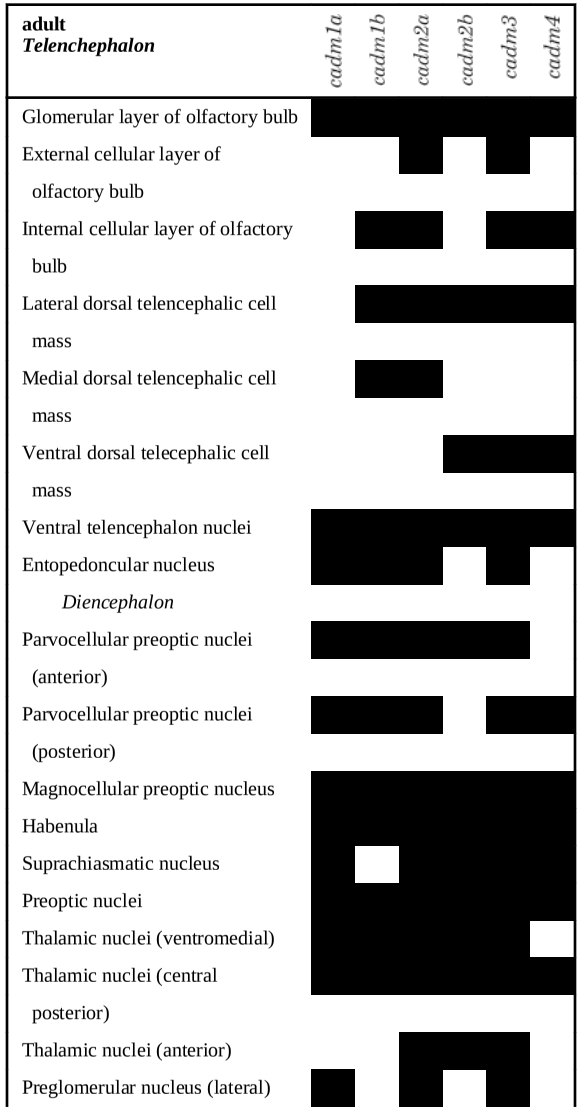
48hpf ***Forebrain***	*cadm1a*	*cadm1b*	*cadm2a*	*cadm2b*	*cadm3*	*cadm4*
Pallium	■	■	■	■	■	■
Olfactory bulb	■	■	■	■	■	■
Epiphysis	■	■	■	■	■	■
Habenula	■	■	■	■	■	■
Subpallium	■	■	■	■	■	■
Olfactory epithelium			■	■	■	
Dorsal thalamus	■	■	■	■	■	■
Ventral thalamus	■	■	■	■	■	■
Griseum tectale			■	■	■	■
Pretectum	■	■	■	■	■	■
Posterior tuberculum (dorsal part)	■	■	■	■	■	■
Posterior tuberculum (ventral part)	■	■	■	■	■	■
Midbrain						
Tectum opticum	■	■	■	■	■	■
Medial longitudinal fasicle nucleus	■	■	■	■	■	■
nucleus						
Midbrain tegmentum	■	■	■	■	■	■
Rostral hypothalamus	■	■	■	■	■	■
Intermediate hypothalamus	■	■	■	■	■	■
Hindbrain						
Valvula cerebelli	■	■			■	■
Cerebellar plate	■	■			■	■
Torus semicircularis	■		■	■	■	■
Trigeminal ganglion			■			
Medulla oblongata	■	■	■	■	■	■
Caudal hypothalamus	■	■		■	■	■
Hypophysis	■		■			■

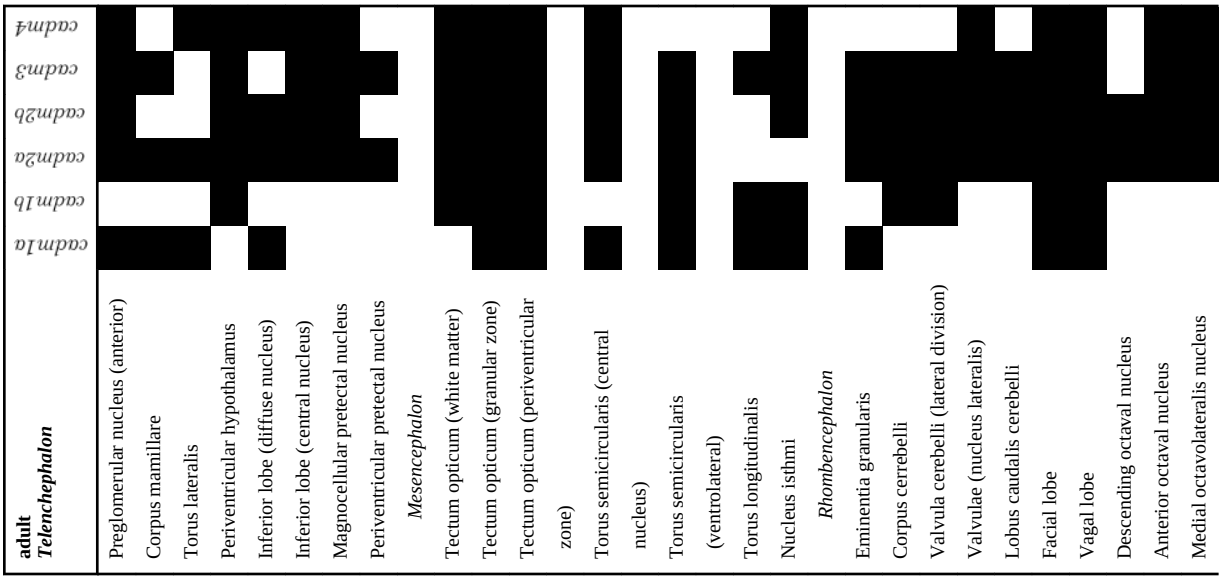
48hpf ***Forebrain***	*cadm1a*	*cadm1b*	*cadm2a*	*cadm2b*	*cadm3*	*cadm4*
Rhombic lip	■		■	■	■	■
Anterior lateral line ganglion				■		
Posterior lateral line ganglion	■		■			
Octaval ganglion	■		■	■	■	■
Facial ganglion						
Glossopharyngeal ganglion						
Vagal ganglion	■		■		■	■

[a] Expression for brain structures are summarized for the six *cadm* genes. A black box denotes the presence of cells expressing the *cadm* gene listed at the top of the columns.

TABLE 3

Summary of *cadm* Expression in Adult Brain





adult ***Telenchephalon***	*cadm1a*	*cadm1b*	*cadm2a*	*cadm2b*	*cadm3*	*cadm4*
Caudal octavolateralis nucleus	■	■	■	■	■	■

[a] Expression for brain structures are summarized for the six *cadm* genes. A black box denotes the presence of cells expressing the *cadm* gene listed at the top of the columns.