

Structural shifts of aldehyde dehydrogenase enzymes were instrumental for the early evolution of retinoid-dependent axial patterning in metazoans

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Aldehyde dehydrogenases (ALDHs) catabolize toxic aldehydes and process the vitamin A-derived retinaldehyde into retinoic acid (RA), a small diffusible molecule and a pivotal chordate morphogen. In this study, we combine phylogenetic, structural, genomic, and developmental gene expression analyses to examine the evolutionary origins of ALDH substrate preference. Structural modeling reveals that processing of small aldehydes, such as acetaldehyde, by ALDH2, versus large aldehydes, including retinaldehyde, by ALDH1A is associated with small versus large substrate entry channels (SECs), respectively. Moreover, we show that metazoan ALDH1s and ALDH2s are members of a single ALDH1/2 clade and that during evolution, eukaryote ALDH1/2s often switched between large and small SECs after gene duplication, transforming constricted channels into wide opened ones and vice versa. Ancestral sequence reconstructions suggest that during the evolutionary emergence of RA signaling, the ancestral, narrow-channeled metazoan ALDH1/2 gave rise to large ALDH1 channels capable of accommodating bulky aldehydes, such as retinaldehyde, supporting the view that retinoid-dependent signaling arose from ancestral cellular detoxification mechanisms. Our analyses also indicate that, on a more restricted evolutionary scale, ALDH1 duplicates from invertebrate chordates (amphioxus and ascidian tunicates) underwent switches to smaller and narrower SECs. When combined with alterations in gene expression, these switches led to neofunctionalization from ALDH1-like roles in embryonic patterning to systemic, ALDH2-like roles, suggesting functional shifts from signaling to detoxification.

Aldehyde dehydrogenase phylogeny | *Branchiostoma floridae* | *Ciona intestinalis* versus *Ciona savignyi* | evolution of retinoic acid signaling | origins of morphogen-dependent signaling

In animal development, major signaling pathways are controlled by morphogens, diffusible molecules whose evolutionary origins are difficult to assess. Aldehyde dehydrogenase (ALDH) enzymes are attractive subjects to study the evolution of morphogen signaling for two main reasons. First, in addition to their acknowledged role in protecting animals by catabolizing reactive biogenic and xenobiotic aldehydes, some ALDHs also synthesize signaling molecules (1–3). Prime examples for these two ALDH enzyme roles are the ALDH2s, which degrade small toxic aldehydes, such as the acetaldehyde derived from ethanol metabolism (1, 2), and the ALDH1s, which process larger aldehydes, including retinaldehyde, a vitamin A-derived precursor of the morphogen retinoic acid (RA). RA plays a critical role during embryonic development of chordates (i.e., amphioxus, tunicates, and vertebrates) and has been suggested to have already been involved in patterning the last common ancestor of bilaterian animals (4–8).

Second, ALDHs are among the best-characterized proteins, and their structure and substrate profiles have been determined with exquisite precision (9–15). Thus, structural modeling of these proteins can be used to study the evolution of substrate specificity without extensive biochemical analyses (16–20).

ALDH1 and ALDH2 enzymes share a high degree of sequence identity, indicating a very close phylogenetic relationship (3). Pioneer observations by Moore et al. on human ALDH2 and sheep ALDH1 (17) suggested that their respective abilities to detoxify small aldehydes and to process large aldehydes are correlated with the size and shape of their substrate entry channels (SECs), the intramolecular cavities that direct aldehydes to the catalytic sites of ALDH enzymes. Human ALDH2 displays a narrow SEC with a constricted entrance, whereas sheep ALDH1A1 exhibits a large SEC with a broad opening (17, 18). Thus, SEC topology influences ALDH1/2 substrate preference. For example, although retinaldehyde is a good substrate for vertebrate ALDH1s and acetaldehyde is a natural substrate of ALDH2s, ALDH2s cannot process retinaldehyde and ALDH1s process acetaldehyde only extremely inefficiently (16, 17–22).

To understand the evolutionary origins of the substrate preferences of ALDH1 and ALDH2 enzymes, as well as to illuminate how signaling and protective functions are connected to these different enzyme activities, we used an integrated approach that combined genomic, phylogenetic, and structural analyses. The resulting comprehensive data set was complemented with information on developmental gene expression of ALDH1/2s in the cephalochordate amphioxus (*Branchiostoma floridae*) and the ascidian tunicate *Ciona intestinalis*. These two invertebrate chordate models possess functional RA signaling cascades and are pivotal models for understanding vertebrate origins from both a genomic and a developmental perspective (4, 23–26). Together, this work provides support for the hypothesis that some intercellular signaling mechanisms evolved from cellular detoxification pathways.

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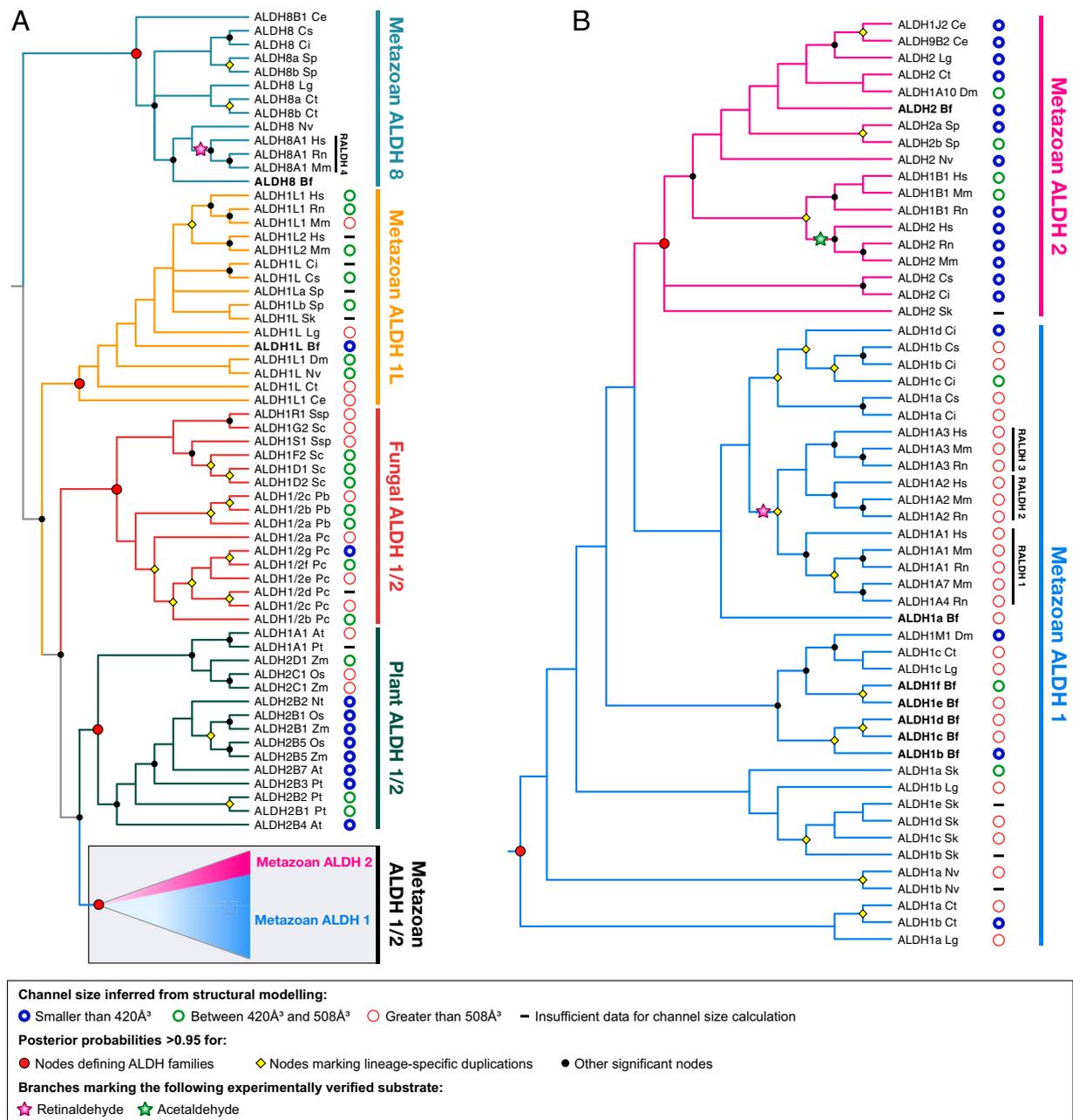


Fig. 1. Phylogeny of ALDH1/2s, ALDH1Ls, and ALDH8s. The overall topology with the ALDH8s used as outgroup is shown in A. The boxed area in A highlights the metazoan ALDH1/2 clade, which is depicted in B. Nodes with significant posterior probabilities (>0.95) are highlighted with icons. The actual values are given in Fig. S2. Channel size categories are shown diagrammatically for ALDH1/2s and ALDH1Ls with sufficient sequence information. ALDH8s are too divergent for these calculations. At, *Arabidopsis thaliana*; Bf, *Branchiostoma floridae*; Ct, *Capitella teleta*; Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis*; Cs, *Ciona savignyi*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Lg, *Lottia gigantea*; Mm, *Mus musculus*; Nv, *Nematostella vectensis*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pc, *Phanerochaete chrysosporium*; Pb, *Phycomyces blakesleeanae*; Pt, *Populus trichocarpa*; Rn, *Rattus norvegicus*; Sc, *Saccharomyces cerevisiae*; Sk, *Saccoglossus kowalevskii*; Sp, *Strongylocentrotus purpuratus*; Ssp, *Schizosaccharomyces pombe*; Zm, *Zea mays*.

channel in amphioxus ALDH1a and ALDH1d, whereas the larger Glu124 (109 Å³) or Ser124 (73 Å³) partially obstruct the channel in the other four amphioxus duplicates, leaving insufficient space to accommodate the β-ionone moiety of retinaldehyde (Fig. 2). The ALDH1s from *C. intestinalis* and *C. savignyi* are also heterogeneous: *C. intestinalis* ALDH1a and ALDH1d, as well as *C. savignyi* ALDH1a, display the small Gly and *C. savignyi* ALDH1b, the small Ala (67 Å³) at position 124, which do not obstruct the channel entrance, whereas *C. intestinalis* ALDH1b and ALDH1c, respectively, display the larger Ser124 and Ile124, which interfere

with retinaldehyde accommodation in the ALDH1 channel (Fig. 2, and Figs. S4 and S5).

In the ALDH2s from amphioxus, *C. intestinalis*, and *C. savignyi*, the amino acid at the second channel signature is Phe459, which constricts the channel neck with its large aromatic ring. As in vertebrate ALDH1s, in some amphioxus ALDH1s, the bulky Phe459 is substituted by smaller amino acids, such as Ile459 in ALDH1a, ALDH1b, and ALDH1d, Thr459 (93 Å³) in ALDH1c or Gly459 in ALDH1e and ALDH1f. In ascidian tunicates, only *C. intestinalis* ALDH1d displays a Leu459, whereas all other

ALDH1s display bulky amino acids, such as Phe and Met at position 459 (Figs. S4 and S5), similar to vertebrate ALDH2s.

As in vertebrates, in amphioxus, *C. intestinalis*, and *C. savignyi*, the amino acids of the third ALDH2 signature at the channel bottom is Cys303. Amphioxus ALDH1s are heterogeneous in that ALDH1a and ALDH1d display the vertebrate ALDH1 pattern (Thr303 and Ile303, respectively), whereas all other amphioxus ALDH1s display the vertebrate ALDH2 pattern (Fig. 2). In ascidian tunicates, only *C. intestinalis* and *C. savignyi* ALDH1a display the vertebrate ALDH1 pattern with Thr303, whereas other ALDH1s show the vertebrate ALDH2 pattern (Figs. S4 and S5). Thus, after lineage-specific duplication, some ALDH1 enzymes of invertebrate chordates incorporated amino acids and/or structural motifs similar to those of the vertebrate ALDH2 channel, shifting from a large, wide open configuration to constricted SEC topologies.

Syntenic analyses in amphioxus and *C. intestinalis* suggest that the ALDH1a genes from both species are the sister groups to the other cephalochordate and ascidian tunicate ALDH1s and that the amphioxus duplicates ALDH1b, ALDH1c, ALDH1d, ALDH1e, and ALDH1f and *C. intestinalis* ALDH1b, ALDH1c, and ALDH1d evolved by duplication from an ALDH1a-like ancestor (Fig. S6). Moreover, reconstructions of ancestral SEC signatures and channel structures indicate that amphioxus and *C. intestinalis* ALDH1 ancestors displayed large SECs, structurally consistent with the capacity to process retinaldehyde into RA (Figs. 1 and 2, and Figs. S2 and S4). The reconstructed amphioxus ALDH1 ancestor already displayed a typical ALDH1 SEC with a 512 Å³-wide opening lined by Gly124, an unobstructed neck flanked by Val459, and a bottom Cys303. The reconstructed *C. intestinalis* ALDH1 ancestor exhibits a large, 540 Å³ SEC, but, curiously, with ancestral Met124, Phe459, and Cys303 SEC signatures, suggesting that large ALDH1 channels emerged independently and by different mechanisms in cephalochordates and ascidian tunicates.

Invertebrate Chordate ALDH1 Channel Switch Is Associated with Transitions Between Restricted and Pleiotropic Expression. Our next goal was to understand the specific developmental contexts in which the invertebrate chordate ALDH1/2s are deployed. We hence assessed developmental expression of the large-channelled ALDH1s structurally capable of accommodating retinaldehyde for RA synthesis, the narrow-channelled ALDH2 genes adapted for small aldehyde detoxification, and the divergent, narrow-channelled ALDH1s.

The ALDH1a and ALDH1d genes of amphioxus and *C. intestinalis* encode enzymes with large and unobstructed SECs. Amphioxus ALDH1a is expressed caudally close to the developing tail bud with a sharp anterior boundary in the neurula (at 12 h) (Fig. 2). In *C. intestinalis*, ALDH1a is expressed in a sharp, posterior mesodermal domain in the early embryo (at 5–8 h) (Fig. S4) (30). At later embryonic stages, ALDH1a is detectable in a distinct domain in the posterior gut endoderm of the amphioxus late embryo (at 24 h) and in the posterior trunk of *C. intestinalis* (at 10–12 h). In amphioxus, expression of ALDH1d overlaps that of ALDH1a at the neurula stage, but diverges from that of ALDH1a in the late embryo. At this stage, amphioxus ALDH1d expression is broad and inconspicuous with a moderate accentuation of the signal in the posterior gut endoderm. In *C. intestinalis*, ALDH1d expression is diffuse and weak in the gastrula and becomes diffusely transcribed in the trunk at 10–12 h of development. Thus, in both amphioxus and *C. intestinalis*, ALDH1a is consistently expressed in patterns that are entirely consistent with a role in anteroposterior patterning and that are reminiscent of expression of vertebrate ALDH1A2 (RALDH2), which defines posterior identity in the vertebrate embryo (31).

In both amphioxus and *C. intestinalis*, there is only a single gene encoding a narrow-channelled ALDH2 enzyme. In amphioxus, ALDH2 expression is restricted to posterior, mesendodermal tissues at 12 h of development and, subsequently, spreads throughout the embryo at 24 h (Fig. 2). In *C. intestinalis*, ALDH2 expression is strong and diffuse at early and late developmental

stages (Fig. S4). Thus, ALDH2 genes are expressed in widespread patterns during development of invertebrate chordates.

There are a total of six genes encoding ALDH1s with narrow channels in amphioxus and *C. intestinalis*: amphioxus ALDH1b, ALDH1c, ALDH1e, and ALDH1f and *C. intestinalis* ALDH1b and ALDH1c. In amphioxus, these duplicates are weakly expressed in posterior domains overlapping that of ALDH1a in the neurula (at 12 h) (Fig. 2). However, by 24 h, they are expressed diffusely and weakly throughout the amphioxus embryo with a weak to moderate concentration of the signal for ALDH1b, ALDH1c, and ALDH1e in the posterior gut endoderm. In *C. intestinalis*, ALDH1b is diffusely transcribed in trunk and tail, whereas ALDH1c is not detectable by in situ hybridization in developing embryos (Fig. S4). Thus, the genes encoding narrow-channelled ALDH1s in amphioxus and *C. intestinalis* generally display either widespread or inconspicuous expression patterns during development, suggesting that these enzymes are not playing major roles in anteroposterior patterning of the embryo.

Discussion

Structural Insights into ALDH1 and ALDH2 Function. Our modeling studies confirm the notion, determined by Moore et al. (17) with only two enzymes, that substrate access channel size is a crucial determinant of ALDH1 and ALDH2 function. Here, we extend this concept to the whole metazoan ALDH1/2 clade and provide insights into the structural adaptations underlying the ability of the narrow-channelled ALDH2s to process small aldehydes and of the large-channelled ALDH1s to process large, bulky aldehydes.

We determined that position 124 at the channel entrance is a selective gate for aldehyde size. Ancestral reconstructions show that, throughout metazoan ALDH1/2 evolution, increases in channel size are associated with substitution of the bulky, ancestral, Met124 by small Ala124 or Gly124 (Table S2). The selective abilities of ALDH1/2 channels are thus regulated by the presence or absence of a steric hindrance to the entry of large aldehydes into the ALDH1/2 channel. Thus, we show that the ALDH2 channel cannot accommodate retinaldehyde, which is consistent with earlier studies (16, 22) showing that retinaldehyde is not an ALDH2 substrate, but a competitive inhibitor of acetaldehyde degradation. This behavior contrasts with the ease with which retinaldehyde is admitted into the ALDH1 channel. Thus, size selection is a fundamental feature of substrate discrimination by the ALDH1/2 channel. We have also shown that, although ALDH2 can keep small aldehydes close to the catalytic Cys302 long enough for catalysis, ALDH1s cannot. Therefore, small aldehyde processing by ALDH2s requires specific structural adaptations to reduce substrate mobility inside their channels, an ability that is lacking in large-channelled ALDH1s (16).

Switches Between Small and Large Substrate Entry Channels Are Common in ALDH1/2 Evolution. The changes in the ALDH1 and ALDH2 SEC that we describe reflect a tendency of eukaryote ALDH1/2 genes to alter the structures of their encoded enzymes after gene duplication (Fig. 2 and Figs. S4, S5, and S7). By accumulating mutations at the mouth, neck, and/or SEC bottom, ALDH1/2s underwent changes in SEC geometry and/or overall volume, which affected their structural abilities to accommodate their original substrates. These changes led to switches from small, constricted channels adapted to the handling of small aldehydes to large, broadly opened channels adjusted to receive large aldehyde molecules or vice versa (Fig. S8). Our results thus provide an important contrast to studies proposing a general nonreversibility of amino acid changes involved in the functional adaptation of proteins (32).

ALDH1/2 Switches and the Origins of RA Signaling. Although ALDH enzymes can catalyze a range of different substrates, the molecular switches between ALDH1 and ALDH2 SECs reported here very likely represent functional transitions between the ability of ALDH1/2s to process small, toxic aldehydes for defense against endogenous and xenobiotic aldehyde aggression and its capacity

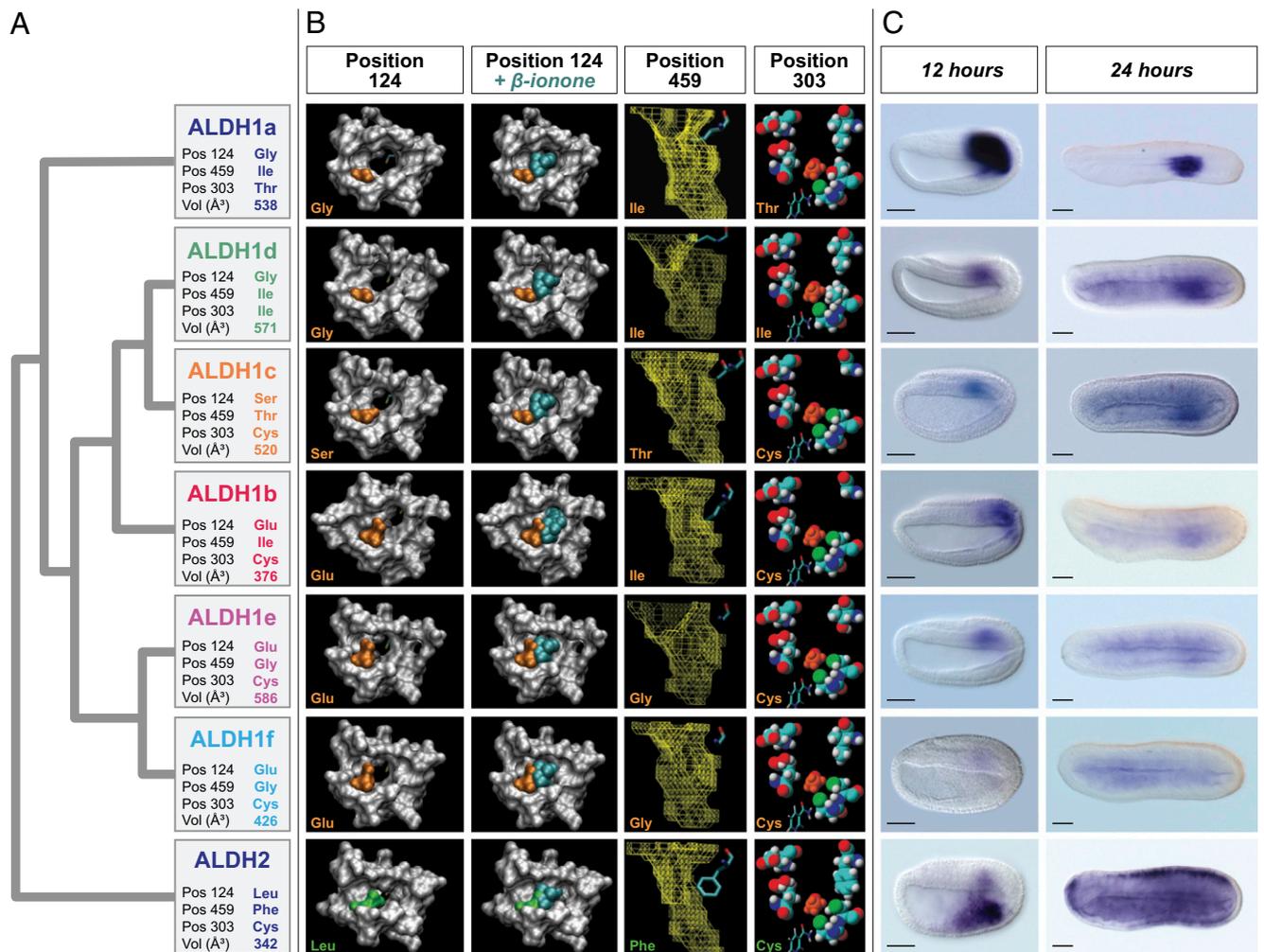


Fig. 2. Amphioxus ALDH1/2 duplicates. Phylogeny (A), channel structure (B), and developmental expression (C). Amino acid signatures of the substrate entry channel at positions 124 (the mouth), 459 (the neck), and 303 (the bottom) are indicated. For the expression analyses, neurulae (12 h) and late embryos (24 h) are shown. (Scale bars: 50 μ m.)

to generate signaling molecules from larger aldehyde precursors. Using ancestral sequence reconstruction, we provide evidence that ALDH1/2 switches were important for the emergence of ALDH1 retinaldehyde dehydrogenases, which probably originated after gene duplication early in metazoan evolution, when a small, narrow-channeled ALDH1/2 ancestor, structurally related to modern ALDH2s, gave rise to a gene encoding a larger SEC capable of accommodating bulkier molecules, including retinaldehyde. This evolutionary scenario supports the view that RA signaling evolved from enzymes implicated in detoxification (3) and, combined with the description of a retinoic acid receptor (RAR) and of other RA signaling cascade members in both protostomes and deuterostomes, pushes the origins of RA signaling to much earlier times than traditionally assumed (4, 7).

ALDH1s Underwent Independent Duplication and Extensive Diversification in Metazoans. Our data substantiate the notion that the metazoan ALDH1 ancestor originated from a eukaryote ALDH1/2 ancestor and underwent duplication before the origin of bilaterian animals. It is also evident that ALDH1s duplicated independently in various animal groups and underwent extensive diversification, which is supported by two ALDH1 genes (one with a large SEC) in the cnidarian *Nematostella vectensis* and, in amphioxus and *C. intestinalis*, by structurally dissimilar ALDH1 ancestors and by the distinct arrangement of ALDH1 SEC signatures. Although duplication and diversification are common in

ALDH1s, the metazoan ALDH2s are typically preserved as single copies, and their SECs have kept the same constricted features of the eukaryote ALDH1/2 ancestor.

Invertebrate ALDH1 Switches Suggest Shifts from Patterning to Detoxification. The presence of ALDH1 duplicates in a given animal raises questions about the roles of each duplicate within the RA signaling cascade (4, 7). ALDH1 duplicates in amphioxus and *C. intestinalis* originated by duplication from an ALDH1 ancestor with a large SEC structurally compatible with RA synthesis (Fig. S6). This structure is present in their ALDH1a paralogs, which display sharp posterior domains, consistent with early embryonic anteroposterior patterning. In contrast, genes encoding ALDH1b, ALDH1c, ALDH1e, and ALDH1f in amphioxus and ALDH1b and ALDH1c in *C. intestinalis* accumulated mutations resulting in constricted ALDH SECs poorly suited to accommodate large molecules, but still capable of admitting small aldehydes. These genes display broad expression patterns, suggesting that they have evolved novel functions probably associated with the processing of small, toxic aldehydes for protection against endogenous or xenobiotic aldehydes (1–3).

A plausible scenario leading from patterning to protective ALDH roles can be derived from the expression patterns of the ALDH1d genes of amphioxus and *C. intestinalis*. The molecular structures of the SECs of these two ALDH1ds are consistent with retinaldehyde processing. However, ALDH1d expression is rather

broad throughout the amphioxus embryo and the *C. intestinalis* embryonic trunk, suggesting that changes in gene regulation of these two genes occurred after duplication and that these changes were not accompanied by structural remodeling of the SEC.

The transition from restricted signaling functions to generalized roles has not been completed to equivalent degrees in each of the divergent amphioxus duplicates. ALDH1b, ALDH1c, ALDH1e, and ALDH1f developed diffuse patterns in the late embryo, while curiously maintaining weak, but restricted, posterior domains during neurulation and, except for ALDH1f, a relative concentration of expression in the posterior gut endoderm, which are likely to represent the ancestral, ALDH1a-like pattern. In *C. intestinalis*, ALDH1b is diffusely and inconspicuously expressed in the trunk, whereas ALDH1c expression is not detectable during embryogenesis, but seems to be restricted to adult tissues, as indicated by EST database searches.

The fate of these ALDH1 duplicates in amphioxus and *C. intestinalis* is consistent with an evolutionary scenario involving neofunctionalization after duplication, with gene duplicates acquiring more generalized functions during embryogenesis and, possibly, in the adult. Therefore, in amphioxus and *C. intestinalis*, some duplicated ALDH1s experienced modifications of gene regulation and protein structure that resulted in neofunctionalization of the duplicates, possibly away from roles in axial patterning, toward generalized, pleiotropic functions similar to those of ALDH2, an enzyme important for protection against small aldehyde toxicity in chordates (33).

The ALDH1/2 Case and Its Implications for Anatomic and Physiological Evolution. Mutations of regulatory regions have been regarded as the major driving force of morphological evolution in development (34), whereas mutations in coding regions are viewed as major determinants of physiological evolution (35). Here, we describe regulatory alterations affecting duplicated ALDH1/2

genes of amphioxus and ascidian tunicates that are accompanied by fundamental structural shifts of the proteins they encode. Therefore, our data suggest that distinctions between anatomical and physiological evolution may not always be so clear cut and that rapid evolution of novel functions can be achieved when regulatory and protein structure mutations are superimposed after gene duplication, a hypothesis that provides a common ground for these two evolutionary mechanisms that have traditionally been thought to depend on distinct mechanisms. In sum, the ALDH1/2 case probably represents one of many examples that are likely to emerge with the incorporation of protein structure analyses into the collection of approaches used to study the evolution of body plans.

Materials and Methods

Whole genomes, EST databases, and trace repositories were mined for ALDH sequences using both signature (InterPro IPR002086) and global similarity searches. Amphioxus and *C. intestinalis* ALDH1/2 clones were obtained, respectively, from cDNA libraries (36) and from the Gene Collection Release 1 (37). ALDH amino acid residue numbers are based on the classical numbering of the mature human ALDH2 enzyme with the catalytic Cys at position 302 (17).

For additional details, see *SI Materials and Methods*.

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