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REVIEW
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Diversification and Functional Evolution of HOX Proteins

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Gene duplication and divergence is a major contributor to the generation of morphological diversity and the emergence of novel features in vertebrates during evolution. The availability of sequenced genomes has facilitated our understanding of the evolution of genes and regulatory elements. However, progress in understanding conservation and divergence in the function of proteins has been slow and mainly assessed by comparing protein sequences in combination with *in vitro* analyses. These approaches help to classify proteins into different families and sub-families, such as distinct types of transcription factors, but how protein function varies within a gene family is less well understood. Some studies have explored the functional evolution of closely related proteins and important insights have begun to emerge. In this review, we will provide a general overview of gene duplication and functional divergence and then focus on the functional evolution of HOX proteins to illustrate evolutionary changes underlying diversification and their role in animal evolution.

Keywords: gene duplication and divergence, protein evolution, HOX proteins, *Drosophila*, mouse

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INTRODUCTION

Evolution has brought an incredible range of morphological and physiological novelties to diverse animals. Centuries of classical research has served to catalog diverse novelties in 1.2 million species and sub-divide them into ~36 phyla and 107 classes, 500 orders, 5500 families and 110000 genera (Mora et al., 2011). These efforts have uncovered the emergence of novelties during the progressive evolution of animals, but we know relatively little about the genetic and genomic changes and mechanisms that underlie this diversity. Technological advances which enabled the systematic sequencing of animal genomes has reenergized this field of research and provided an opportunity for comparative genomics of the diverse animals to probe the underlying genetic causes of their morphological and physiological differences (Rogers and Gibbs, 2014). These genome-wide analyses have highlighted common origins and similar physiological functions but have found it challenging to uncover the genetic changes and mechanisms that underlie animal diversity. Comparative genomic analyses reveal a very similar number of genes in diverse animals (**Figure 1**), indicating that the total gene number does not reflect diversity (Hahn and Wray, 2002; Copley, 2008). Furthermore, many of the same genes and gene families are present in a broad range of animal species, suggesting there is a shared or common "gene toolkit."

The discovery of a very similar number of genes and a common "gene toolkit" in animal genomes appeared to refute the hypothesis that gene duplication and diversification is a major contributor to animal diversity. This led to a shift in the focus of research from analyses of coding regions to identifying and characterizing diversification of *cis*-regulatory (non-coding) regions and gene regulatory networks embedded in the genome (Biemont and Vieira, 2006). A broad array of

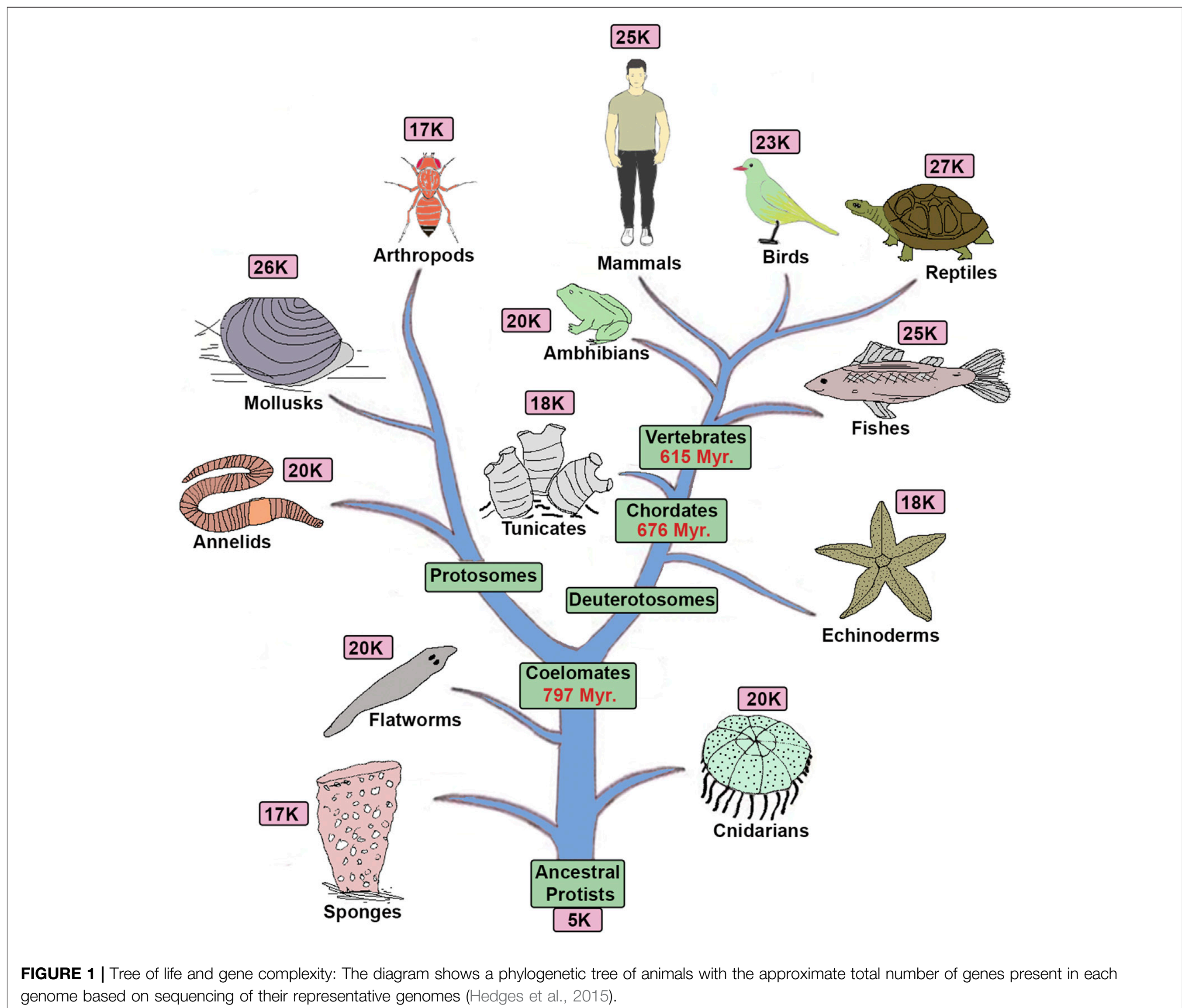


FIGURE 1 | Tree of life and gene complexity: The diagram shows a phylogenetic tree of animals with the approximate total number of genes present in each genome based on sequencing of their representative genomes (Hedges et al., 2015).

technological advances have also revolutionized this field and enhanced our ability to identify and functionally validate the *cis*-regulatory code embedded in the genome by the integration of comparative genomics, transgenic analyses, CRISPR/Cas9 genome modifications and genome-wide approaches (i.e., ChIP-seq, ATAC-seq, massive parallel reporter assays and single cell transcriptomics) (Zhen and Andolfatto, 2012; Paul et al., 2014; He et al., 2015; Gasperskaja and Kucinskas, 2017; Avsec et al., 2021). Application of these approaches, has revealed that diversity in non-coding *cis*-regulatory regions of the genome has played a major role in the emergence of animal diversity (Carroll, 2008; Rubinstein and de Souza, 2013; Reilly and Noonan, 2016; Franchini and Pollard, 2017; Xie et al., 2019; Roberts Kingman et al., 2021a; Roberts Kingman et al., 2021b).

Evaluating how changes in protein sequence impact *in vivo* function following gene duplications in animal evolution has been challenging to investigate in the absence of technologies for precise manipulation of endogenous genes and quantitative and qualitative functional assays to evaluate activity in an *in vivo* context. The development of CRISPER/Cas9 gene editing technology for precise manipulation of endogenous genes in the genome of diverse animals has opened the door for more direct cross-species comparisons of homologous protein functions (Doudna and Charpentier, 2014). This approach has the advantage of expressing the proteins being compared at the same physiological levels in their normal spatial, temporal and tissue-specific contexts under control of regulatory components of the endogenous loci. In the past, functional studies have primarily relied on comparing the degree of conservation in amino acid sequences, *in silico* structure predictions, *in vitro* assays for activity and ectopic over-expression assays *in vivo*. The *in vitro* assays of protein activity, such as ligand binding, enzymatic activity, transcription factor binding properties, can be limited by *ex-vivo* conditions, which often lack key co-factors

or components important *in vivo*. Hence, they may provide a limited perspective on a subset of functional activities relevant to their *in vivo* roles. To overcome some of the limitations of *in vitro* assays, transgenic approaches have been used to ectopically express genes in the animals and compare the *in vivo* properties of candidate proteins (Mcginnis et al., 1990; Quiring et al., 1994; Hanks et al., 1998). However, this approach often involved broad over-expression of proteins at high levels and ectopic sites, making it difficult to compare activities in normal physiological and developmental contexts. CRISPR/Cas9 technologies now offer possibilities to manipulate the genome to precisely compare function of homologous genes *in vivo*. As a result, cross-species analyses of gene function are beginning to uncover unexpected changes and mechanisms that contribute to conservation and divergence of protein functions contributing to animal diversity (Enard et al., 2009; Truong and Boeke, 2017; Laurent et al., 2020; Singh et al., 2020). In addition, recent advances in cryo-EM and computational approaches for predicting protein structures are rapidly changing our ability to analyze and compare the properties of proteins (Assaiya et al., 2021; Jumper et al., 2021).

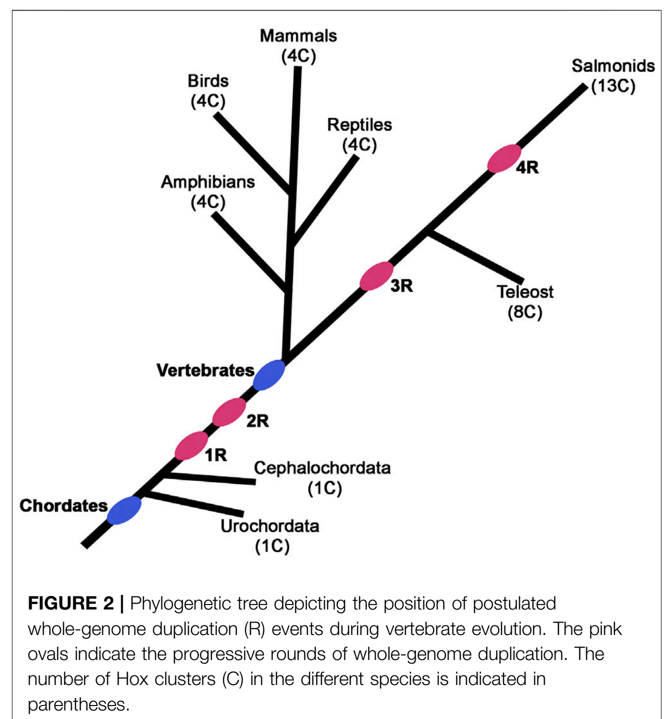


FIGURE 2 | Phylogenetic tree depicting the position of postulated whole-genome duplication (R) events during vertebrate evolution. The pink ovals indicate the progressive rounds of whole-genome duplication. The number of Hox clusters (C) in the different species is indicated in parentheses.

GENE DUPLICATION AND DIVERGENCE

Sequence analyses have revealed a high level of conservation of many domains in proteins with very diverse functions across the animal phylum (Laity et al., 2001; Ponting and Russell, 2002; Noyes et al., 2008). This implies that during evolution, the appearance of novel functions is not associated with widespread *de novo* evolution of new genes and that novel functional activities most likely arose by diversification of existing genes (Holland et al., 1994; Hughes, 1994; Friedman and Hughes, 2001; Blomme et al., 2006). Altering the function of an essential gene could be detrimental to the survival or fitness of a species, but gene duplication events provide a mechanism to circumvent this limitation. Generating multiple copies of a gene provides a range of opportunities to maintain essential functions, releasing selective pressure on a single essential gene, while also producing new substrates that can diversify and evolve novel functions. Analyses of gene sets across the animal kingdom revealed that vertebrate genomes have multiple copies of many invertebrate genes, including those that regulate development, differentiation and physiological processes, such as transcription factors, cell signaling pathways, odorant receptor genes etc. (Paps and Holland, 2018; Richter et al., 2018; Fernandez and Gabaldon, 2020). Large genome duplication events followed by gene losses are considered as a critical step in the emergence and evolution of vertebrates. Susumu Ohno suggested that two rounds of whole-genome duplications (2R-WGDs) could be a major source of gene amplification and functional diversification in vertebrate lineage (Ohno, 1970; Panopoulou et al., 2003; Vandepoele et al., 2004; Dehal and Boore, 2005). While this hypothesis was used to explain a major cause of gene duplications, comparing all homologous gene families between *Drosophila* and humans showed that less than 5% of these families display a predicted 1:4 gene ratio (Friedman and Hughes, 2001). This was not consistent with Ohno's hypothesis and lead to an alternative idea, suggesting that selective regional duplications (segmental duplications) created a mixed repertoire of duplicated genes in the genome.

Despite these conflicting models, which remain challenging to resolve, there is evidence for whole genome duplications in many plants, yeast, and vertebrate species, such as teleost fish (3R), salmonid fish (4R) and *Xenopus laevis* (Wendel, 2000; De Bodt et al., 2005; Scannell et al., 2006; Pascual-Anaya et al., 2013; Session et al., 2016) (**Figure 2**). Advocates of the 2R hypothesis have further refined the model to suggest that during vertebrate evolution frequent gene loss after duplication, as a consequence of redundancy, contributed to the observed digression from the expected 1:4 gene ratio. Analyses of many known genome duplications indicate that gene loss is the most common fate of duplicated genes (Nadeau and Sankoff, 1997; Albalat and Canestro, 2016). Loss of many duplicated genes and nonessential genes could also explain how a similar number of total genes are present in higher animal genomes despite differences in the whole genome or segmental duplication events. Despite the controversy on the underlying mechanisms for widespread amplification of gene families (Braasch et al., 2018; Sandve et al., 2018), the importance of gene duplication in creating a major substrate for functional divergence and emergence of novel functions is widely accepted in the field (Lynch and Conery, 2000).

FATE OF THE DUPLICATED GENES

The contribution of gene duplication and divergence to the emergence of novel protein functions in animal evolution has gained more traction as gene functions have been characterized in different animals across the phylogenetic spectrum (Blomme

et al., 2006). Comparing classes of genes based on their functions revealed that genes involved in animal development and cell signaling pathways are highly amplified during the evolution, while many other classes of genes have been lost. Based on these analyses several hypotheses came to explain the forces that shaped the future of a duplicated genes. Loss of function is the most common fate of the duplicated genes. Studies have shown that only half of duplicated genes are retained while others lose functional activities by processes that include deletion, rearrangement, point mutations, and pseudogene formation (Nadeau and Sankoff, 1997; Blomme et al., 2006; Scannell et al., 2006; Albalat and Canestro, 2016; Guijarro-Clarke et al., 2020a; b). Even in the absence of duplicated genes, a large number of genes (90% in bacteria, 80% in yeast, 65% in *C. elegans* and 85% in *Drosophila*) are dispensable for animal survival, which provides a large set of substrates for evolutionary change. Gene duplication events further expand the repertoire of substrates for change and generate opportunities for functional redundancy that allows for non-deleterious functional diversification of genes.

Essential genes and their functional roles need to be retained to ensure animal survival and fitness. Hence, at least one member of a duplicated gene family must retain the key ancestral functions during evolution. Other members are free to accumulate mutations that potentiates diversification and the emergence of novel activities, which is called neofunctionalization (Ohno, 1970; Clark, 1994; Holland et al., 1994; Lundin, 1999; Friedman and Hughes, 2001; Mazet and Shimeld, 2002; Sandve et al., 2018). A variation of this idea is that the ancestral functions of essential genes maybe collectively retained by partitioning sub-sets of the functional roles between different duplicated family members, which is termed subfunctionalization (Ohno, 1970; Force et al., 1999; Lynch and Force, 2000; Sandve et al., 2018). These are not mutually exclusive processes. A study by Le and Zhang demonstrated that neofunctionalization or subfunctionalization alone do not adequately explain the diversification of protein function. They proposed that many duplicated genes may go through a combination of subfunctionalization and neofunctionalization to produce duplicated genes that possess new and retain some ancestral roles (He and Zhang, 2005; Marcussen et al., 2010). Various theoretical and functional studies have explored these ideas and confirmed that the retention of duplicated genes appears to be mediated by a varying combination of these processes (Force et al., 1999; Lynch and Force, 2000; Vandenbussche et al., 2003; Walsh, 2003; Burki and Kaessmann, 2004; Escriva et al., 2006; Perry et al., 2007; Kleinjan et al., 2008; Innan, 2009; Truong and Boeke, 2017; Zimmer et al., 2018; Singh et al., 2020).

HOX GENES AS A PARADIGM FOR DUPLICATION AND DIVERGENCE OF FUNCTION

Hox genes, encode a broadly conserved family of transcription factors in animals, and represent an interesting paradigm for examining the duplication and divergence of gene functions. The HOX proteins are involved in patterning and specification of the anterior-posterior (AP) axis of all bilaterian animals (Mcginnis and Krumlauf, 1992; Krumlauf, 1994; Carroll, 1995; Pearson et al., 2005). The temporal and spatial order of *Hox* gene expression and function across the embryo is “colinear” and correlated with their organization along the chromosome (Lewis, 1978; Duboule and Dolle, 1989; Graham et al., 1989; Duboule, 1998; Kmita and Duboule, 2003). These genes are typically found to be tightly clustered in the genome except for some animals where evolution has led to the disintegration of the ancestral complex (Kaufman et al., 1980; Akam et al., 1994; Seo et al., 2004; Sekigami et al., 2017). Each gene in a cluster specifies distinct cellular identities along AP axis during very early embryonic development, which ultimately patterns tissues and structures in adult animals. Evidence of *Hox* genes in animal genomes is traced back to Cnidarians, however, their role in patterning the AP axis is observed only in bilaterians, as they have roles in patterning radial segmentation in cnidarians (Pascual-Anaya et al., 2013; Arendt, 2018; He et al., 2018). Mutations that affect the expression and function of *Hox* genes in bilaterians lead to homeotic transformation of one part of the body into another (Lewis, 1994). Furthermore, diversification of *Hox* gene number and function correlates with increased diversity in the evolution of animals (Wagner et al., 2003; Lemons and Mcginnis, 2006). There are fewer *Hox* genes in lower invertebrates as compared to higher invertebrates, chordates, and vertebrates, as illustrated by the 5 *Hox* genes in nematodes (*C. elegans*), 8 in arthropods (*Drosophila*), 14 in chordates, and 39 in mammals (Human) (Ikuta, 2011; Pascual-Anaya et al., 2013; Irie et al., 2018) (**Figure 3A**).

Despite their diverse and distinct functional activities, sequence analysis indicates that *Hox* gene cluster was generated by tandem duplication of a single gene. Analysis of *Hox* gene clusters in invertebrate genomes suggests that they expanded from a common ancestral gene through tandem regional duplication events to form a maximum of fifteen alloparalogs in cephalochordates (ex. *Branchiostoma floridae)* (Garcia-Fernandez and Holland, 1994; Koonin, 2005). There are four Hox clusters in mammalian genomes with a maximum of 14 genes in each cluster suggesting two rounds of whole complex duplication (2R) from a common invertebrate ancestor with 14 genes (Maconochie et al., 1996; Hoegg and Meyer, 2005; Lemons and Mcginnis, 2006; Duboule, 2007; Kuraku and Meyer, 2009; Pascual-Anaya et al., 2013; Holland and Ocampo Daza, 2018; Smith et al., 2018). However, there is a maximum of 13 *Hox* genes in each cluster of tetrapod genomes, such as mouse and human, indicating a loss of 14th paralog during vertebrate diversification (**Figure 3A**). There is also evidence for two additional lineage specific whole-genome duplications events in vertebrates, one in teleosts (3R) (Pascual-Anaya et al., 2013) and an additional round (4R) in salmonid lineages to further amplify HOX genes (**Figure 2**) (Soshnikova et al., 2013; Vieux-

Rochas et al., 2013). These data suggest that genome duplication events have dramatically increased the number of *Hox* genes in vertebrate genomes and provide opportunities for evolution of novel functions. Further, functional compensation or redundancy after gene duplication events allowed for loss and diversification of these genes which have a critical role in patterning the AP axis and the properties of tissues in a manner that has been remarkably conserved across the bilateral animals (Wagner et al., 2003).

CONSERVATION AND DIVERSIFICATION OF *HOX* GENE FUNCTION

The correlation between the expansion of *Hox* genes, a master regulator of development, and greater complexity of vertebrates highlights the importance of studying the functional evolution of HOX proteins. There are reports to suggest both conservation and diversification of *Hox* gene function through evolution (Lawrence and Morata, 1994; Saurin et al., 2018). Early work

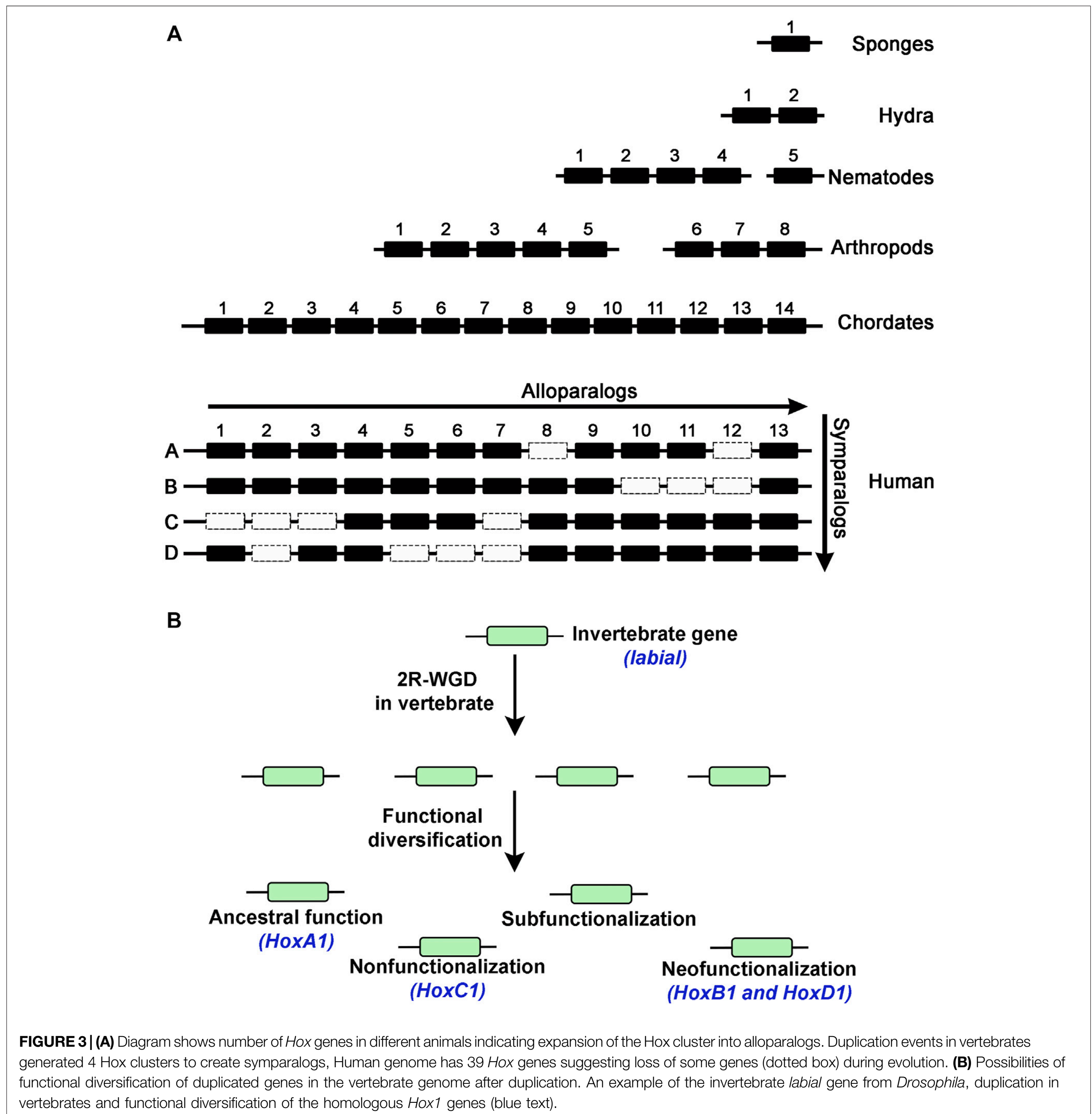


FIGURE 3 | (A) Diagram shows number of *Hox* genes in different animals indicating expansion of the Hox cluster into alloparalogs. Duplication events in vertebrates generated 4 Hox clusters to create symparalogs, Human genome has 39 *Hox* genes suggesting loss of some genes (dotted box) during evolution. **(B)** Possibilities of functional diversification of duplicated genes in the vertebrate genome after duplication. An example of the invertebrate *labial* gene from *Drosophila*, duplication in vertebrates and functional diversification of the homologous *Hox1* genes (blue text).

in the field has demonstrated that HOX proteins from different alloparalogs groups defines distinct morphological features along the AP body axis implying that each regulates a distinct set of gene regulatory networks and developmental pathways (Garcia-Bellido et al., 1973; Mcginnis and Krumlauf, 1992; Lawrence and Morata, 1994; Averof, 2002; Maeda and Karch, 2006). However, HOX proteins appear to share many similar biochemical properties. They display nearly identical DNA binding specificities *in vitro*, as a consequence of the presence of a highly conserved 60 amino acid long homeodomain (HD) (Scott et al., 1989; Gehring et al., 1994a; Piper et al., 1999). HOX proteins have the ability to interact with a PBC class (e.g., PBX, MEIS, PREP) of homeodomain transcription factors, which serve as cofactors in binding DNA and modulating gene expression (Desplan et al., 1988; Gehring et al., 1994a; Knoepfler and Kamps, 1995; Mann and Chan, 1996; Noyes et al., 2008). The interaction of HOX proteins with PBC factors is primarily mediated by a conserved six amino acid domain, referred to as the hexapeptide (HP). HOX-PBX cofactor interactions modify the affinity and specificity of HOX DNA binding on target sites in the genome (Slattery et al., 2011; Merabet and Mann, 2016). These generic DNA binding properties of HOX proteins and interactions with shared cofactors, such as PBX, make it difficult to explain the paralog-specific functions of HOX proteins. Hence, the distinct differences in the functional roles and genome-wide binding preferences of HOX proteins *in vivo* is likely to be a consequence of additional unidentified features and interactions of HOX proteins that impact and modulate their context-dependent activities.

Conservation

Early studies on *Hox* genes explored the evolutionary conservation of functions among homologous genes across the animal evolution by using transgenesis to express vertebrate *Hox* genes in *Drosophila* (Malicki et al., 1990; Mcginnis et al., 1990; Zhao et al., 1993; Lutz et al., 1996). These studies uncovered deep conservation of *Hox* gene function in specifying regional identity along the AP body axis over 600 million years of animal evolution. Protein sequences of alloparalogs, formed by tandem duplication of the ancestral *Hox* genes, show high diversification and each of them are known to drive distinct developmental and differentiation programs to regulate regional identities in specific tissues along the AP axis (Lamka et al., 1992; Mclain et al., 1992; Yokouchi et al., 1995; Carapuco et al., 2005). Despite these differences in functional roles, many alloparalogs show redundancy or overlaps of function in regulating developmental events in some specific tissues (Saurin et al., 2018). For example, it has been observed that ubiquitous expression of many *Hox* genes is important in *Drosophila* larval fat body cells, which appears distinctly different from their roles along the AP axis facilitated by their nested collinear expression patterns in embryos. Furthermore, functional analyses in autophagy inhibition uncovered no paralog specificity and suggested redundant functions of many HOX proteins (Banreti et al., 2014). Similarly, *Drosophila Hox* genes have been also shown to have redundant activity in specification of tritocerebrum identity, endocrine ring gland development, dorsal DA3 muscle lineage specification and head repression etc. (Hirth et al., 2001; Coiffier et al., 2008; Enriquez et al., 2010; Sanchez-Higueras et al., 2014). The redundant role of *Hox* genes is not limited to *Drosophila*, vertebrate HOX proteins have also been shown to have overlapping or redundant functions during development of several tissue types (Young et al., 2009; Lacombe et al., 2013; Denans et al., 2015). For example, vertebrate *Hox6* paralogs (*Hoxa6*, *Hoxc6*, and *Hoxb6*) are required to specify lateral motor column motoneurons and functional studies displayed that Hox paralogs 5, 7, and 8 can all substitute for this function (Lacombe et al., 2013). These observations suggest that despite evidence for sequence and functional diversification among HOX alloparalogs, which underlies their distinct roles in axial patterning, some aspects of their functional activities have been conserved during evolution and play roles in specific tissue contexts during development.

As expected, the functional redundancy among the *Hox* genes is more common among the symparalogs formed more recently after whole cluster duplications in vertebrate lineage (**Figure 3A**). Deletion of a single gene or even a whole cluster does not show dramatic consequences on embryonic development, consistent with the idea of extensive functional redundancy between *Hox* genes (Medina-Martinez et al., 2000; Suemori and Noguchi, 2000; Spitz et al., 2001; van Den Akker et al., 2001; Hunter and Prince, 2002; Soshnikova et al., 2013). Gene swap experiments in mouse models have also demonstrated that symparalogs, formed after duplication of ancestral invertebrate cluster, are functionally equivalent (Horan et al., 1995; Manley and Capecchi, 1997; Greer et al., 2000; Tvrdik and Capecchi, 2006; Iacovino et al., 2009). An interesting example is a gene swap of mouse *Hoxa3* and *Hoxd3* that resulted in adult mice with no detectable developmental defects (Greer et al., 2000). These observations suggests that HOX paralogs have retained similarity in their activity through millions of years of evolution, which has been attributed to conservation of DNA binding properties of the homeodomain and a shared hexapeptide domain that mediates interaction with PBC factors.

Diversification

Over expression studies have provided evidence that Hox alloparalogs regulate development of specific organs across the body axis, indicating that they can drive distinct gene regulatory networks (Schneuwly et al., 1987; Lamka et al., 1992; Mclain et al., 1992; Yokouchi et al., 1995; Carapuco et al., 2005). Comparative analyses of gene expression profiles upon ubiquitous expression of *Drosophila Hox* genes show a very small number (1.3%) of common changes, and most of changes are unique to each gene, suggesting they individually regulate distinct set of targets (Hueber et al., 2007). This data implies that despite the high conservation of homeodomain region, HOX proteins regulate distinct set of genes to specify unique cellular identities across the AP body axis during animal development (Mcginnis and Krumlauf, 1992; Averof and Akam, 1993; Lawrence and Morata, 1994; Patterson et al., 2001; Averof, 2002). The functional diversity among the HOX proteins may be

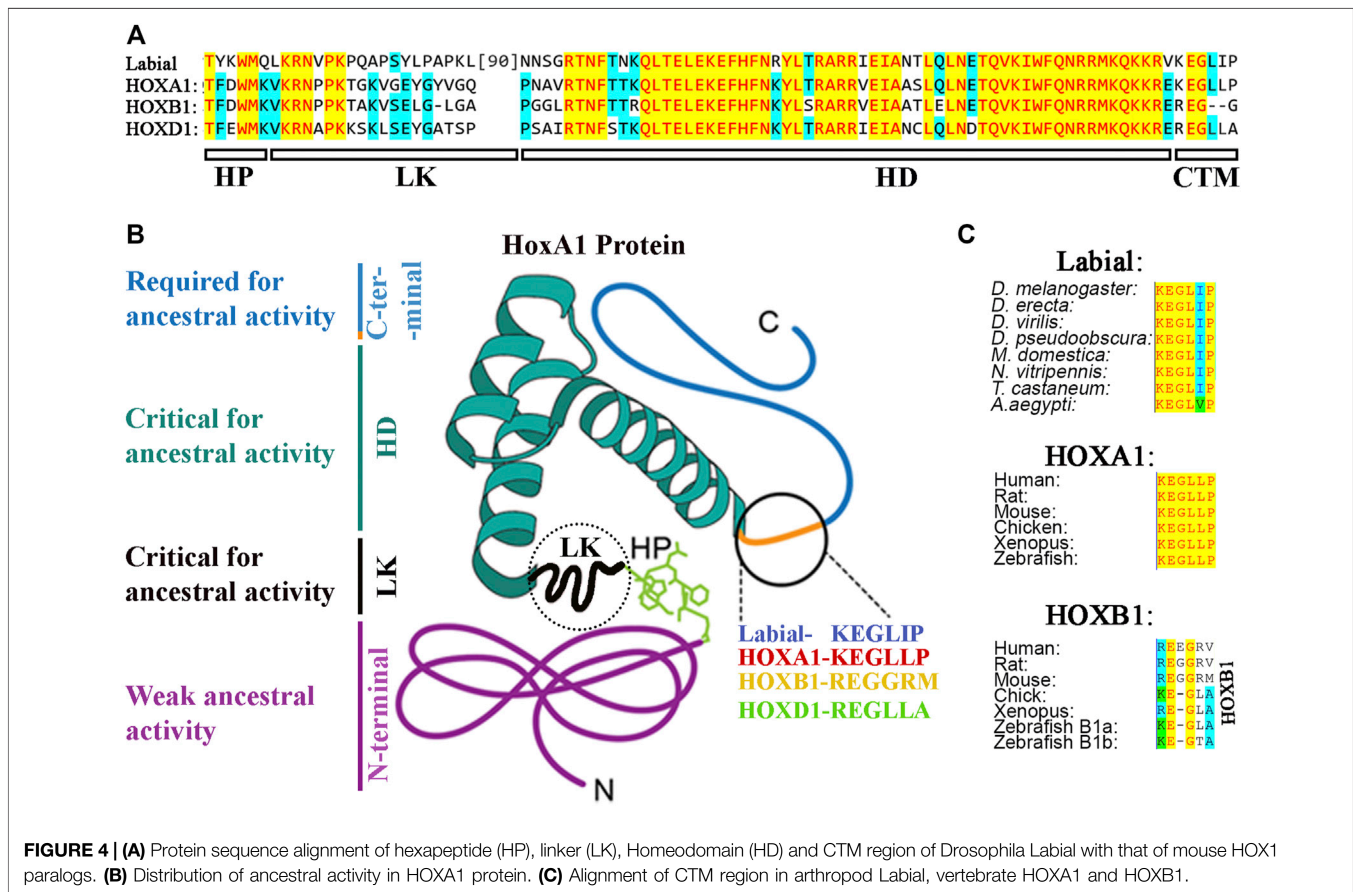
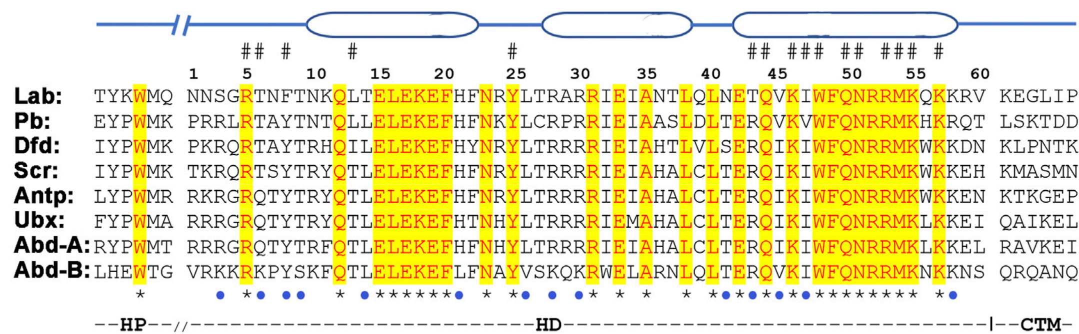


FIGURE 4 | (A) Protein sequence alignment of hexapeptide (HP), linker (LK), Homeodomain (HD) and CTM region of Drosophila Labial with that of mouse HOX1 paralogs. **(B)** Distribution of ancestral activity in HOXA1 protein. **(C)** Alignment of CTM region in arthropod Labial, vertebrate HOXA1 and HOXB1.

generated in multiple ways. Domain swaps of the HD, the most conserved region of HOX proteins, can result in functionally distinct activity in some developmental contexts. This suggests that even a small number of changes can lead to diversification of the DNA binding properties and transcriptional activity of HOX proteins (Zhao and Potter, 2001; 2002). In addition, studies have shown that amino acid differences among the Hox alloparalogs may not alter DNA binding preference but change their ability to recruit different coactivators or corepressors (Li and Mcginnis, 1999; Gebelein et al., 2004; Joshi et al., 2010). Another mechanism for generating diversity among HOX proteins is through its interaction with PBC group of cofactors, which alters DNA binding specificity. In fact, a high throughput study revealed that DNA binding specificities of HOX-Exd complex are only revealed upon heterodimerization (Slattery et al., 2011). These observations suggest that HOX proteins are subject to a variety of ways that can diversify or modulate their functional properties.

As discussed earlier, there are many examples of functional redundancy among the HOX symparalogs (Horan et al., 1995; Chen and Capecchi, 1997; Greer et al., 2000; Wahba et al., 2001; Tvrdik and Capecchi, 2006; Iacovino et al., 2009). However, several studies have also found functional diversification among symparalogs (Fromental-Ramain et al., 1996; Miguez et al., 2012; Singh et al., 2020; Singh et al., 2021). Analyses of *Hoxa9* and *Hoxd9* mutants in mouse revealed that these two symparalogous genes have both specific and redundant functions in lumbosacral axial skeleton patterning and in limb morphogenesis (Fromental-Ramain et al., 1996). Similarly, *Hoxa2* and *Hoxb2* symparalogs show synergistic interactions in regulation of gene expression in the hindbrain, however, during oligodendrogenesis in the mouse hindbrain *Hoxb2* antagonizes *Hoxa2* function (Davenne et al., 1999; Miguez et al., 2012). These analyses indicate that symparalogs have retained a lot of overlapping functions during evolution, but they have also diversified their functional roles thought changes in the patterns of expression and protein structure. An important understudied question in the field is how changes in amino acid sequences of symparalogous after duplication from the ancestor homolog relate to altered functions. Analyses of HOX1 proteins in Xenopus suggests they have redundant roles of HOXA1, B1 and D1 in hindbrain development (Mcnulty et al., 2005). Gene swaps of HoxA1 and HoxB1 in mouse also suggest they have largely overlapping or redundant roles (Tvrdik and Capecchi, 2006). However, in a recent study, we utilized CRISPR/Cas9 technology to replace the *Drosophila Hox* gene *labial* with its mouse homologs *HoxA1*, *HoxB1* and *HoxD1* to investigate conservation of ancestral functions and assess diversification during evolution (Singh et al., 2020). Despite similar degrees of protein sequence diversification of mouse HOX1 proteins from *Drosophila* Labial, our results revealed that among the HOX1 symparalogs (HOXA1, HOXB1 and HOXD1) only *HOXA1* is able to rescue *labial* function, as HOXB1 and HOXD1 failed to do

A **Alignement of Drosophila Hox proteins:**



B

Alignment of mouse HOXA proteins:

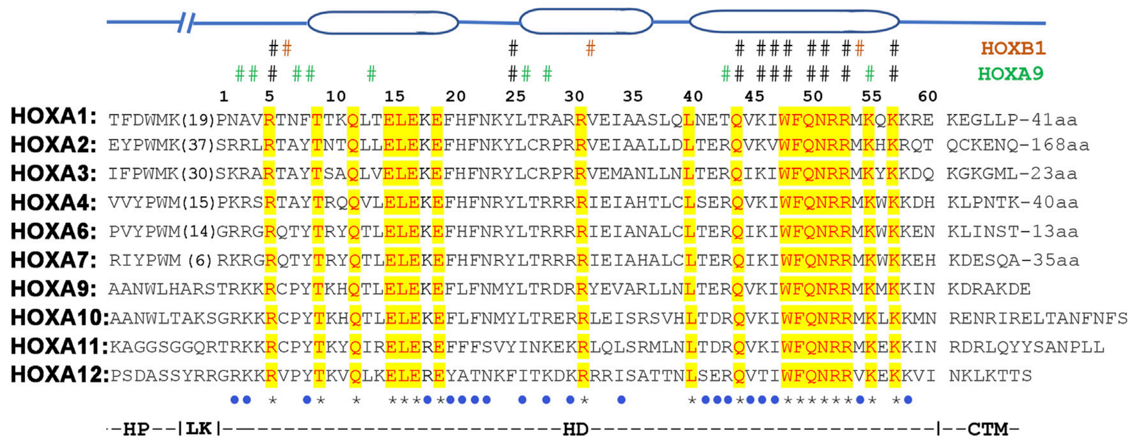


FIGURE 5 | Alignment of hexapeptide (HP), linker (LK), homeodomain (HD) and CTM regions from Drosophila **(A)** and mouse **(B)** HOX proteins. The three ovel regions on the top of the alignment shows the position of alpha-helices. The # sign above the alignment depict amino acids that contact DNA, while star (*) and blue dot under the alignment show conservative and semi conservative amino acids respectively.

so (**Figures 3B**, **4A**). This demonstrates a remarkable conservation of ancestral activity by HOXA1 and indicates that HOXB1 and HOXD1 have diversified through 600 million years of evolution (**Figure 3B**). Furthermore, consistent with their share ancestral activities, comparative genome-wide DNA binding properties revealed that HOXA1 and Labial have similar patterns of binding in mouse genome, while HOXB1 binds to a distinctly different set of targets. This adds support for neofunctionalization of HOXB1 by regulating a district gene regulatory program (**Figure 3B**) (Singh et al., 2020; Singh et al., 2021). Studies on mouse HOXD1 have shown that it has lost ancestral activities and appears to have undergone neofunctionalization through expression in new tissue types and altered activities in regulating novel gene regulatory programs (Guo et al., 2011). These distinct functional properties of mouse HOX1 symparalogs illustrate the diversification of HOXB1 and HOXD1 function and loss of ancestral activity in mammalian lineage. Mapping of the protein sequences that underlie functional diversification of HOXA1 and HOXB1 proteins revealed that a small number of changes across the protein cause functional diversification. It will be important to examine similar changes in other paralogy groups of HOX proteins to determine if this is a common means for modulating functional activities.

CRITICAL REGIONS UNDERLY HOX PROTEIN FUNCTION AND FUNCTIONAL DIVERSIFICATION

The deep conservation of HOX protein function across bilaterians and their redundant role in some tissue types suggest that the essential roles of HOX proteins in AP patterning has restricted their diversification. Preservation of

many of their ancestral functions is likely mediated through conservation of the homeodomain and hexapeptide regions. The homeodomain of *Drosophila* HOX proteins is known to bind directly to DNA (Galant et al., 2002; Merabet et al., 2003). However, on many *in vivo* target sites, binding affinity and specificity is enhanced by interaction with PBC factors (Loker et al., 2021). The regions outside these domains are highly diversified among the HOX proteins that can impact activation or repression of transcription of potential target genes (Li et al., 1999; Li and Mcginnis, 1999). In this section of the review, we will discuss evidences on what is known about various domains of HOX proteins and how they may have diversified to adopt novel functions during evolution.

Homeodomain

The HD stands out as the most conserved region of all HOX proteins (**Figure 5**). Three-dimensional structural studies using X-ray crystallography and NMR spectroscopy have revealed the presence of three alpha-helix regions in the HDs (Gehring et al., 1994b; Passner et al., 1999; Piper et al., 1999). The third helix, also known as the recognition helix, directly contacts the DNA through the major groove of DNA, while the region between the first and second helices establishes specific contact with the phosphate backbone. In addition, sequences adjacent to the N-terminus of the HD, referred to as the N-terminal extension region, also makes specific contact with DNA in the minor groove. These regions that insert into the minor groove have been shown to confer specificity to the HDs of *Drosophila* Ultrabithorax and Sex combs reduced Hox proteins imparting distinct DNA recognition properties (Joshi et al., 2007). The interactions of the N-terminal extension along with contacts mediated by the third helix are the primary determinates of DNA binding specificity and serve as a key constraint, maintaining the high level of sequence conservation.

Genetic and biochemical data suggest that the HD of HOX proteins is not freely interchangeable with each other. Functional assays of chimeric HOX proteins with swapped homeodomains of paralogs have been found to alter their functions, suggesting that homeodomains are not equivalent to each other (Zhao and Potter, 2001; Zhao and Potter, 2002). This indicates that the small differences between HD sequences contribute to differences in their DNA binding properties and functional activities (Furukubo-Tokunaga et al., 1993; Phelan et al., 1994; Zappavigna et al., 1994; Noyes et al., 2008; Breitinger et al., 2012). For example, 28 out of 60 amino acids are identical between all *Drosophila* HOX proteins and 18/60 are identical between all mouse HOXA paralogs (**Figures 5A,B**). In addition, many differences among the paralogs are conservative amino acid replacements that would be expected to preserve biochemical properties. This means that there are a series of non-conservative amino acid replacements or changes (19/60 in *Drosophila* and 22/60 in mouse HOXA paralogs), that have the potential to alter the DNA binding properties of HDs. For example, the crystal structures of HOXB1 and HOXA9 display many paralog specific amino acid contacts with DNA (**Figure 5B**) (Piper et al., 1999; LaRonde-LeBlanc and Wolberger, 2003). On the basis of sequence alignments and specific amino-acid residues within the homeodomain along with their relative positions in *Hox* clusters, *Hox* genes have been assigned to 14 different alloparalogous groups (PG1-14). These fall into three general classes: the anterior class contains PG1-5, the central class PG6-8 and the posterior class genes (PG9-14) (Domsch et al., 2015; Frobius and Funch, 2017). Hence, despite high conservation between homeodomain sequences there are paralog-specific differences that may alter DNA binding properties and preferences of HDs to modulate HOX function. The paralog specific changes in homeodomains may also alter the interaction of conserved amino acids with the DNA (Gruschus et al., 1997).

Hexapeptide

The PBC group of proteins, *Drosophila* Extradenticle (Exd) and Homothorax (Hth) proteins and their vertebrate orthologs Pre-B-cell leukemia transcription factor (PBX1, PBX 2, PBX3 and PBX4) and myeloid ectopic leukemia virus integration site (MEIS1, MEIS2 and MEIS3) respectively, are well characterized cofactors of HOX proteins (Chan et al., 1994; Chang et al., 1995; Lu et al., 1995; Mann, 1995; Ryoo et al., 1999). Interactions with PBC proteins change DNA binding affinity and specificity of HOX monomer proteins (Slattery et al., 2011). *In vitro* binding analyses have demonstrated that the interaction between most HOX proteins and PBX are highly dependent on the HP motif, which contains the four core residues YPWM. This interaction alters the DNA binding properties of each protein, leading to the recognition of a bipartite HOX-PBC consensus site, and it enhances affinity and specificity of HOX target selection (van Dijk and Murre, 1994; Chang et al., 1995; Phelan et al., 1995; Popperl et al., 1995; Chan and Mann, 1996; Hudry et al., 2012).

The presence of the HP is important for HOX function, as loss of this motif has been implicated in the eliminating homeotic functions in several genes (*fushi-tarazu*, *zerknüllt*, *bicoid*) formed after duplication of *Hox* genes (Alonso et al., 2001; Panfilio and Akam, 2007). There is also evidence for sequence diversity among HP regions of different HOX proteins, which may impact the DNA binding properties and function of HOX proteins (Chang et al., 1995; Neuteboom et al., 1995; Medina-Martinez and Ramirez-Solis, 2003; Remacle et al., 2004). While the four core amino acids in the HP motif have been shown to be critical for its activity, only the tryptophan (W) residue in the 4th position is highly conserved. The rest of the amino acids are highly variable among the HOX protein paralogs, which may alter their interactions with PBX (**Figures 5A,B**) (Neuteboom et al., 1995; Neuteboom and Murre, 1997; Mann et al., 2009). The divergence of the HP region away from YPWM motif in HOX proteins from paralogous groups 8–13 suggests a progressive change in their function through evolution of this motif (**Figure 5B**).

Consistent with the divergence of the HP regions, *in vitro* DNA binding experiments show that HOX proteins from paralog groups 1–10 mainly interact with PBX, while paralogy groups 11-13 preferentially interact with MEIS (Chan et al., 1994; Shen W. F. et al., 1997; Shen W.-F. et al., 1997). HOX proteins also form trimeric complex with PBX and MEIS proteins upon binding to target genes (Berthelsen et al., 1998; Shanmugam et al., 1999;

Ferretti et al., 2000; Penkov et al., 2013; Amin et al., 2015). The presence of MEIS proteins have also been shown to induce remodeling of HOX-PBX interactions that leads to changes in the requirements for motifs that drive trimeric complex formation (Dard et al., 2019). High throughput SELEX-seq technology, which measures the relative affinities of transcription factor complexes with all possible DNA sequences, has shown that interaction of HOX proteins with Exd-Hth dimers alters the DNA binding properties of all eight *Drosophila* HOX proteins. Based on this data, the DNA binding specificities of HOX proteins can be subdivided into three main classes with similar DNA binding preferences: 1) anterior, containing Labial and Proboscipedia; 2) middle with Deformed and Sex comb reduced; and posterior, with Antennapedia, Ultrabithorax, Abdominal-A and Abdominal-B. These observations are consistent with the idea that the nature of interactions with PBX and MEIS varies among different HOX proteins and this directly impacts their DNA binding properties and functions.

Recent research has opened new insights into the complex nature of interactions between HOX and PBC proteins. Studies have revealed that novel HOX-PBX cofactor interactions arise through the loss or gain of other interacting domains beyond the HP and these have the potential to further modify HOX DNA binding properties and functions (Shen W. F. et al., 1997; Liu et al., 2008; Noro et al., 2011; Slattery et al., 2011; Hudry et al., 2012; Merabet and Mann, 2016; Dard et al., 2019; Singh et al., 2020). Use of sensitive *in vivo* assays to quantify HOX-PBX interactions have revealed that, in the presence of MEIS, the HP motif is dispensable in all HOX proteins except those from anterior paralog groups 1 and 2 (Dard et al., 2018). Furthermore, detailed analyses uncovered alternative PBC interaction motifs in human HOXB3, HOXA7 and HOXC8 proteins that are critical for HOX-PBC interaction in specific cell contexts and DNA-binding site topologies. While in the case of HOXA9, the HOXA9-PBX-MEIS interaction is dependent on the activity of the HP motif and two paralog-specific residues of the homeodomain region. These observations suggest that HOX-PBC interactions are not rigid and may behave in a dynamic manner that vary based on specific cellular and genomic contexts. The array of HOX- PBC interactions might have evolved independently in novel ways as a common regulatory node or mechanism to diversify DNA binding properties of HOX proteins. The highly conserved W residue in the HP region has been shown to be required for binding on HOX–PBX consensus motifs, while HOX protein binding on non-consensus motifs and low-affinity binding sites may be altered through HP diversity in combination with other novel interaction domains (Foos et al., 2015; Dard et al., 2018; Singh et al., 2020).

Linker Region

HP region of HOX proteins is connected to the N-terminal of the HD through a linker (LK) region (**Figures 5A,B**). The sequence and size of the linker region is highly variable among the HOX paralogs, ranging from 3 to 50 amino acids in the vertebrate HOX proteins. Since the HP region of HOX proteins interacts with a highly conserved three amino acid loop extension domain in the HD region of PBX cofactors, the size and sequence of the linker region may constrain these interactions and further modulate DNA binding properties. Structural studies have revealed that the LK region of the *Drosophila* Sex Combs Reduced (Scr) HOX protein is critical for binding at some target sites, but on other binding targets it is disordered and makes minimal contribution to binding (Joshi et al., 2007). Converting the LK region of *Drosophila* Antp to that of Scr changes the DNA binding preference of the protein such that it binds very similar targets to those of Scr (Abe et al., 2015). Furthermore, comparative functional studies of mouse HOXA1 with HOXB1 proteins, Drosophila Ubx with Abd-A and Dfd with Scr show that the linker region is required for some aspects of the paralog specific functions of these HOX proteins (Merabet et al., 2003; Joshi et al., 2010; Singh et al., 2020). This indicates that the LK sequence has a role in modulating DNA binding preferences of HOX proteins and diversity in this region may be a determinant that underlies aspects of the paralog-specific functions of HOX proteins.

C-Terminal Region

The C-terminal region flanking the homeodomain of HOX proteins is highly variable in size and sequence. The importance of this part of HOX proteins has been generally ignored because of the high degree of variability among the HOX paralogs. This region has also been left out of the studies that analyzed the three-dimensional structure of HOX proteins with PBX and DNA. Sequence analyses show that this region varies from 7 amino acids in HOXA13 to 168 amino acids in HOXA2 (**Figure 5B**). This is also highly variable between the HOXA alloparalogs (HOXA1 to HOXA13) and symparalogs among Hox A, B, C and D clusters. Our recent cross-species functional analyses revealed that a highly conserved CTM motif (KEGLLP) is a key determinant involved in maintaining the homologous ancestral functions of *Drosophila* Labial in the mouse HOXA1 protein (**Figures 4A,C**) (Singh et al., 2020). Diversification of this motif in mammalian homologs, HOXB1 and HOXD1led to a loss of the ancestral activity. Furthermore, structure prediction analyses suggested that the CTM region may establish another interaction domain with PBX1 on DNA. Consistent with this idea, *in vitro* DNA binding analyses revealed that the CTM region is not sufficient for HOX1 interaction with PBX1, but it can modulate the ability of HOXA1 to interact with PBX1 when bound to a target site (Singh et al., 2020). Similarly, small, conserved regions in the C-terminal domains of HOX proteins have also been observed in *Drosophila* HOX proteins Ubx and Abd-A (Lelli et al., 2011). *In vitro* DNA binding analyses and three-dimensional structures show that this region in Ubx (called Ubd-A region) is required for direct physical interaction with Exd, a homolog of PBX1, and affects DNA binding properties (Foos et al., 2015). Hence, it may play an analogous role to the CTM, identified in HOXA1. The UBD-A region is highly conserved among insect orthologs of Ubx, but it is absent from other arthropods and onychophorans (Galant and Carroll, 2002; Ronshaugen et al., 2002). Functional analyses of this domain with a transgenic reporter line displayed a repressive role on a Distal-less (*Dll*) *cis*-regulatory element that is involved in promoting limb development. These results suggests

that evolution of the UBD-A domain suppressed limb formation in abdominal segments and provided an evolutionary transition to hexapod limb pattern. Furthermore, *in vitro* DNA binding and *in vivo* reporter assays show cooperation between linker and UBD-A region of the Ubx protein, which suggests subtle changes in HOX–PBC complexes have played a major role in the diversification of HOX protein function in evolution (Saadaoui et al., 2011). These data illustrate that flexible extensions outside of the HD helix have the potential to mediate additional contacts between HOX proteins and their cofactors in concert with those mediated by the HP motif on the opposing side of the DNA. Together these findings demonstrate that small differences in sequences outside of HDs, which do not contact DNA themselves, may be a common mechanism for modulating protein-protein interactions that impact DNA binding specificity of HOX proteins.

SUMMARY

A small number of changes in key amino acids may affect DNA binding properties and protein-protein interactions of transcription factors that can influence their DNA binding targets and potential for transcriptional activation or repression (Lai et al., 2001; Johnson et al., 2003; Chi, 2005; Sakazume et al., 2007; Shoubridge et al., 2012; Webb et al., 2012; Jubb et al., 2017; Singh et al., 2020; Chi, 2005). This indicates that sequence conservation alone may be a poor determinant in predicting the functions of HOX proteins. Genome-wide binding and gene expression analyses have revealed both overlapping and paralog-specific targets of HOX proteins (Hueber et al., 2007; Sorge et al., 2012; Beh et al., 2016; Bulajic et al., 2020; Singh et al., 2020). This suggests that there could be many common downstream targets in the genome, but the unique targets might have evolved by diversification of HOX proteins, resulting in selective alterations in their downstream target genes and inputs into novel gene regulatory programs. Paralog specific binding at unique target sites could arise through small differences in DNA binding domain and associated regions that alter interaction with cofactors such as PBX. Studies have shown that HOX-PBX interactions have diversified by altering interactions through the HP region and evolving novel contact points beyond it (Merabet et al., 2011; Saadaoui et al., 2011; Rivas et al., 2013). Altered HOX-PBX interactions may affect both DNA binding specificity of the HOX proteins and the transcriptional state of the target site. The diversification in the function of HOX proteins can be also introduced by changes outside the homeodomain and hexapeptide region (Chauvet et al., 2000; Gebelein et al., 2002; Singh et al., 2020). Several conserved short linear motifs (SLiMs) have been identified in HOX proteins that can often restrain the interaction potential of HOX proteins (Baeza et al., 2015). Deletion of SLiM motifs leads to loss, gain or interestingly enhanced interaction with cofactors that can alter regulatory potential of HOX proteins in a context specific manner. These dynamic changes in interaction with cofactors may alter Hox activity in tissue and cell type-specific manners which vary depending upon the cellular context (Capovilla et al., 1994; Joshi et al., 2010; Jung et al., 2014). These observations illustrate that a small number or subtle changes in multiple regions of HOX proteins can have a dramatic effect on their activity and may be an important feature that underlies the paralog specific functions by modifying DNA binding specificity and/or protein-protein interactions. Investigating the *in vivo* functional roles and evolution of other domains of HOX proteins beyond the HD should help to unravel how such similar proteins can exert diverse functions and be relevant in determining if this is a general mechanism used by other transcription factor families in the generation of diversity and evolution of novel functional activities of proteins.

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