An Ancient Adenosine Receptor Gains Olfactory Function in Bony Vertebrates

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Abstract

Nucleotides are an important class of odorants for aquatic vertebrates such as frogs and fishes, but also have manifold signaling roles in other cellular processes. Recently, an adenosine receptor believed to belong to the *adora2* clade has been identified as an olfactory receptor in zebrafish. Here, we set out to elucidate the evolutionary history of both this gene and its olfactory function. We have performed a thorough phylogenetic study in vertebrates, chordates and their sister group, ambulacraria, and show that the origin of the zebrafish olfactory receptor gene can be traced back to the most recent common ancestor of all three groups as a segregate sister clade (*adorb*) to the *adora* gene family. Eel, carp, and clawed frog all express *adorb* in a sparse and distributed pattern within their olfactory epithelium very similar to the pattern observed for zebrafish that is, consistent with a function as olfactory receptor. In sharp contrast, lamprey *adorb*-expressing cells are absent from the sensory region of the lamprey nose, but form a contiguous domain directly adjacent to the sensory region. Double-labeling experiments confirmed the expression of lamprey *adorb* in nonneuronal cells and are consistent with an expression in neuronal progenitor cells. Thus, *adorb* may have undergone a switch of function in the jawed lineage of vertebrates towards a role as olfactory receptor.

Key words: adenosine receptor, lamprey, olfaction.

Significance

The adenosine receptor *A2c/adorb* is known as olfactory receptor in zebrafish, but the origin of this gene and of its olfactory function were unknown. We have identified the origin of *adorb* in the most recent common ancestor of deuterostomes, much earlier than other olfactory receptor families. We show in representative species that jawed vertebrates express *adorb* in sparsely distributed olfactory receptor neurons. In sharp contrast, the jawless lamprey expresses *adorb* in a contiguous region of nonneuronal cells, which could be neuronal progenitor cells. Thus the *adorb* receptor appears to have acquired an olfactory function in bony vertebrates, a long time after the birth of this gene. These findings constitute an important step in understanding the evolution of olfactory receptor function.

Introduction

The sense of smell is both evolutionary ancient and of primary importance in many different animal species, who employ it to detect prey or food, evade predators, guide mating behavior, and sculpt manifold social interactions. In vertebrates perception of odors is mediated by four main families of olfactory receptors, the odorant receptors (ORs) proper, the trace-amine-associated receptors (TAAR and TAAR-like), and the vomeronasal receptors types I and II—V1R/ORA and V2R/OlfC, respectively (Korsching 2016). The evolutionary origin of these four families differs, with ORs already observed in chordates (Churcher and Taylor 2009) but see (Nordström et al. 2008), V1R and TAAR-like receptors first reported in vertebrates, and V2Rs and TAARs only found in jawed vertebrates (Korsching 2016). Additionally, several smaller receptor families or even single genes have been repurposed for an olfactory function at differing evolutionary stages, in some cases rather recently, for example, some

formyl peptide receptors in rodents, but not in other mammals (Dietschi et al. 2017).

For most of the main odor classes, we have a good understanding of their respective receptors. The OR families of tetrapods often recognize volatile that is, hydrophobic ligands, whereas the TAAR families of both tetrapods and teleost fish appear to be specialized for detection of amines (Korsching 2020). Tetrapod and teleost V1R/ORA are assumed to detect pheromones, whereas the V2R/OlfC family appears to detect peptide pheromones in tetrapods, but amino acids (food odors) in teleosts and amphibians (Korsching 2020). Another important component of food odors in aquatic animals are nucleotides/nucleosides, but for a long time the molecular nature of their olfactory receptors was unknown. There are three well-described purinergic receptor families (metabotropic P1 and P2Y, ionotropic P2X; Fredholm et al. 2011), but none of their genes seems involved in olfactory detection of nucleotides or nucleosides in zebrafish (Wakisaka et al. 2017). However, the same study identified a novel gene as an olfactory adenosine receptor. This gene was found to be related to **ad**en**o**sine **r**eceptors type A, *adora* genes, and the name A2C, for *adora2c*, was suggested (Wakisaka et al. 2017). A preliminary phylogenetic analysis has detected the presence of this gene in many other vertebrate species (Wakisaka et al. 2017), but its evolutionary origin is currently unknown, and it is also unclear at what point in its evolutionary lineage it acquired an olfactory function.

Here, we report that this novel gene is already present as a sister clade to *adora* genes in the common ancestor of chordates and echinoderms that is, close to the origin of deuterostomes (one of two superphyla in bilateral animals), which prompted us to (re)name the gene clade *adorb* (**ad**en**o**sine **r**eceptors **B**), in close analogy to the accepted naming of the **ad**en**o**sine **r**eceptor **A** genes (*adora*). Interestingly, a role as olfactory receptor seems to have been acquired only much later in evolution: we observed an olfactory receptor-like expression pattern for the *adorb* gene in several teleost and one amphibian species, but not in lamprey, consistent with *adorb* first becoming an olfactory receptor in jawed vertebrates. The expression pattern of *adorb* in lamprey is consistent with a role in differentiation of olfactory neuron progenitor cells.

Results

The Adenosine Receptor *adorb* (Synonym A2c) Is Already Present in the Common Ancestor of Chordates and Echinoderms, That Is, Close to the Origin of Deuterostomes

We have performed an in-depth phylogenetic analysis of the evolutionary origin of the *adorb* gene, making use of the recent availability of genomes representing many early-derived lineages within vertebrates (three jawless and three cartilaginous fish), chordates (five urochordates and four cephalochordates) and even the sister clades of chordates (10 echinoderms and two hemichordates). We report a single clade with very high branch support comprising orthologs for the zebrafish gene named *A2c* (Wakisaka et al. 2017) in reptiles, amphibians, teleost fish, ray-finned and lobe-finned fish, cartilaginous and jawless fish, cephalochordates, hemichordates, and echinoderms (figs. 1 and 2). At all evolutionary levels analyzed these zebrafish *A2c*-related genes form a separate sister clade to the *adora* clade encompassing the well-known *adora1*, *adora2a*, *adora2b*, and *adora3* genes. This result differs from that of a preliminary study that was restricted to vertebrates, used neighbor-joining algorithm for construction of the gene tree, and a less diverse outgroup (Wakisaka et al. 2017). We have employed well over 100 genes representing all major subgroups of rhodopsin class G protein-coupled receptors (GPCRs) and even rhodopsin-associated genes including many genes from early-diverging species as outgroup (see supplementary files 1–3, Supplementary Material online). In our experience, it is essential to use large and diverse outgroups in the phylogenetic analysis to obtain a robust tree topology. We consequently suggest renaming the *adora2c* (A2C) gene as *adorb* for **ad**en**o**sine **r**eceptor **B** to accurately reflect the phylogenetic position and distance to the **ad**en**o**sine **r**eceptor **A** clade (*adora*). An analysis of splice sites confirms the inferred segregation of *adora* and *adorb* genes: all vertebrate *adora* genes exhibit the same, conserved splice site, whereas *adorb* genes are mostly monoexonic, with occcasional intron gains at positions differing from that of *adora* genes (figs. 1–3, and supplementary file 2, Supplementary Material online).

The tree topology of the *adorb* clade generally reproduces the known species relationships faithfully, with hemichordate (acorn worms) and echinoderm (sea stars and sea urchins) *adorb* genes forming a common sister clade to chordates. Within chordates, cephalochordates (lancelets) form a sister clade to vertebrates, and within vertebrates, jawless fish *adorb* genes are basal to those of cartilaginous fish, which themselves are basal to bony fish orthologs. Thus, the origin of the zebrafish adenosine receptor *adorb* (formerly *A2C*, Wakisaka et al. 2017) can be traced back to the MRCA of deuterostomes that is, well beyond the chordate branch of the tree of life.

Rare Gene Duplications and Several Losses Characterize the Evolution of the *adorb* Gene

To better understand the evolution of the *adorb* gene, we have also searched for its closest homologs outside of the *adora/adorb* clade. The *adorb* gene is distinct from *adora* as far back as the MRCA of deuterostomes (see above), but both genes share a common ancestor, which may be named *ador* as it is the ancestor of both *adora* and *adorb* genes; fig. 1 and supplementary files 1–4, Supplementary Material online). Our search for the origin of this deduced *ador* gene has identified a sister gene, *sador* (**s**ister of **ador**) in all 12 ambulacraria
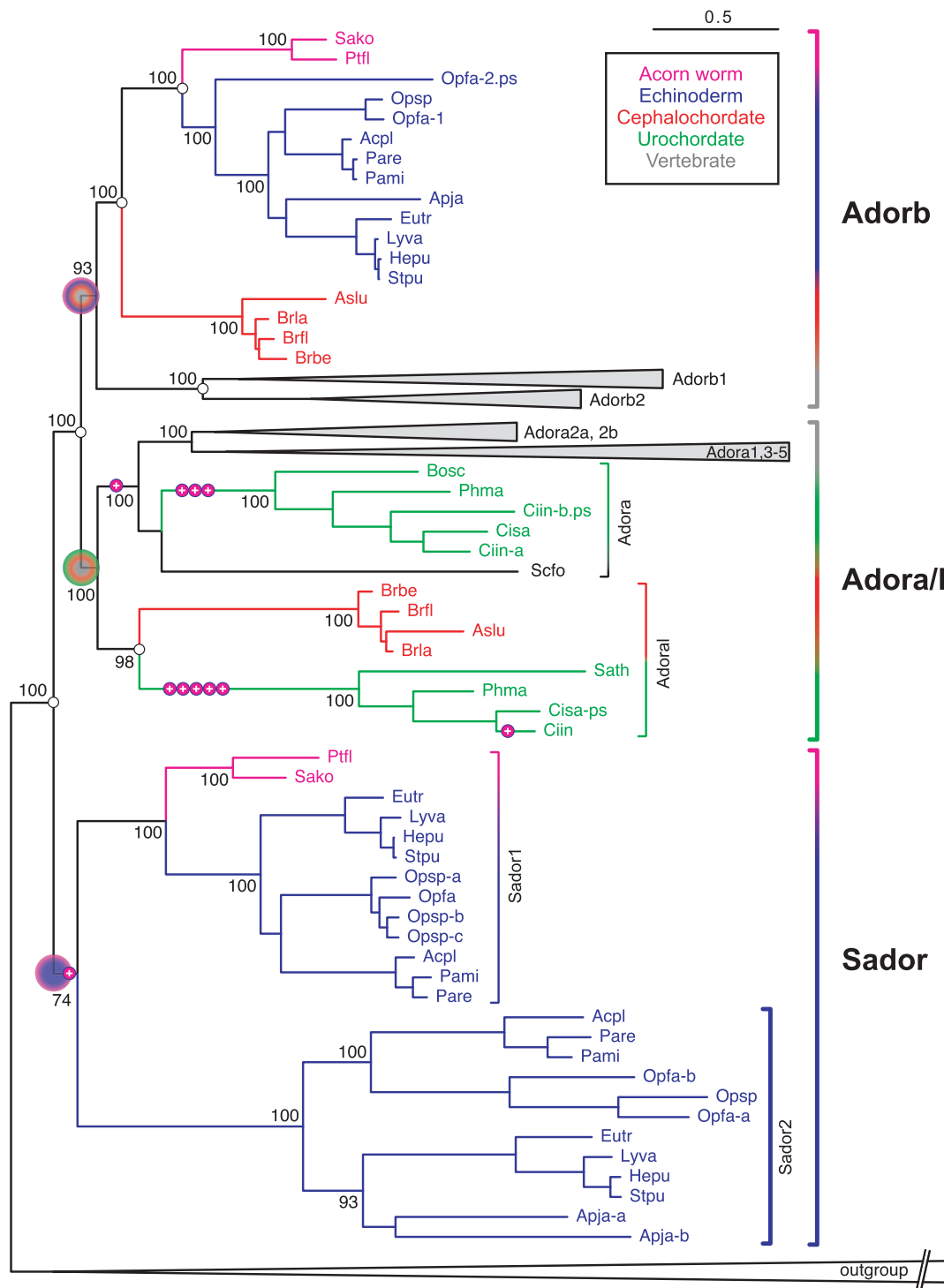
Fig. 1.—The evolutionary origin of the nucleotide receptor *adorb* in the MRCA of deuterostomes. Maximum likelihood tree for *adora*, *adorb*, and related receptors. Branch support is given in %, scale bar denotes number of amino acid substitutions per site. Species groups are color-coded as indicated, all vertebrate clades are collapsed. Magenta circles with plus sign indicate intron gains, small empty circles denote absence of introns. Gene clades are visualized by angular parentheses, color-coded according to the species represented in that clade. Species are indicated by four letter abbreviation, for full names see supplementary file 3, Supplementary Material online. The root of the *adora*, *adorb*, and *sador* clade is denoted by large circles, color-coded according to species represented within that clade. *Adora, adoral, adorb*, and *sador* clades are well separated with high to maximum branch support. *Adorb* is present in all deuterostome groups analyzed except urochordates. The outgroup (collapsed) consists of over 100 genes encompassing all subgroups of rhodopsin class GPCRs as well as rhodopsin-associated class genes (see supplementary file 3, Supplementary Material online).
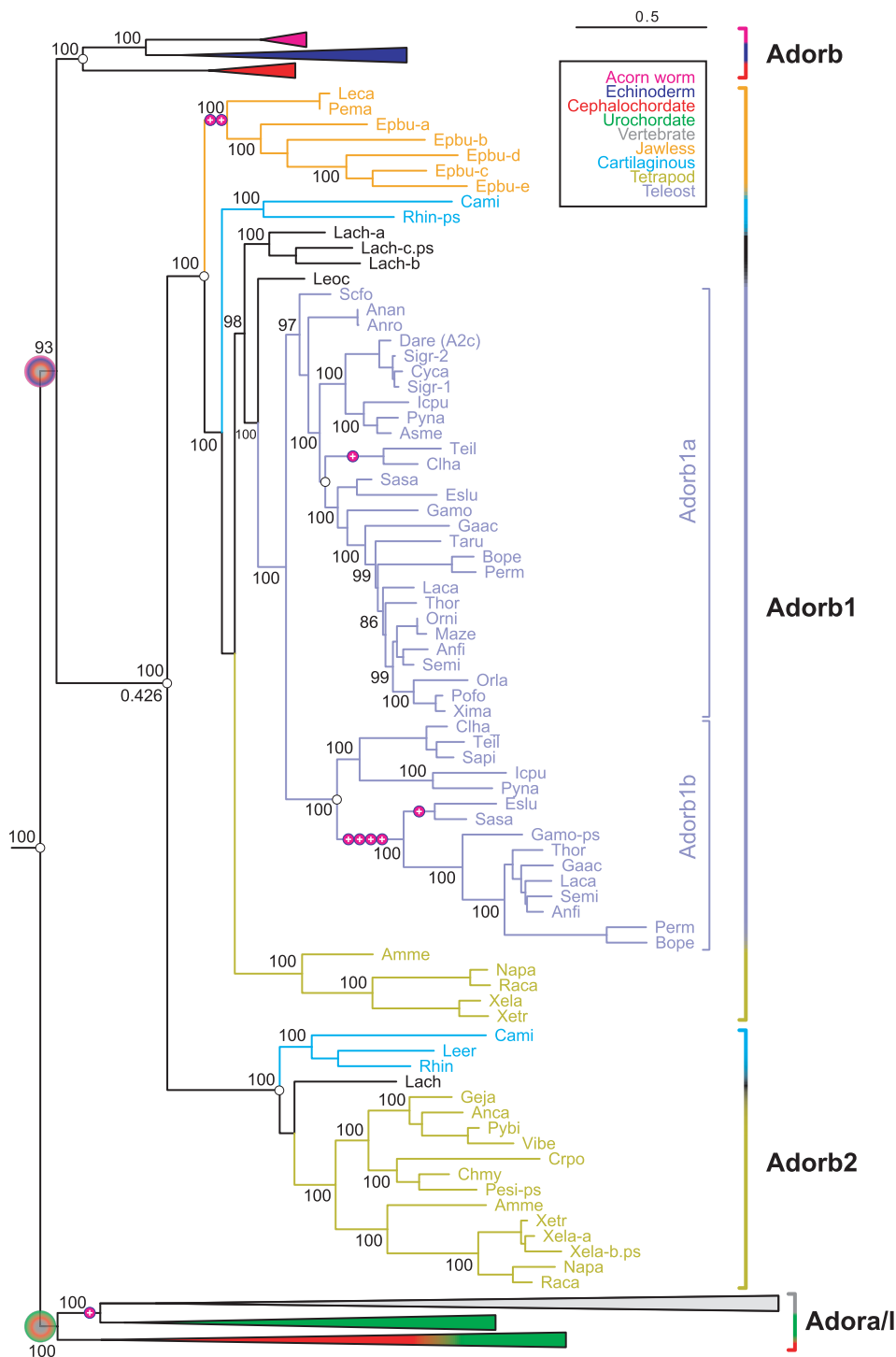
Fig. 2.—Evolution of the *adorb* gene clade in vertebrates. Maximum likelihood tree for *adora*, *adorb*, and related receptors, same tree file as figure 1, only vertebrate *adorb* clade expanded here. Branch support is given in %, for the root of the adorb clade also the common branch length is given (0.426); scale bar denotes number of amino acid substitutions per site. Species groups are color-coded as indicated, all nonvertebrate nodes and the vertebrate adora node are collapsed. Magenta circles with plus sign indicate intron gains; small empty circles denote absence of introns. Gene clades are visualized by angular parantheses, color-coded according to the species represented in that clade. Species are indicated by four letter abbreviation, for full names see supplementary file 3, Supplementary Material online. The outgroup (not shown) consists of over 100 genes encompassing all subgroups of rhodopsin class GPCRs as well as rhodopsin-associated class genes (see supplementary file 3, Supplementary Material online). The root of the *adora* and *adorb* clade is denoted by large circles, color-coded according to species represented within that clade. The vertebrate *adorb* clade is well separated with maximum branch support. Coelacanth (Lach) and hagfish (Epbu) show small gene expansions, the remaining increases in gene number result from two whole genome duplications or polyploidy (Sigr, Xela).
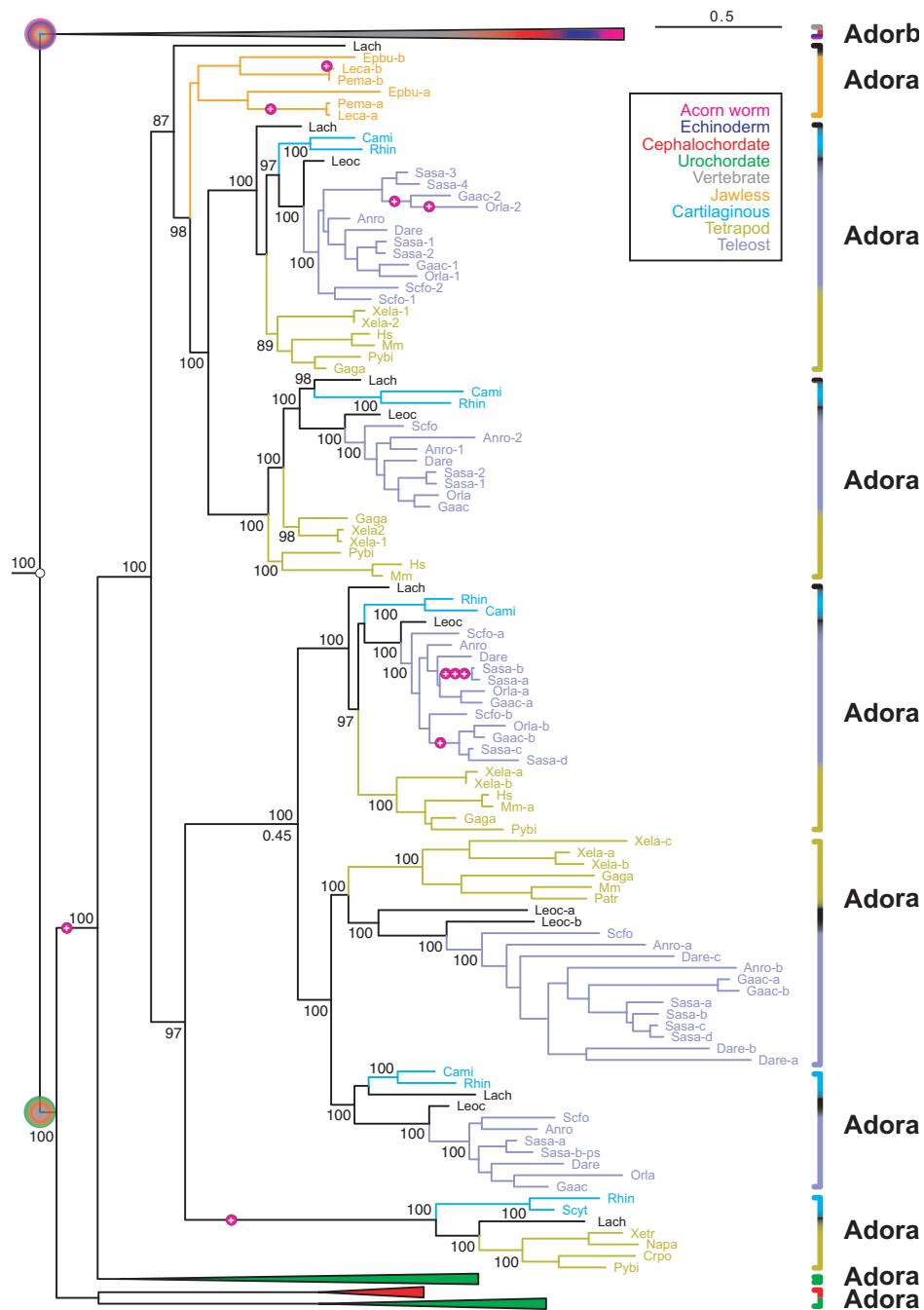
Fig. 3.—Two novel *adora* genes in vertebrates. Maximum likelihood tree for *adora*, *adorb* and related receptors, same tree file as figure 1, only vertebrate *adora* clade expanded here. Branch support is given in %, scale bar denotes number of amino acid substitutions per site. Species groups are color-coded as indicated; all nonvertebrate nodes and the vertebrate adorb node are collapsed. Magenta circles with plus sign indicate intron gains; small empty circles denote absence of introns. Gene clades are visualized by angular parantheses, color-coded according to the species represented in that clade. Species are indicated by four letter abbreviation, for full names see supplementary file 3, Supplementary Material online. The outgroup (not shown) consists of over 100 genes encompassing all subgroups of rhodopsin class GPCRs as well as rhodopsin-associated class genes (see supplementary file 3, Supplementary Material online). The root of the *adora* and *adorb* clade is denoted by large circles, color-coded according to species represented within that clade. The vertebrate *adora* clade is well separated with maximum branch support. Orthologs for mammalian genes *adora1, 2a, 2b,* and *3* are present in teleosts, for the first three also in cartilaginous fish. Two novel clades, *adora4* and *5*, are present in cartilaginous fish, lobe-finned fish, and ray-finned fish, respectively amphibians. Remnants of the teleost-specific whole genome duplication are visible, some other increases in gene number result from polyploidy, for example, salmon.

genomes analyzed (two hemichordates and ten echinoderms), which together form the sister group of chordates. Despite extensive searches, no chordate orthologs could be identified, suggesting a loss of *sador* early in the chordate lineage. *Sador* itself has undergone an early duplication event in ambulacraria resulting in *sador1* and *sador2*, the latter not found in either of the two acorn worm genomes available that is, presumably lost. *sador1* and *sador2* mostly have remained singletons in ambulacraria, except two gene duplications in a starfish *sador1* (*Ophiothrix spiculata*) and one gene duplication each in *sador2* from another starfish and a sea cucumber (*Ophionereis fasciata* and *Apostichopus japonicus*, respectively). Ligands for *sador* genes are unknown.

The *sador/adora/adorb* monophyletic clade is supported with maximal branch support and a common branch of considerable length (0.34 amino acid substitutions/site), segregating it clearly from other rhodopsin alpha group genes including aminergic neurotransmitter receptors, as well as from the other rhodopsin family subgroups (beta, gamma, and delta, Fredriksson et al. 2003), which were used as further outgroup (fig. 1 and supplementary files 1–3, Supplementary Material online).

Within the *adorb* node, we identified echinoderm, ambulacraria, cephalochordate, and vertebrate representatives, but never observed any urochordate genes (urochordate *adora* genes were localized easily). We conclude that *adorb* has been lost in urochordates, which are closer related to chordates than cephalochordates (Delsuc et al. 2006). Within vertebrates *adorb* has duplicated to *adorb1* and *adorb2*, presumably as a consequence of a whole genome duplication in early vertebrates (Nakatani et al. 2007), since in species with chromosome annotation these two genes lie on different chromosomes (data not shown). *Adorb1* and *adorb2* show very diverging evolutionary histories. *Adorb1* is present in jawless fish (three species), cartilaginous fish (two species), coelacanth, a ray-finned fish, all representative teleost fish species analyzed (28 species), and in amphibians (five species), but absent in reptiles, suggesting widespread conservation, but a loss in terrestrial species. *Adorb2* is not found in jawless fish, absent in all ray-finned fish and teleosts analyzed, but present in cartilaginous fish (three species), coelacanth, amphibians (five species), and reptiles (seven species), suggesting losses in jawless and in ray-finned fish, and a somewhat later loss inside the terrestrial lineage (no avian or mammalian orthologs were found). The whole genome duplication in the MRCA of teleost fish (Nakatani et al. 2007) led to another duplication of *adorb1* into *adorb1a* and *adorb1b*, which again have a very different evolutionary history. Although *adorb1a* has been retained in all but one teleost species (and the absence in the sardine genome may well have technical reasons), *adorb1b* has been lost repeatedly in different sublineages. Interestingly, the *adorb1b* gene has gained four introns in the MRCA of Euteleosteomorpha and a fifth in salmonids, but has remained intronless in Otomorpha

(herring, sardine, catfish, and piranha), whereas herring/sardine *adorb1a* is the only *adorb1a* gene with an intron gain (fig. 2). Two unrelated intron gains have occurred in the MRCA of jawless fish in *adorb1* (fig. 2).

A Thorough Delineation of the *adora* Family Reveals Two Novel Clades

Unexpectedly, our control searches for *adora* genes revealed the presence of two novel clades, *adora4* and *adora5* (fig. 3). To substantiate our results for the adorb clade being separate from all *adora* genes, we considered it essential to perform a thorough delineation of the *adora* clade in all basal and some of the derived species we had examined for the presence of *adorb* orthologs. *Adora1, 2a, 2b*, and *3* were identified in a ray-finned and a lobe-finned fish (spotted gar and coelacanth [minus adora3], respectively) as well as in early-derived (dragonfish, eel, and zebrafish) and later-derived (salmon, medaka, and stickleback) teleost fish (fig. 3). Some species-specific, late gene births were observed, as well as partial retention of genes duplicated in the whole genome duplication occurring in the MRCA of teleost fish (Nakatani et al. 2007), in particular for *adora1* and *adora2a* (fig. 3). Additionally, the polyploidization of salmon increased the total number of *adora1–3* genes to 14. A*dora1, -2a, -2b, and -3* genes were present in an amphibian (clawed frog), a reptile (python), and a bird (chicken), consistent with earlier reports (Wakisaka et al. 2017).

A fifth clade is apparent in the phylogenetic tree as sister clade to *adora3* and was named *adora4*. This clade contains shark, coelacanth, spotted gar, and teleost fish orthologs, but seems to have been lost in tetrapods, as no amphibian, reptilian, or avian representative could be uncovered (fig. 3). Finally, we describe another novel clade basal to *adora1*, *3*, *4*, which we named *adora5*. This clade contains shark, coelacanth, amphibian, and reptilian representatives, but seems to have been lost in ray-finned fish, birds, and mammals (fig. 3). Ligands for adora4 and adora5 are unknown but may be expected to include adenosine, which is the physiological ligand both for the four previously described *adora* genes (Fredholm et al. 2011) and the recently described zebrafish *adorb* gene (Wakisaka et al. 2017, here named *A2c*).

All gene birth events leading to *adora1–5* have taken place within the vertebrate lineage, but the ancestral *adora* gene can be followed back further to the MRCA of urochordates and vertebrates (figs. 1 and 3). A sister gene (adora-like, *adoral*) is present in urochordates as well as cephalochordates, but not in vertebrates, consistent with a loss of *adora* in cephalochordates and a loss of *adoral* in vertebrates (figs. 1 and 3). Ligands for *adoral* genes are unknown, but could include adenosine, the ligand for *adora* and *adorb* genes (Fredholm et al. 2011; Wakisaka et al. 2017). No ambulacraria *adora* genes were detected despite intensive searches, consistent with a loss of *adora* in ambulacraria or, alternatively,

with a later origin of *adora* as gene duplication of an earlier *adorb* gene within the chordate lineage.

An Olfactory Receptor-like Expression Pattern of *adorb1* in Representative Teleost and Amphibian Species

Next, we wished to analyze the expression pattern of *adorb* in representative species. The zebrafish *adorb1a* gene has been shown to be expressed in sparsely distributed olfactory receptor neurons (Wakisaka et al. 2017), the characteristic spatial expression pattern of an olfactory receptor (Korsching 2020). We chose common carp as a close relative to zebrafish, European eel as a rather distantly related and much earlier-diverged teleost, and the African clawed frog as an amphibian representative. Zebrafish was included as control. For all four species, we performed in situ hybridization (ISH) on cryostat sections of the olfactory organ. We observe sparsely

distributed labeled cells in each of the olfactory organs (fig. 4), in the typical pattern observed for olfactory receptor genes (Weth et al. 1996; Syed et al. 2013; Korsching 2020), suggesting that the *adorb1* gene has served an olfactory function throughout the evolution of bony fish, from the MRCA of the ray-finned (carp, eel, and zebrafish) and the lobe-finned (frog) lineage, about 430 Ma (Kumar et al. 2017), up to its loss in nonamphibian tetrapods. A quantitative analysis of the spatial distribution of *adorb1*-expressing cells in zebrafish shows the expression domain of *adorb1* to be of comparable width with those of *OR* genes, with a center of gravity rather close to the center of the olfactory epithelium, and within the range covered by *OR* genes (supplementary files 1 and 6, Supplementary Material online). Thus, zebrafish *adorb1* expression conforms to the spatial pattern observed for and characteristic for other olfactory receptor gene families (Weth et al. 1996; Ahuja et al. 2018).
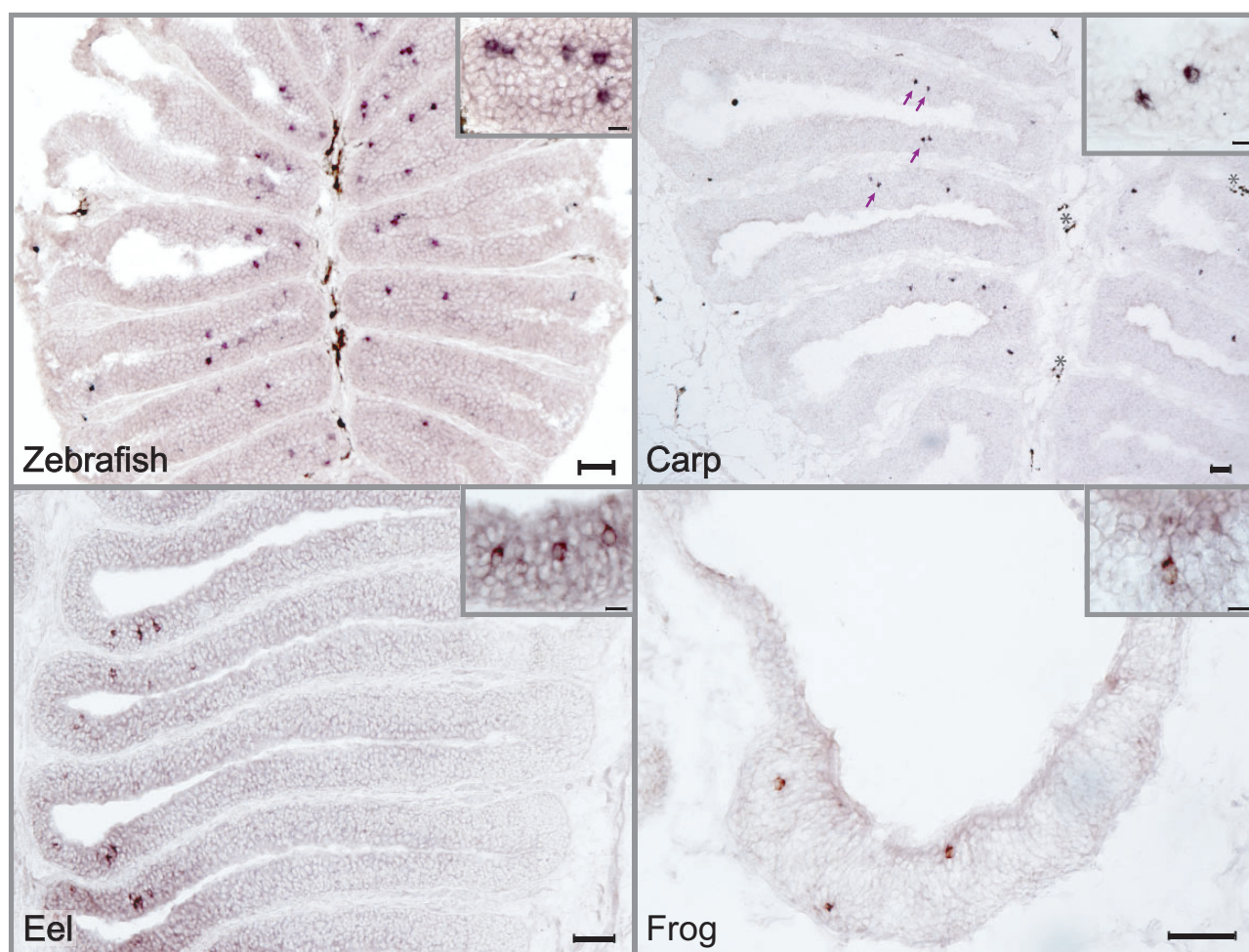

Fig. 4.—Across a wide evolutionary range *adorb* is expressed in sparsely distributed cells within the olfactory epithelium. Species-specific *adorb1* probes were used to perform chromogenic ISH of horizontal cryostat sections of olfactory epithelia for three fish (zebrafish, top left panel; carp, top right panel; eel, bottom left panel) and one amphibian species (clawed frog, bottom right panel). Insets at higher magnification; scale bars in main panels 50 μm, in insets 10 μm.

Lamprey *adorb1* Is Expressed in a Nonsensory Region of the Olfactory Organ, but Absent in Olfactory Sensory Neurons

We then went on to investigate whether the lamprey *adorb1* gene would also show an olfactory expression pattern. Much to our surprise, we found a completely different situation than that observed for bony fish and frog. The lamprey *adorb1* gene is indeed expressed in the nose, but as one contiguous region within a lamellae, which appeared to be adjacent to the sensory area (fig. 5A and B). Together, these regions form an outer "ring." In some cases, a second zone of expression is observed close to the inner tip of lamella (fig. 5C), and in rare cases additional intermediate zones are seen (fig. 5D). In all cases, expression is in contiguous areas, not in isolated cells. For comparison, a lamprey olfactory receptor (Berghard and Dryer 1998) shows the sparsely distributed expression pattern typical for olfactory receptor genes (fig. 5E). The accessory olfactory system (Ren et al. 2009, Green et al. 2017) does not express *adorb1* (supplementary file 5, Supplementary Material online).

The unusual expression pattern of lamprey *adorb1* suggests that it is not expressed in olfactory sensory neurons.

To substantiate this hypothesis, we performed double labeling with the panneuronal marker HuC (Kim et al. 1996) and established markers for ciliated and microvillous neurons, the two main subpopulations of olfactory sensory neurons in vertebrates, including lamprey (Laframboise et al. 2007; Korsching 2020). The HuC antibody staining delineates the known sensory region of the olfactory lamella, all of which is negative for *adorb1* expression (fig. 6). Even at high magnification no coexpression is seen (fig. 6). We then proceeded to perform double ISH for *adorb1* and *golf*, a marker for ciliated neurons (fig. 7). *golf* has been shown to label a subpopulation of OSNs also in lamprey and thus demarkates the sensory area also in this species (Frontini et al. 2003). The expression zones for *golf* and *adorb1* are adjacent, but clearly segregated, and no overlap is seen. As a control, double-labeling was performed with *golf* and HuC. As expected, there was extensive overlap of staining observed (fig. 7), confirming the suitability of Golf to delineate the sensory area. Next, we performed double ISH for *adorb1* and *trpc2*, an accepted marker for microvillous neurons of bony vertebrates (fig. 7).
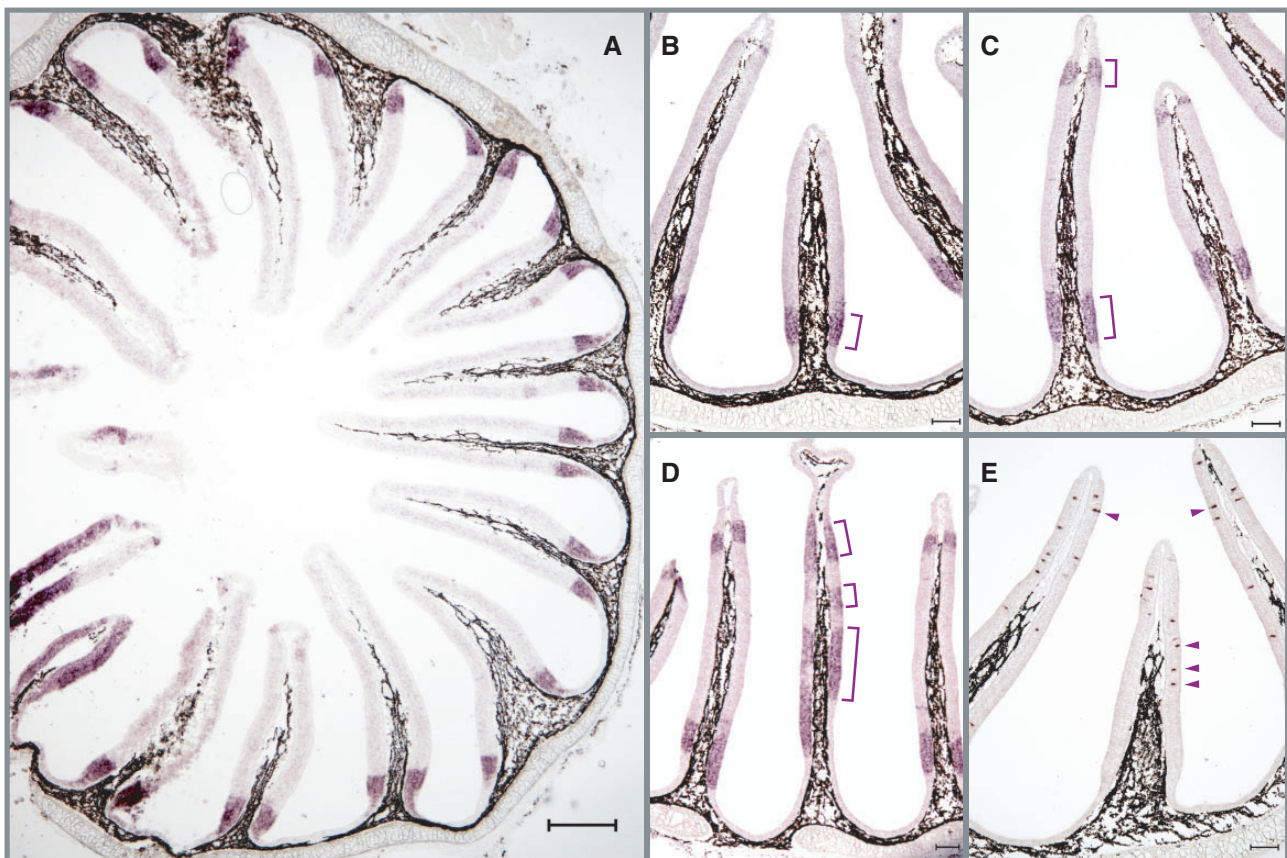

Fig. 5.—Expression of lamprey *adorb* in contiguous segments of the nose is distinctly different from that observed in jawed vertebrates. ISH was performed in horizontal cross-sections of the olfactory organ of *L. fluviatilis* using NBT/BCIP for visualizing *adorb1* (A–D) and *tarl7a* (aka *Lor3*) expression (E). *Adorb1* is expressed in a contiguous region adjacent to the olfactory sensory area (A, B), in some cases close to the inner tip (C) and rarely in intermediate zones (D) of the OE. In contrast, a known olfactory receptor (Lor3, Berghard and Dryer 1998), is sparsely distributed in the olfactory sensory area (E). Scale bar (A) 300 μm, (B–E) 100 μm.
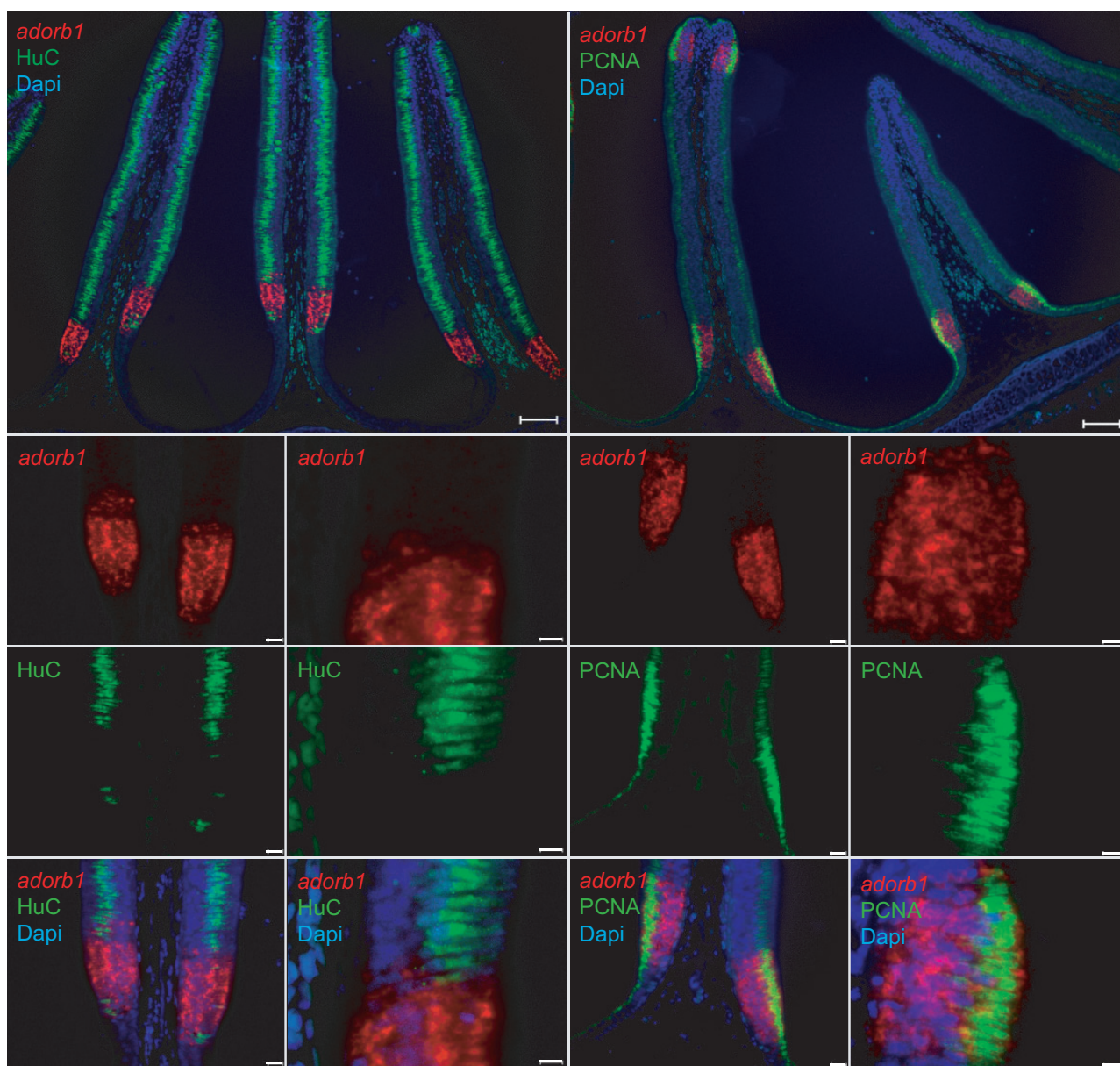
Fig. 6.—Lamprey *adorb1* expression zone borders the sensory area of the olfactory organ. Double labeling of horizontal cross-sections of *L. fluviatilis* olfactory epithelium with HuC antibody and PCNA antibody confirms the expression of *adorb1* (visualized by ISH) in a nonsensory region. Genes and color as indicated on the respective panels, nuclei are visualized by DAPI staining (blue). The neuronal marker HuC (left columns, green) labels the olfactory sensory area of the lamella, whereas PCNA (right columns, green) labels the adjacent mitotic regions. *Adorb1* (red) is expressed in a contiguous manner in the PCNA-positive region, but absent from the sensory region. Note that PCNA labeling (green) is restricted to the apical layer, whereas *adorb1*(red) staining is distributed more broadly. Top panels show an overview (scale bars 100 μm); the smaller bottom panels are enlarged (scale bars 50 and 10 μm, respectively).

Unexpectedly, *trpc2* expression was not restricted to the sensory segment as defined by HuC labeling and thus does not seem to be a specific marker for microvillous olfactory neurons in lamprey, in contrast to bony vertebrates (Sato et al. 2005). Nevertheless, we observe no overlap of *trpc2* with *adorb1* expression at the cellular level (fig. 7).

In summary, *adorb1*-positive cells express neither a general neuronal marker nor markers characteristic for ciliated and microvillous neurons, and thus are clearly nonneuronal, in sharp contrast to the expression patterns observed in bony fish (figs. 4–7 and supplementary files 1 and 6, Supplementary Material online).

Lamprey *adorb* Expression Is Consistent with Expression in Neural Progenitor Cells

So, what *are* these *adorb1*-expressing cells? Interestingly they are present in a region directly adjacent to the neuronal
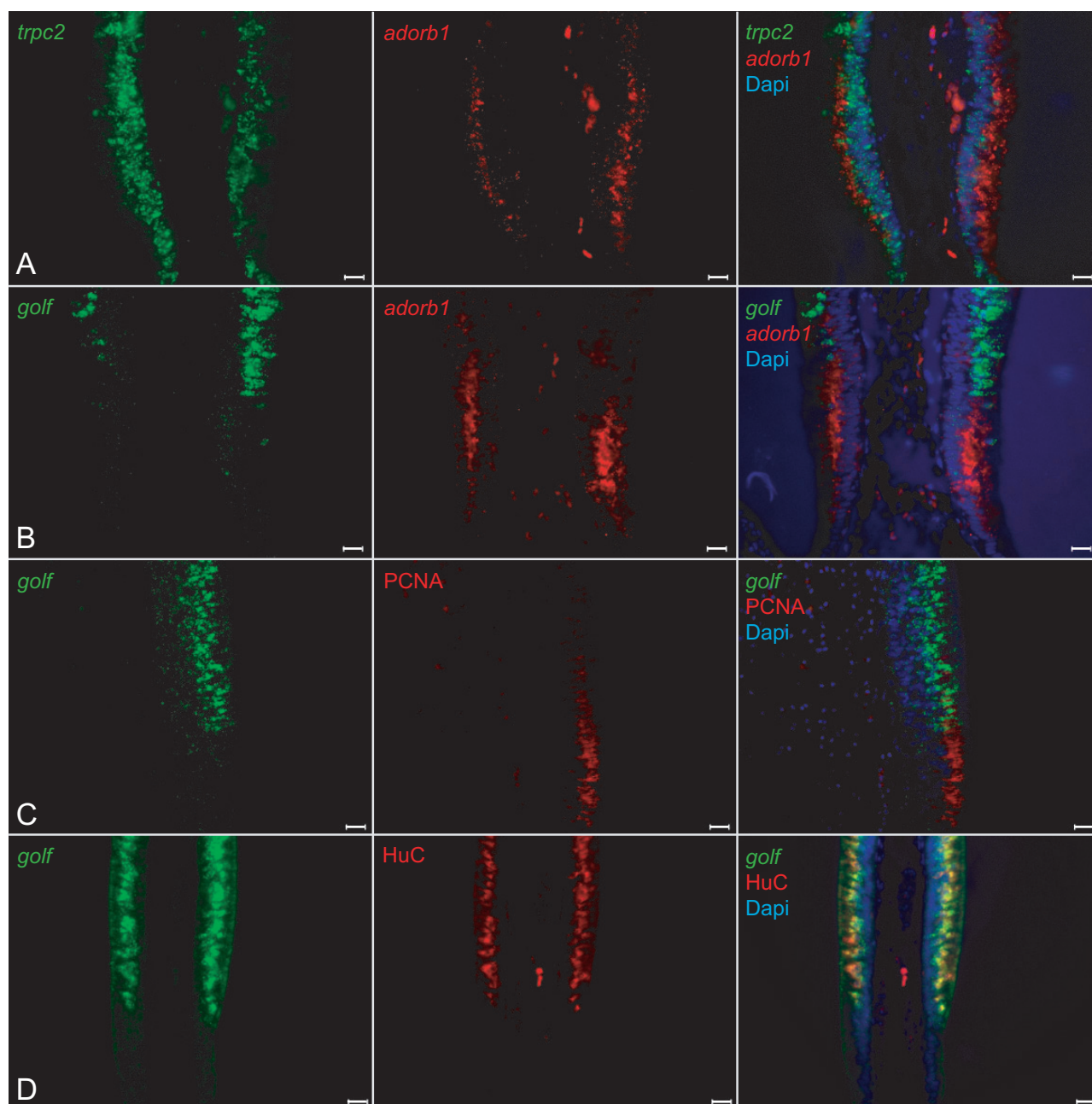
Fig. 7.—*Adorb1*-expressing cells in lamprey are negative for several neuronal markers. Horizontal cross-sections of *L. fluviatilis* olfactory epithelium were double labeled with OSN markers in several combinations; nuclei are visualized with DAPI. Left and middle columns, single label; right column, merged pictures. Orientation of panels: the *adorb1*-positive region is always below the adjacent sensory region. All scale bars are 20 μm. (*A, B*) Double ISH with neuronal markers for microvillous and ciliated OSNs (*trpc2*, and *golf*, respectively) and *adorb1* shows no coexpression with either marker. (*A*) Unexpectedly, *trpc2* (green) is expressed both in the sensory region and the *adorb1*-positive (red) segment. However, no double-labeled cells are seen. The nature of the *trpc2*-positive cells in the nonsensory region is unclear. (*B*) *golf* (green) is expressed in the sensory area, and exclusive of *adorb1* expression (red). (*C, D*) Double labeling of *golf* with HuC and PCNA antibodies shows extensive overlap of *golf* and HuC at the cellular level, and complete absence of overlap for *golf* and PCNA, as expected.

segment of the olfactory lamellae, suggesting this region to be a potential source of newly born olfactory receptor neurons. Similar mitotic zones have been shown in the zebrafish olfactory organ and migration from this zone into the sensory region of the nose has been demonstrated (Oehlmann et al. 2004; Bayramli et al. 2017). The morphology of the lamprey olfactory organ is somewhat different from that of jawed fish, as it consists of a single, medially fused structure. However,

olfactory lamella that are fused to outer nonsensory structures and merge in the center of the organ occur in lamprey as well as jawed fish, and topology-wise the outer and inner *adorb1*-expressing regions would correspond to the outer and inner "ring" of mitotically active cells in the zebrafish nose (Oehlmann et al. 2004). In zebrafish, these rings express pro-liferating cell nuclear antigen (PCNA), and no overlap with the *adorb1*-expressing sensory region is seen (supplementary files 1 and 7, Supplementary Material online). In contrast, in lamprey double labeling with PCNA stains exactly those segments of the olfactory lamella, which are expressing *adorb1* (fig. 6 and supplementary files 1 and 7, Supplementary Material online). Nevertheless, at the cellular level, there is little overlap, with PCNA-expressing cells forming a contiguous apical layer, whereas the *adorb1* expression is centered in more basal layers of the lamella (fig. 6). PCNA is essential for replication and an established marker for neuroepithelial cells (Ino and Chiba 2000; Bayramli et al. 2017), that is, a marker of stem cells in the neuronal lineage. *Adorb1*-expressing cells might represent a different (later) stage in the neuronal lineage, but our attempts at identifying suitable markers for such stages in lamprey have so far not been successful. However, the close association with PCNA-expressing cells and the absence of neuronal markers in *adorb1*-expressing cells is consistent with these cells representing an intermediate stage in the differentiation from neuroepithelial stem cells to mature neurons.

Discussion

Nucleotides and nucleosides constitute important food-derived odors for aquatic organisms including fishes. The recent description of the first olfactory receptor for a nucleoside (Wakisaka et al. 2017) prompted us to investigate the evolution of this receptor and its olfactory function as estimated by the presence or absence of the characteristic expression pattern—sparsely distributed cells within the sensory region of the olfactory epithelium. We report that the receptor itself is very ancient and already present in the common ancestor of deuterostomes, since it is found in hemichordates, echinoderms, cephalochordates, and vertebrates, in the latter as a sister clade to the *adora* gene family (fig. 8). In contrast, the *adora* genes are only observed in vertebrates and their closest relatives, the urochordates, consistent with either a loss in hemichordates, echinoderms, and cephalochordates, or a later birth of *adora* in the MRCA of vertebrates and urochordates. Thus, we suggest to replace the initial designation (*adora2c*; Wakisaka et al. 2017) with *adorb* (adenosine
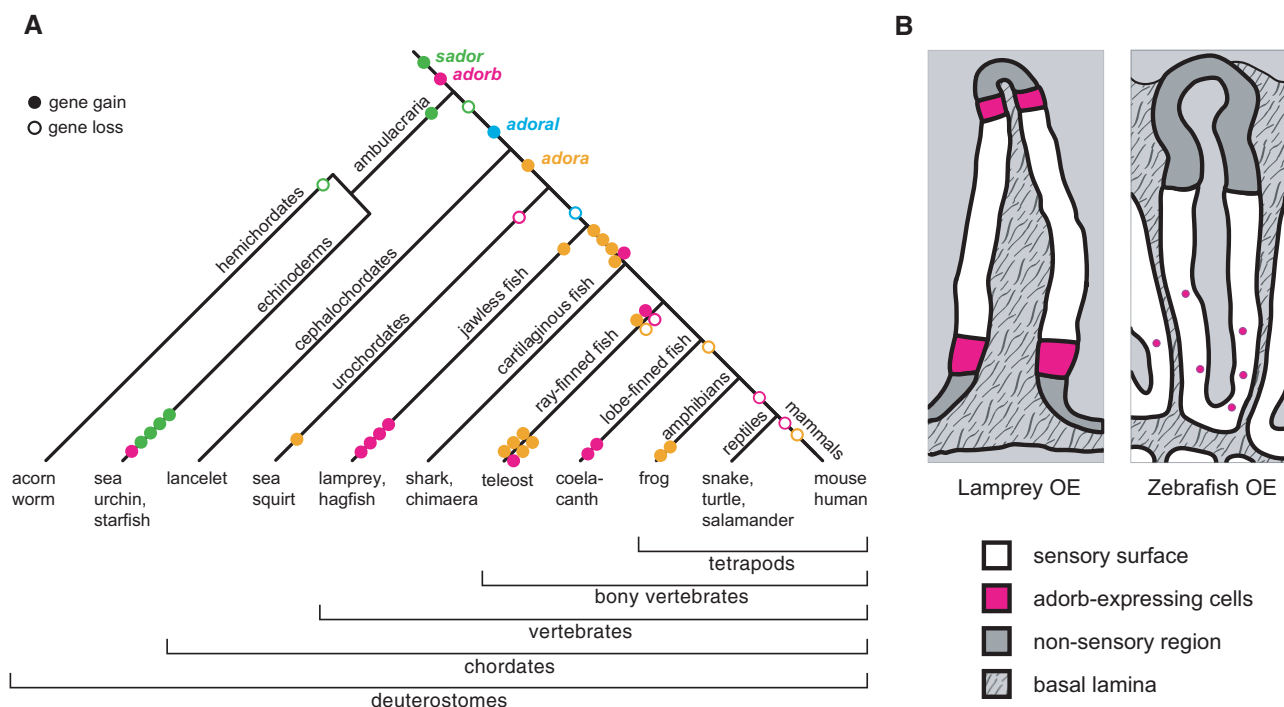

Fig. 8.—Evolutionary dynamics of *adorb* and related clades and transition in *adorb* expression patterns between jawless and jawed fish. (*A*) Dynamic evolution in the *adora*, *adorb* and related clades inferred from the phylogenetic analysis. The evolutionary history is depicted using maximum parsimony. Genes are color-coded as indicated and gene name is given at the birth of the respective clade. Each gene duplication event is shown as filled circle, an empty circle denotes a gene loss in the respective species group. Late gene duplication events are shown close to the tips, species-specific gene losses are not shown to keep the figure legible. Gene duplications resulting from a whole genome duplication are counted as one event. (*B*) Schematic representation of *adorb* expression in lamprey and zebrafish. Cross sections of a lamella are shown for lamprey (left panel) and zebrafish (right panel). Magenta, cells expressing *adorb*; white, sensory surface; light gray, lumen; medium gray, nonsensory region; stippled gray, basal lamina.

receptor b), which appears better suited to the phylogenetic position as sister clade to *adora* (adenosine receptor a).

We identified several related genes in the nonvertebrate clades analyzed, which we named according to their phylogenetic position, *adoral* for adora-like gene, and *sador* for sister of *ador*, *ador* being the deduced ancestral gene for *adora* and *adorb*. The *sador* clade is present in ambulacraria (both echinoderms and hemichordates), but absent from chordates, and the *adoral* clade is present inside the chordate lineage (both cephalochordates and urochordates), but absent from vertebrates, consistent with losses of these genes in the respective lineages (fig. 8). Such an evolutionary dynamic parallels findings for other receptors when analyzing the lineage of a known vertebrate receptor family outside of vertebrates (see e.g., Ramos-Vicente et al. 2018).

As a proxy for a function as olfactory receptor gene, we examined the spatial expression of *adorb* genes in an array of vertebrate species. Most olfactory receptors, including the zebrafish *adorb1a* gene (Wakisaka et al. 2017) have a highly characteristic expression pattern consisting of isolated cells dispersed between cells expressing other olfactory receptors (Korsching 2020)—for lamprey see Berghard and Dryer (1998). We find such sparsely dispersed neurons expressing *adorb* in fishes closely (common carp) and not so closely (eel) related to zebrafish, as well as in a tetrapod (frog). This suggests that the function of the *adorb* gene as an olfactory receptor is at least as old as the MRCA of the ray-finned and the lobe-finned lineage, about 430 Ma (Kumar et al. 2017). In contrast, the contiguous expression of the lamprey *adorb* gene in a region adjacent to, but clearly different from the sensory surface, argues strongly against a function as an olfactory receptor (fig. 8). The regions expressing *adorb* in lamprey are colabeled with a marker for proliferating cells (PCNA) and appear to be identical to the proliferative regions of the lamprey nose described in an earlier study (Thornhill 1970). Olfactory sensory neurons have a finite life time of a few weeks and have to be replenished regularly from dividing precursor cells throughout adult life in both mammals and fishes (Mackay-Sim and Kittel 1991; Ferrando et al. 2010). In zebrafish, it has been shown that immature neurons migrate from sensory-adjacent regions into the sensory surface (Oehlmann et al. 2004; Bayramli et al. 2017), so it is conceivable that the proliferative regions in the lamprey nose might serve the same purpose. Thus, the *adorb* expression pattern is consistent with a role in the development of olfactory sensory neurons. In this context, it is worth noting that purines (ATP and ADP, adenosine triphosphate and adenosine diphosphate, respectively) are in fact mitotic signals for olfactory stem cells in frog and fish (Hassenklöver et al. 2009; Demirler et al. 2020).

It is tempting to suggest that lamprey might represent the ancestral situation, in which the *adorb* gene may have served a function in proliferating precursor cells of the olfactory organ, and that at some time point after the divergence of

jawed fish and before the emergence of ray- and lobe-finned fish a function as olfactory receptor may have been acquired. In another case of neofunctionalization—immune system peptide receptors of the formyl peptide receptor family became olfactory receptors in rodents—it seems to have been sufficient to come under control of transcription factors regulating olfactory receptor-like expression patterns (Dietschi et al. 2017). Even the beta adrenergic receptor, expressed as a knock-in under olfactory receptor promoter control, was able to generate a specific target glomerulus like proper OR genes (Feinstein et al. 2004).

Alternatively, the nonsensory expression of lamprey *adorb* could constitute the derived pattern, in analogy to what has been observed for several OR genes, whose coding regions came under regulatory control of nonolfactory genes, resulting in extra-nasal expression (Feingold et al. 1999). However, this appears less likely for several reasons, among them the function of the presumptive ligands ATP and adenosine as mitotic signals acting on proliferating progenitor cells (Hassenklöver et al. 2009; Demirler et al. 2020). A final answer will require expression analysis in a larger array of chordate, hemichordate, echinoderm, and additional early-derived vertebrate species.

In an initial attempt to shed light on the change in *adorb* function between jawless and bony fish, we analyzed the genomic surrounding of the *adorb1* gene in bony, cartilaginous, and jawless fish. The *adorb1* gene is situated inside the large fourth intron of the *parp1* gene for spotted gar, zebrafish, medaka, and Western clawed frog (Wakisaka et al. 2017)—incidentally this has led to many misannotations of the *adorb* gene as *parp1* by automated gene prediction methods. We observed the same genomic location inside the fourth *parp1* intron in whale shark, elephant shark, sea lamprey, and arctic lamprey (data not shown), the latter being a very close relative of the lamprey species we used for expression studies (*Lampetra fluviatilis*). However, chordate and ambulacraria *adorb* genes do not exhibit this association with *parp1*, which often only possesses small introns in these species (data not shown). Thus the genomic surrounding of *adorb* changed in vertebrates compared with chordates, but not between jawless and jawed vertebrates. In other words, the association with *parp1* predates the olfactory function of the *adorb* gene, and consequently cannot explain the aquisition of olfactory function in jawed vertebrates.

Taken together, we have shown the presence of the *adorb* gene in the MRCA of ambulacraria and chordates, which makes this gene more ancient than even the OR genes, previously known as the most ancient olfactory receptor genes, which are limited to chordates (Churcher and Taylor 2009). However, the olfactory function itself appears to have been acquired much later in jawed vertebrates, because the jawless vertebrate lamprey expresses *adorb* in nonolfactory cells, albeit directly adjacent to the olfactory region (fig. 8). The evolutionary process converting the olfactory-adjacent

contiguous expression in lamprey to the expression in scattered olfactory sensory neurons in jawed vertebrates remains to be elucidated.

Materials and Methods

Animal Handling

Experiments were performed with tissue from adult river lampreys (*L. fluviatilis*) in the anadromous stage, African clawed frog (*Xenopus laevis*) in the larval stage 50–54, juvenile Common carp (*Cyprinus carpio*), juvenile European eel (*Anguilla anguilla*), and adult zebrafish (*Danio rerio*) of either sex. Lampreys were kept at 4 °C and zebrafish at 28 °C both with a 12 h/12 h light/dark cycle. Animal housing and maintenance was licensed by the office for environment and consumer protection of the city of Cologne, Germany. Eel and carp were anesthetized and dissected direct after delivery. The experimental procedures were approved by the Federal ministry for nature, environment and consumer protection of Nordrhine-Westfalia, Germany, and the local ethics committee in Stockholm (Northern Stockholm Animal Review Board), Sweden, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996 revision).

Dissection of Olfactory Tissue

Lamprey

Lampreys were handled according to (von Twickel et al. 2019). In short, they were anesthetized with MS-222 (ethyl 3-minobenzoate methanesulfonate; 100 mg/l water; Sigma, St Louis, MO) dissolved in water from the aquarium at 4 °C. Animals were then decapitated with a scalpel caudal to the gills and moved to a preparation tray on ice (containing Sylgard gel; Dow Corning, Midland, MI) filled with 4 °C artificial cerebral spinal fluid, with the following composition (in mM): NaCl 125, KCl 2.6, MgCl2 1, CaCl2 2, NaHCO3 25, and Glucose10. After dissection of an olfactory epithelium, it was placed for 17–40 h in 4% paraformaldehyde (PFA), then 3 h in 20% sucrose, and 15 min in Tissue-Tek O.C.T. Compound before freezing.

Zebrafisch, Carp, and Eel

Before the preparation of olfactory epithelia, the fish was anesthetized with the appropriate amounts of MS-222 (300 mg/l for zebrafish and carp, double the amount for eel). After this the fish was decapitated with a sharp razor blade and the head was fixed from the top with a sterile forceps in a petridish with prechilled phosphate-buffered saline (PBS) ph 7.4. To simplify the removal of the olfactory epithelia, both eyes were taken out and the gills were pulled into an anterior direction. Next, the skin at the top of the head and around the nose holes was removed to expose the olfactory epithelia. Then as much cartilage as possible was removed around the olfactory epithelia before final removal. For ISH, the complete epithelia were stored in Tissue-Tek O.C.T. Compound at −80 °C. In case of immunohistochemistry (IHC), the epithelia were fixed in 4% PFA in PBS pH 7.4 for 5 min and then stored in tissue tec-OCT Compound at −80 °C.

Frog

Frog olfactory tissue was prepared as published in Syed et al. (2017).

mRNA Isolation, cDNA Synthesis, and ISH Probes

After dissection, noses were immediately placed in RNALater (Sigma-Aldrich). Total mRNA was extracted using the easy-spin Total RNA Extraction Kit (iNtRON Biotechnology) resulting in highly concentrated mRNA (OD260 > 0.9). Complementary DNA (cDNA) was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, No. 18064022), with forward (FW) and reverse (RV) primers for probe synthesis as listed (table 1). The T3 promoter sequence (ATTAACCCTCACTAAAGG) was added 5′ to the RV primer for generation of RNA probes. The lamprey primers were originally developed based on a cDNA of *Petromyzon marinus* and tested in *L. fluviatilis*. For *tarl7a* (aka *Lor3*) a previously published primer-pair was used (Berghard and Dryer 1998). All PCR products were confirmed by sequencing.

Table 1
Primer Sequences

Species	Gene	Forward 5′–3′ primer	Reverse 5′–3′ Primer	No. of bp
Carp	*adorb1*	TCTACGCGCAGATCTTCGTC	TTGTTCATCGCCATCACCGA	362
Eel	*adorb1*	AACAGGAAGATGCACACCGT	ATGAGGAAGGACAGGACCCA	308
Frog	*adorb1*	TTAACGTCTCTGTCCGTGGC	AGAGGGGTTTATGCCAGCC	314
Zebrafish	*adorb1*	TTCGTCACGGTTAAGCGTCA	ATTCCCCTGCAGCACATGAA	329
Lamprey	*adorb1*	TCTACGCGCAGATCTTCGTC	TGTTCATCGCCATCACCGA	530
Lamprey	*Golf*	ATTTTTCGACCACGTGAAGG	AGGATGATCGAGATGGTTCG	432
Lamprey	*trpc2*	GAGTGCAAGGAGGTGTGGAT	ATGAGCATGTTGAGCAGCAC	599
Lamprey	*tarl7a*	CCGCAACGCGTGGTCCTGAT	TCCTAAAGTTGAATAGATCCGTC	961

In table 1, species, genes, and primer direction as indicated. Number of bp denotes the length of the PCR product used for generation of the RNA probes.

ISH and ISH Combined with IHC

Ten-micrometer cryosections of olfactory epithelia were placed onto Superfrost Plus slides. ISH and IHC were performed as described (von Twickel et al. 2019). Primary antibodies used in the IHC are mouse anti-PCNA (1:200, Merck) and mouse anti-HuC (1:200, Invitrogen). A strong PCNA signal in proliferating neuronal precursor cells is easily distinguished from very faint labeling of neuronal cells, similar to observations by (Ino and Chiba 2000). The HuC signal is highly specific for the neuronal region of the nose, whereas acetylated tubulin antibody (1:200, Sigma) stained both sensory and nonsensory regions of the adult lamprey nose (data not shown), in contrast to more specific staining of the olfactory sensory surface of larval lamprey (Frontini et al. 2003). 4′,6-diamidino-2-phenylindole (DAPI) was used as counterstain for fluorescent detection. Micrographs were taken using a Keyence BZ-9000 fluorescence microscope, and absence of crosstalk between channels was confirmed.

Data Mining and Phylogenetic Trees

Initial searches using zebrafish adorb and adora amino acid sequences as queries and tBLASTn (Gertz et al. 2006) in Whole Genome Shotgun (WGS) databases available at NCBI showed widespread presence of adorb in fish, amphibians, and reptiles. Representative species for detailed analysis of adorb evolution were selected as follows (numbers for adora after the slash): 3/3 jawless fish, 3/2 cartilaginous fish, 1/1 lobe-finned fish (Latimeria chalumnae), 1/1 nonteleost ray-finned fish (Lepisosteus oculatus), 28/6 early- and later-derived teleost fish, 5/1 amphibians, and 7/1 reptiles (supplementary files 1 and 3, Supplementary Material online). However, in case of a predicted gene loss all available genomes from the respective phylogenetic group were analyzed. To investigate the evolutionary origin of adorb and adora genes all nonvertebrate deuterostome genomes available at NCBI were examined—ten echinoderm, two hemichordate, four cephalochordate, and five urochordate genomes (supplementary files 1 and 3, Supplementary Material online). Blast hit regions were extracted with 0.5–1 kb flanking sequence. For monoexonic genes Expasy (Artimo et al. 2012) was then used to identify the appropriate open reading frame, for multiexonic genes genewise (Madeira et al. 2019) was used with close homologues as template. Most candidates could be obtained as full length, but all candidates down to 100 amino acid length were evaluated further. In few cases, gene predictions were made by splicing several contigs together. This was only done, when a template with

high homology was available, and when no ambiguity was possible. Searches were performed recursively using more closely related genes as query once they had been validated in phylogenetic trees with reference and outgroup genes (see below). BLAST hits were ordered by decreasing % identity, and evaluated until several consecutive candidates fell into the outgroup. Candidate genes were validated in phylogenetic trees with known reference genes and a large outgroup consisting of representative members from all subgroups of rhodopsin (alpha, beta, gamma, and delta) and rhodopsin-associated receptors including genes from early-diverging species (Fredriksson et al. 2003; Nordström et al. 2011; supplementary files 1 and 3, Supplementary Material online). Phylogenetic tree construction was performed as described (Dieris et al. 2021). In short, MAFFT version 7 with E-INSI setting (Katoh and Standley 2013; Katoh et al. 2019) was used to generate the multiple sequence alignment, which was then degapped at 90% threshold. The tree was constructed using the maximum likelihood algorithm PhyML-aLRT with smart model selection (resulting in substitution model JTT+G+F), subtree grafting and chi square-based aLRT to estimate branch support (Dereeper et al. 2008; Guindon et al. 2010; Lefort et al. 2017).

Naming of Genes

Adora genes newly identified in this study were named according to phylogenetic relationship with already named genes, whenever possible. Adora genes outside the known clades of adora1, 2a, 2b, and 3 fell into two clades segregating with maximal branch support, which were named adora4 and adora5. An urochordate sister group of vertebrate-urochordate adora genes was named adoral, for adora-like. The zebrafish gene initially named a2c (adora2c; Wakisaka et al. 2017) belongs to a sister clade of [adora (adenosine receptor a) + adoral genes] and was therefore renamed as adorb (adenosine receptor b). Adora and adorb genes together possess a sister clade, which was named sador (sister of ador). Subdivisions of adorb, adoral and sador were named first by number and then by letter.

Inference of Gene Birth and Death Events

The topology of the gene tree was generally similar to that of the taxonomic tree, for example, within ambulacraria the hemichordate genes always formed a sister group to the echinoderms, and within vertebrates the jawless fish were more basal than cartilaginous than bony fish. Thus, it was mostly straight-forward to infer gene birth and death events by eye, and no program was used. In a few exceptions, we considered maximum parsimony to infer gene birth/death events, not the tree topology, since in our experience a small number of genes in a particular clade can distort its position in the tree (Dieris et al. 2021).

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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Data Availability

The data underlying this article are available in the article and in its Supplementary Material online.

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