ISSN 0006-2979, Biochemistry (Moscow), 2024, Vol. 89, No. 6, pp. 987-1001 © The Author(s) 2024. This article is an open access publication.

REVIEW

Irreducible Complexity of Hox Gene: Path to the Canonical Function of the Hox Cluster

Milana A. Kulakova[1,a]*, Georgy P. Maslakov[1], and Liudmila O. Poliushkevich[1]

[1]*Department of Embryology, Faculty of Biology, St. Petersburg State University, 199034 St. Petersburg, Russia*
[a]*e-mail: m.kulakowa@spbu.ru*

Received November 19, 2023
Revised March 22, 2024
Accepted March 27, 2024

Abstract—The evolution of major taxa is often associated with the emergence of new gene families. In all multicellular animals except sponges and comb jellies, the genomes contain Hox genes, which are crucial regulators of development. The canonical function of Hox genes involves colinear patterning of body parts in bilateral animals. This general function is implemented through complex, precisely coordinated mechanisms, not all of which are evolutionarily conserved and fully understood. We suggest that the emergence of this regulatory complexity was preceded by a stage of cooperation between more ancient morphogenetic programs or their individual elements. Footprints of these programs may be present in modern animals to execute non-canonical Hox functions. Non-canonical functions of Hox genes are involved in maintaining terminal nerve cell specificity, autophagy, oogenesis, pre-gastrulation embryogenesis, vertical signaling, and a number of general biological processes. These functions are realized by the basic properties of homeodomain protein and could have triggered the evolution of ParaHoxozoa and Nephrozoa subsequently. Some of these non-canonical Hox functions are discussed in our review.

DOI: 10.1134/S0006297924060014

Keywords: homeodomain, ANTP, Hox genes, non-canonical functions of Hox genes, Metazoa, ParaHoxozoa, Nephrozoa, neurogenesis, developmental autophagy, oogenesis, vertical signaling

INTRODUCTION

The history of multicellular animal emergence is tightly linked to the appearance of a new class of transcription factors – Antennapedia (ANTP), members of the superclass of homeodomain proteins [1]. The rapid structural and functional evolution of the ANTP genes led to the emergence of the most numerous and diverse clade in the animal kingdom, now referred to as ParaHoxozoa [2]. This clade includes approximately 7 million species of bilaterians (Bilateria), about 10 thousand species of cnidarians (Cnidaria), and several species of placozoans (Placozoa). The name of the clade indicates an evolutionary boundary within Metazoa, which separates taxa with Hox/ParaHox genes from comb jellies and sponges, which lack these genes [2-5].

Hox (homeotic homeobox) genes were the first genes shown to be involved in development and evolution [6]. Their discovery led to the emergence of a new science – evolutionary developmental biology (evo-devo) and made Hox genes the most studied group among all homeobox genes in animals. The homeobox is a conserved region of the primary sequence that encodes a DNA-binding motif of the homeodomain, which is required for the Hox protein to interact with enhancers of downstream target genes [7, 8]. Hox genes are organized in a cluster, i.e., they are physically linked. Traditionally, they are divided into 9 paralogous groups (PG1-8 and PG9/14). This classification is based on the differences in Hox protein sequences and their relative positions in clusters. The level of evolutionary conservation within a paralogous group (e.g., between lab (PG1) of the fruit fly and Hox1 (PG1) of the amphioxus) is always higher than outside of it (between lab (PG1) and pb (PG2) of the fruit fly). Overall, the structural expansion of the Hox cluster and the formation of the most paralogous

Abbreviations: ANTP, Antennapedia; HOX, homeotic homeobox; GRN, gene regulatory network; PG, paralogue group; Ubx, ultrabithorax.

* To whom correspondence should be addressed.

groups occurred before the appearance of the three major Bilaterian clades [9, 10].

The main feature of the Hox cluster is the ability to exhibit a colinear expression. Colinearity refers to the correspondence between the location of genes on a chromosome and the order of their expression along the anterior-posterior axis of the body [11, 12]. The closer is a Hox gene to the 3′-end of the cluster, the closer to the anterior end of the embryo it will function. This is called spatial colinearity. Colinearity can also be temporal, when genes are expressed sequentially in time, starting from the 3′-end of the cluster [13].

The Hox gene cluster is the result of tandem *cis*-duplications of the ancestral sequence, starting from the single proto-Hox gene that belonged to the NK family [14, 15]. This event occurred before the sister branches Bilateria and Cnidaria were formed because the Hox genes belonging to the paralogous groups PG1, PG2, and PG4/14 are already present in both these branches [16]. These paralogous groups emerged as a result of the diversification of *cis*-duplicates under two scenarios: neofunctionalization and subfunctionalization. In the former case, a copy acquires a new function, while in the latter, the ancestral function is shared between two copies [17]. The cluster can be intact (compact or relaxed) or contain rearrangements and discontinuities (breakages) up to complete atomization [18-21]. Integrity of the Hox cluster is essential for maintaining temporal colinearity but is not crucial for spatial colinearity [22].

Paradoxically, Hox genes are both highly conserved and functionally flexible. Their functions are universal during the establishment of the bilateral body plan and species-specific at the level of local patterns, such as the formation of bristles on the legs of different *Drosophila* species [23]. This systemic property is known as scalability. In the case of Hox genes, it is manifested at both ontogenetic and phylogenetic levels.

The staggering diversity of the bilaterian animals results from the rapid evolution of developmental programs, which are simultaneously stable and flexible. Bilaterian animals maintain a common body plan due to the conserved members and parts of gene regulatory networks (GRNs), which start functioning shortly after cleavage and are necessary for regionalization and patterning. This is particularly evident in vertebrates and other segmented animals, which undergo a phylotypic period or “zootype” stage [24]. During this period, representatives of a particular phylum (or subphylum) are morphologically very similar to each other (for example, all vertebrates at the pharyngula stage). A the molecular regulation level, the similarity is even broader, as segmented animals from different phyla (vertebrates, arthropods, annelids) exhibit orderly expression of Hox genes shortly before or during gastrulation [25-27]. The zootype concept is graphically represented as the hourglass model, where the constricted waist corresponds to the onset of colinear transcription of the Hox clusters along the anterior-posterior axis of the body. Such expression is conceptually similar in all segmented Bilateria, despite significant differences in implementation mechanisms. Hox proteins determine the fate of cells in broad spatial domains of the embryo along the anterior-posterior axis of the body. During this early period, their targets are genes of signaling pathways and transcription factors, the sets of which will qualitatively and quantitatively differ depending on the Hox code. This difference ultimately will lead to morphological and functional differences between embryo regions.

This function is traditionally considered basic, i.e., canonical. It is this function that is implied when discussing the role of Hox genes in development, and there are several reasons for this. Firstly, animals in which we observe early colinear transcription of Hox genes in broad spatial domains (i.e., regionalizing function) belong to the three superphyla – Deuterostomia, Ecdysozoa, and Lophotrochozoa, which are grouped into the clade Nephrozoa. The likelihood that such a function of the Hox genes arose independently (convergently) in these groups appears lower than the likelihood of its direct inheritance from a common ancestor of all Nephrozoa. Secondly, the sequential in-time early activation of Hox cluster genes is always associated with regionalization, and such a mode of activation depends on the intactness or minimally damaged state of the cluster [22, 28]. If the structure of the cluster determines its regionalizing function and if a whole cluster is *a priori* considered primary, it is logical to assume that this function itself is inseparably linked to the emergence of the Hox cluster.

The constricted waist in the hourglass model indicates a lack of variability available for selection, so it can be confidently stated that the action of Hox genes determines the organization of the body plan, at least in the segmented Bilateria. However, with this approach, questions about the early stages of system evolution remain unsolved. The coordinated temporal and spatial early colinear expression of Hox clusters looks very complex. It is difficult to imagine the primary and intermediate steps that formed this hyper network. Moreover, if the canonical function of Hox genes is primary, then the last common ancestor of Nephrozoa is a complex animal, not inferior in terms of organization to the amphioxus, fruit fly, or *Platynereis*. This leads us to the old paradox of irreducible complexity. Perhaps among the multitude of functions of Hox genes, which are not canonical ones, there are those that provide a clue to the primary state of Hox regulation in the Para-Hoxozoa lineage and its subsequent evolution. Some of them are discussed in our review.

HOX GENES AND NEUROGENESIS

The older the trait is, the more likely it is to be found in the phylogenetically distant descendants of the species that acquired this trait. The idea that the ancestral function of Hox genes is patterning of the nervous system was first suggested by Jordi Garcia Fernandez [29], and not without reason. If we put the canonical function of Hox genes out of consideration, we are left with the most widespread and most damage-resistant function – control of neurogenesis [30-32]. Control persists even in the animals that have abandoned early regionalizing function (leeches, appendicularia, rotifers), or use it in isolation from the anterior-posterior axis specification (mollusks) [19-21, 33-36]. It is noteworthy that in most of the studied mollusks, the ganglia of the nervous system show signs of Hox colinearity [21, 35, 36].

Modern experimental methods make it possible to locally turn on or off selected genes at different stages of development of model animals (nematodes, *Drosophila*, mouse). These experiments revealed an important pattern. It turned out that the Hox genes do not simply determine the cellular territories in which neuroblasts are laid down. But they control pathways of their differentiation and, more interestingly, establish terminal specificity of the mature postmitotic neurons [32]. Terminal specificity (neuronal terminal identity) brings the neuron to a functional state. It begins to form neurites, synthesize neuropeptides, proteins necessary for the production of neurotransmitters, receptors to them, and components of ion channels. All these and many other changes in the postmitotic neurons occur due to unrelated regulatory proteins called terminal selectors. One such selector in the nematode *Caenorhabditis elegans*, Unc-3 (an ortholog of EBF/Olf/Collier), determines the terminal differentiation of the cholinergic motor neurons. The Unc-3 protein binds directly to the cis-regulatory sites of acetylcholine biosynthesis genes, ion channels, and numerous other genes. It does not work alone but in cooperation with various Hox proteins that act as its cofactors. Different Hox proteins (depending on the site of the body) determine differences in the number and length of neurites, synaptic connections, and electrical activity of the motor neurons. This general scheme is also valid for other types of neurons (sensory, motor, and intermediate) with other terminal selectors [37]. It is important that the Hox proteins are needed by the worm not only for the correct tuning of the neuron at the time of its terminal differentiation but also for its further work. It has been shown that the Hox protein of *C. elegans* Lin-39 (PG 4/5) is necessary in adulthood to maintain the terminal specificity of the motor neurons [38, 39].

C. elegans is a simply organized animal. An adult worm (hermaphrodite) has only 302 mature neurons [40]. An adult *Drosophila* brain contains about 200,000 neurons [41], but their differentiation, targeting, and number of synapses are determined by similar processes. The fly neuroblasts acquire unique fates under the action of Hox proteins [42], and, more surprisingly, neuromuscular synapse formation is under the Hox control [31, 43]. It is hypothesized that the assembly of synaptic contact between a neuron and a muscle cell is possible if they express the same Hox protein (or set of Hox proteins). This is true for at least one model system, where the Hox protein Dfd (PG4) directly turns on the expression of ankyrin (Ankyrin2-XL; synaptic protein) and turns off the expression of Con (an adhesive protein that selectively works in neuromuscular synapses of another type) in the motor neurons and muscles they innervate [43].

Finally, a type of neurons (leucokinergic neurons) has been described in *Drosophila* for which Hox proteins from the BX-C complex [Ubx (ultrabithorax), abd-A (abdominal A), Abd-B (abdominal B)] are direct terminal selectors because they turn on (Ubx, abd-A) and turn off (Abd-B) synthesis of the neuropeptide leucokinin [44].

Without going into details, it should be noted that mammals (mouse, human) have fundamentally similar rules for establishing proneural territories, differentiation of neurons, and their postmitotic settings under the control of Hox genes [30]. Mammals have also been shown to have Hox proteins that function as terminal selectors of motor neurons [45] and are required in adulthood. Hindbrain development is controlled by 24 Hox genes, and they continue to work in the mature brains of adult mice, while forebrain development in vertebrates is a Hox independent process [46].

This is all the more surprising that the Hox genes from several anterior paralogical groups (PG1,3-5) begin to be expressed in the postnatal neocortex and thalamus of the mouse [46].

Thus, modern experimental data obtained with different model animals lead us to the idea that the ancestral function of Hox genes is the terminal differentiation of neurons, most likely motor neurons. This hypothesis has a strong theoretical and evidence base. Firstly, it has recently become known that the homeobox-containing factors in general tend to trigger and maintain neurogenic differentiation. The *C. elegans* genome encodes 102 homeodomain-containing proteins from different families that selectively and combinatorially function as terminal selectors or their partners in the mature neurons [47, 48]. Therefore, the specification of neurons using Hox proteins is a special case of a general principle.

Secondly, in the GRNs, direct linkage between high-level regulatory genes and terminal differentiation genes may indicate the ancestral state of the system. Within the GRNs master genes, target genes and

intermediate regulatory genes are distinguished. These intermediate genes form a complex architecture of the developmental GRNs, and according to the hypothesis of intercalary evolution, they are the result of evolutionary intercalations (insertions) between the master gene and its target. Such as in the case of the homeobox gene *Pax6* and the light-sensitive transmembrane protein rhodopsin [49-51]. The Hox genes are universally implicated in the establishment and maintenance of the terminal neuronal specification in Protostomia and Deuterostomia, allowing for the existence of a simple organized ancestor of all Nephrozoa, which used Hox genes for the same purpose. Initially, the simple GRNs of such ancestor gradually and independently became more complex in different evolutionary lineages due to the involvement of the new clade-specific genes under the control of the Hox cluster. In the course of evolution, heterochronies shifted the activity onset of all participants to earlier developmental stages and brought their expression to the canonical state. It is possible that in the first stages of this evolutionary process, Hox genes coordinated the formation of synapses between the motor neurons and muscles. Therefore, the general principle of colinear transcription of Hox genes relates *Drosophila* and mammals at the level of two germ layers, ectodermal and mesodermal. Importantly, the principle of intercalary evolution allows the GRN growth by duplication of the master gene and subfunctionalization of the descendant genes with partial preservation of the ancestral function [51].

This attractive hypothesis has internal contradictions. Firstly, the physical linkage of terminal selectors is not necessary to specify neurons. Most homeobox genes that create the neural code in nematodes are not clustered [47, 48]. Secondly, the level of complexity of the last common ancestor of all Nephrozoa remains in question because its Hox cluster already consisted of at least 7 or 8 genes – five anterior (PG1-5), one or two middle (PG6/8), and one posterior (PG9/14) [9]. It is known that functions of the genes from different paralogous groups overlap significantly [52], which means that the ancestral cluster formed very quickly before its members began to differ greatly in the spectra of their targets. If the quantitative information realized by the Hox proteins was at some point more important than the qualitative (paralog-specific) information, this could have pushed the Hox cluster to a rapid structural expansion with minimal divergence of participants. However, the paralog-specific functions began to appear later. This explanation looks logical but raises the following questions: why are the Hox proteins from different paralogous groups qualitatively important for the neuron specification, and why do these proteins differ structurally, while some of their paralog-specific functions are conserved (common to Nephrozoa)? It seems that selection drove the evolution of the Hox cluster in several directions at once, and this could be explained by the scenario when the neurogenic function was not the only one. Whether this is true or not, we could figure it out by referring to basal taxa.

Outside the Nephrozoa group, expression of the Hox genes has been studied less, but it is known that small and disjointed clusters of Acoelomorpha (a sister branch of Nephrozoa, previously classified as a clade within flatworms) operate in the nervous, muscular, and reproductive systems [53-56]. In a single study on embryos [53], three Hox genes of *Convolutriloba longifissura* (PG1, PG5, and PG9/14) colinearly turn on in the proneural territories shortly after gastrulation. Two of these three genes operate in parenchymatous internal domains slightly later or simultaneously with this event.

The cnidarian Hox genes have been extensively studied [57-59], and expression of some of them can be associated with the nervous system, e.g., *Hox1* (PG1) of *Clytia hemisphaerica* functions in statocysts and *Anthox1* (PG9-like) in the apical tuft of the *Nematostella vectensis* planula larva. However, in comparison with the diverse neural differentiation involving other homeobox genes in cnidarians, this is a very modest outcome [60, 61]. Surprisingly, many cnidarian neurotransmitters (including acetylcholine) and enzymes of their biogenesis are synthesized not in neurons but in gastrodermal cells [62]. Analyzing expression data is also difficult because direct correspondence of the genes from Hox/ParaHox classes in cnidarians and bilaterians is not obvious due to the high divergence or loss of orthologs [63]. However, there are no direct regulators of neurogenesis among the genes, which exactly match the category of “PG1-like” or “PG2-like”, but there are genes with broad expression domains at the level of ectoderm and endomesoderm.

Thus, before the origin of the last common ancestor of Nephrozoa, at the level of Acoelomorpha, Hox genes were already engaged in several different developmental programs. Their functions in cnidarians are diverse and not associated with terminal neuronal specification. The path from the common ancestor of cnidarians and bilaterians to modern Nephrozoa was accompanied by structural expansion of the Hox cluster and complex rearrangements of the regulatory relationships between the ancient developmental programs hidden to us. Some of these programs may have relied on the shared paralog-nonspecific functions of Hox genes, which are realized in isolation from the spatial colinear transcription. We hypothesize that these functions have remained in modern animals, and to investigate them we need to look at the general biological processes in which Hox genes are involved. There are several examples where the paralog-nonspecific function of Hox genes is realized particularly clearly. We will discuss them in the following sections.

HOX PROTEINS CONTROL DEVELOPMENTAL TIMING THROUGH AUTOPHAGY

Autophagy is a cellular degradation process necessary for maintaining cell homeostasis and renewing its cytoplasmic components. It is highly conserved, and its influence on various biological functions has been described in a wide range of organisms, from plants and yeast to humans [64]. For bilaterians, autophagy is an important tool in early development, as it participates in cellular differentiation and tissue remodeling [65, 66]. For instance, in the *Drosophila* larvae, autophagy activity is very high in the fat body during the wandering L3 (L3W) stage, when the larva is rapidly growing and undergoing metamorphosis, but not in the younger feeding L3 (L3F) stage. It has been shown that the transition from the L3F to L3W stage is controlled by ecdysone, and the main regulators of autophagy in this case are Hox proteins, which suppress premature autophagy at the L3F stage [67]. During normal development, colocalization of Hox proteins from multiple paralogous groups is observed in the fat body of L3F larvae, but it is not colinear. Here, the Hox proteins suppress the expression of the *atg* genes (18 genes), which are responsible for autophagy. It has been shown that inactivation of the individual Hox genes (*Dfd*, *Scr*, *Ubx*, *abd-A*, *AbdB*) does not lead to premature induction of autophagy. Only the simultaneous shutdown of all Hox paralogs in the L3F larval experiment initiates this process [67]. Conversely, prolonged expression of the investigated Hox genes inhibits autophagy in the larval fat body cells. Such animals enter the wandering stage 6-7 days later than the controls, indicating that the forced maintenance of the Hox gene expression delays development in *Drosophila*.

Thus, in the larval fat body, the universal activity of Hox proteins carries temporal rather than spatial information, regulating the onset of autophagy at the required stage of development. It is worth noting that in the culture of mammalian fibroblasts, HoxB8 and HoxA9 also inhibit autophagy. The same proteins exhibit a similar effect on the *Drosophila* larvae after transgenesis [67]. These preliminary studies do not rule out the paralog-nonspecific involvement of Hox genes in autophagy control in the last common ancestor of insects and vertebrates but require additional analysis across a wide range of models.

WHY DO HOX GENES WORK BEFORE DIFFERENTIATION BEGINS?

In multicellular animals, Hox genes are not expressed in totipotent and pluripotent cells because this expression induces differentiation. In mammalian embryonic stem cells, Hox loci have an ambivalent epigenetic status. Their histone code contains both repressive and permissive tags [68]. These cells do not express Hox genes, but expression can start rapidly in case of additional permissive signals, which will lead the cells to the beginning of the differentiation path.

Despite the blockage of Hox genes activity in totipotent cells, their maternal transcripts have been found in oocytes of mammals (mouse, cow, and human [69], amphibians (*Xenopus laevis* [70]), annelids (*Platynereis dumerilii* [71]), myriapods (*Strigamia maritima* [72]; *Trigoniulus corallinus* [73], hymenopterans (ants of the tribe *Camponotini* [74]), decapods (*Macrobrachium olfersii* [75]), and even in oocytes of hydroid polyp *Clitya hemisphaerica* [58]. In all animals, except *Xenopus laevis*, the genes from several paralogous groups are expressed in oocytes.

The structure of oocyte transcripts of Hox genes may provide clues about their functions. The example of centipede *Strigamia maritime* (Chilopoda) [72] showed that the maternal RNAs of Hox genes are polyadenylated, but some of them do not contain an open reading frame. Possibly, part of the maternal RNAs of the centipede belongs to the class of regulatory (protein-noncoding) RNAs. On the other hand, the Hox gene transcripts in mammalian oocytes are deadenylated [69]. This is reasonable if such maternal transcripts are required for later developmental stages and stored in a stable (non-translated) form [76]. In addition, the HOXB9 protein was detected in the nuclei of oocytes and the cells of early mammalian (mouse, cow) embryos [77], and the Ubx and AbdA proteins were found in ant oocytes [74].

The function of Hox genes in oocytes differs from the canonical one since transcripts of different paralogous groups are localized in a single cell. The widespread nature of this phenomenon suggests that it is not random. So far, there are no successful experiments unambiguously indicating the functions of the Hox gene transcripts in oocytes, but several hypotheses can be put forward.

Most of the maternal transcripts of the Hox genes may not be translated. They may represent an element of epigenetic tuning of the zygotic genome. It is known that the non-coding RNAs often function as scaffolds for the assembly of chromatin remodeling proteins, targeting them to subordinate loci. This function has been described for the regulatory RNAs that are read from the vertebrate Hox clusters [78] and it is consistent with the presence of transcripts without an open reading frame in the *Strigamia* oocytes. The importance of the non-coding RNAs in the first cleavage divisions was shown in the mouse embryos [79].

On the other hand, the mRNAs of Hox genes can be translated. For example, the HoxB9 protein is present in the nuclei of oocytes (both mature and immature) and the cell nuclei of early embryos [77].

This does not exclude an early function of Hox genes directed to oogenesis. It is known that in the mouse several transcripts of Hox genes and their cofactors are already present at the stage of the growing oocyte [80]. Interestingly, the homeodomain protein Nobox from the family of the same name, which is close to both the Antp and PRD classes [81], is present in the mouse oocytes and regulates the functioning of the genes important for oogenesis [82]. It cannot be ruled out that the oocyte RNAs and proteins of the Hox genes are required for the transcriptional control of the early zygotic genes [83].

The Hox proteins can be not only transcriptional regulators, but also regulators of the cell cycle, RNA splicing, DNA replication and repair [84-86]. For example, some Hox proteins have recognition sites for the serine/threonine kinase ATM [87], whose role in the DNA double-strand break repair is widely known [88]. At least one vertebrate Hox protein, HoxB7, has been experimentally shown to be involved in this process [85]. In the mammary epithelial cells, HoxB7 increases the probability of nonhomologous DNA end joining by binding to the complex of Ku70/80 heterodimers (proteins that recognize double-stranded breaks) and DNA protein kinase [85]. HoxB7 expression stimulates the DNA-protein kinase activity, which correlates with the repair efficiency, whereas this effect is lost when HoxB7 is knocked out. In addition, Hox proteins promote the assembly of pre-replicative complexes. For example, HoxD13 and HoxC13 in different cell cultures interact through homeodomains with the proteins of ORC and Cdc6 pre-replicative complexes [86, 89, 90]. Although the mentioned functions of Hox proteins were not described in embryogenesis, we assume that they can still participate in the first stages of the development of multicellular animals. In the early development, synchronous divisions of blastomeres occur with a minimum interval (there are no G1 and G2 phases of the cycle), therefore, a very precise adjustment of the molecular machinery of the oocyte is necessary for the successful completion of the cleavage stage. In this case, Hox proteins in oocytes may accelerate the assembly of pre-replicative complexes and enhance DNA repair to maintain the integrity of the embryo's genetic material.

Undoubtedly, the role of Hox genes in oogenesis will not be clarified without functional tests on a wide range of models. It cannot be ruled out that the oocyte RNAs and proteins of the Hox genes could be a "transcriptional noise" or by-products of the previous stages of oogenesis [91].

DOSE-DEPENDENT FUNCTIONS OF HOX PROTEINS

There are dose-dependent functions of Hox proteins when their concentration determines the morphology of the anlage [92]. In mammals, Hox proteins specify vertebral morphology in a dose-dependent manner [93] and set the number and length of digits [94]. In both cases, a gradual decrease in the dose of Hox proteins increases intensity of morphological changes. For instance, when the dose of any protein from the posterior paralogs of HoxA and HoxD clusters (Hoxd11, Hoxd12, Hoxa13, Hoxd13) is gradually decreased, the digits shorten linearly and paralog-independently depending on the proportion of mutant alleles of the Hox genes [94].

In invertebrate animals, the most obvious example of a dose-dependent function of Hox proteins is the regulation of wing morphology. This function has been described in insects from different clades and, apparently, is universal for the diversification of the wing shape and size in the second (T2) and third (T3) thoracic segments [92]. It involves the Antp and Ubx proteins in its realization. In the wild-type Drosophila, the Antp protein is present only in T2 and the Ubx protein in T3, with a lower concentration of Antp in T2 than of Ubx in T3 (Fig. 1a) [95]. In the $Ubx^{-/-}$ mutants, a pair of wings is formed at T3 instead of halteres (Fig. 1b). If the dose of Antp in T3 is increased to the level of Ubx in such mutants, the normal phenotype is restored (halteres are formed on T3 (Fig. 1c) [95]. On the contrary, when the Ubx dose is decreased, wings grow instead of halteres at T3 (Fig. 1d) [95]. Likewise, when the Antp dose is increased in T2, halteres grow instead of wings (Fig. 1e) [95].

HOX FACTORS CAN BE SECRETED BY CELLS

The difficulty faced by a researcher who decides to elucidate the ancestral function of the ANTP-class homeobox genes is related to the fact that such function was not originally a single function, at least at the level of Metazoa. This follows from the arrangement of multitasking ANTP-class homeodomain proteins (Fig. 2). At the end of the last century, it was discovered that a synthetic homeodomain protein of 60 amino acid residues, repeating the sequence of the *Drosophila* Antp homeodomain, can penetrate the membranes of rat nerve cells without the mediation of any receptors. After penetration, it is transported into the nucleus and increases the level of differentiation of the recipient cells [96]. Later it was found out that the natural homeodomain proteins Emx1, Emx2, Engrailed-2 (En2), Hoxa5, Hoxb4, Hoxc8, Knotted1, Otx2, Pax6, and Vax1 are present in the cells that do not express their mRNA [97].

Apparently, most of the homeodomain proteins, including those beyond the boundaries of the ANTP class [97-100], have the ability for intercellular transport like a signaling or morphogen molecule. The mechanism

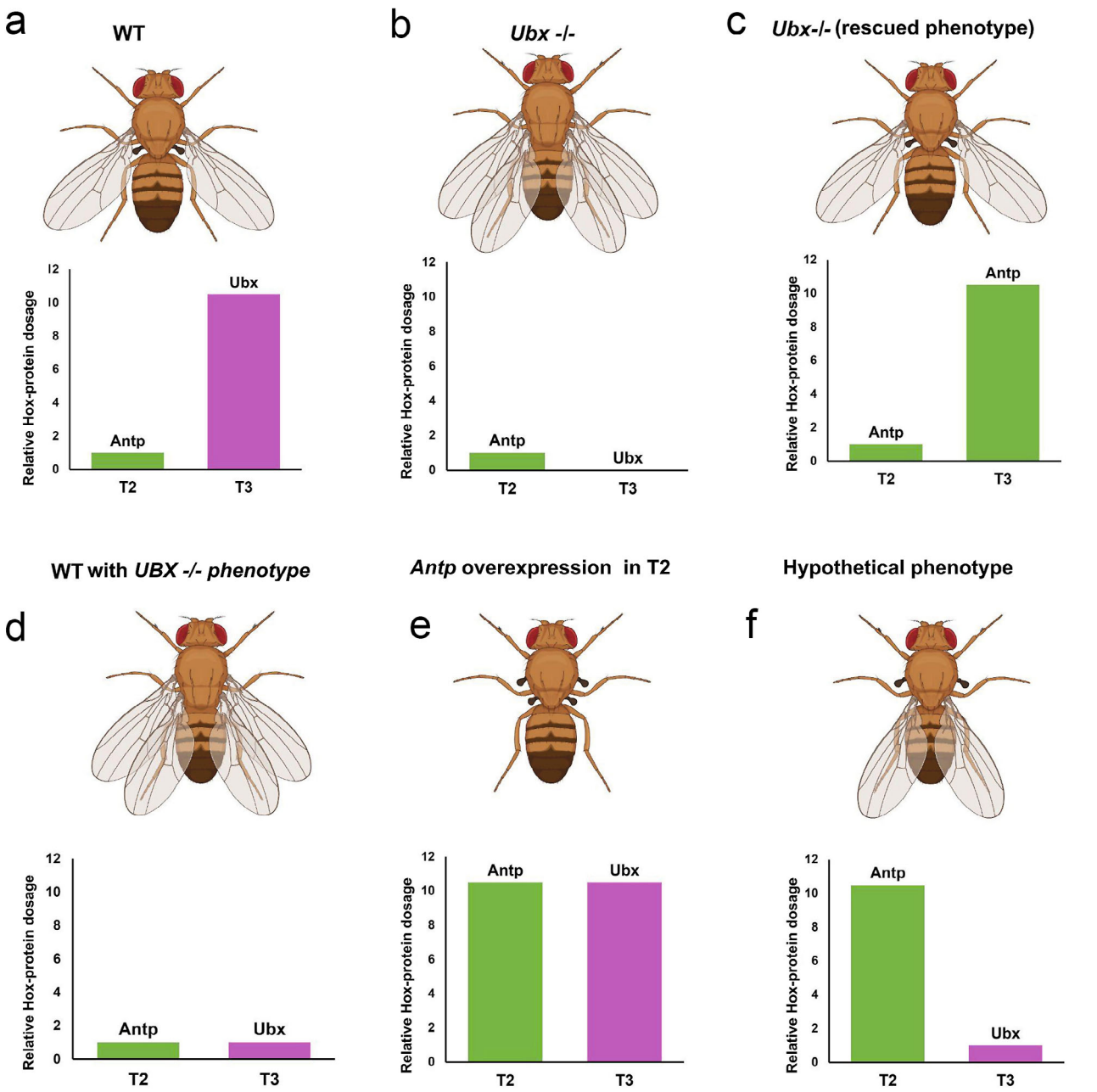

Fig. 1. Effect of Antp and Ubx protein dose on the *Drosophila* phenotype. a) "Wild-type" (WT); b) Ubx mutant –/–; c) Antp dosage increase in T3 to the Ubx level restores normal phenotype; d) decrease of Ubx dosage in T3 to the Antp level in T2 leads to wing formation; e) Antp dosage increase in T2 to the Ubx level in T3 leads to halteres formation; f) hypothetical phenotype that could be modeled on *Drosophila*. It is characteristic of insects from the order Strepsiptera. The illustration is based on the data of Paul et al. (2021) and Merabet and Carnesecchi (2024) [92, 95].

by which this is realized is not yet fully understood. It is known that secretion and internalization depend on two overlapping motifs localized in the most conserved regions of the homeodomain [100]. In addition, secretion depends on individual hydrophobic amino acids outside of the homeodomain (Fig. 2) [97]. It seems that homeodomain proteins can enter any cell type through macropinocytosis, but the efficiency of the process depends on the structure of the glycocalyx of the receiving cell [97, 101].

In the impressive study conducted in 2019 [97], 162 human homeodomain proteins from different classes were tested for their secretion and transfer abilities, and the test was performed simultaneously on three different cell cultures (secretion – HEK 293T, GT1-7, and MDCK; internalization – HeLa). It was shown that secretion efficiency strongly depends on the cell type and characteristics of the primary sequence of the homeodomain proteins themselves. For example, the proteins EN2, HOXC8, PAX6, and VAX1 were secreted

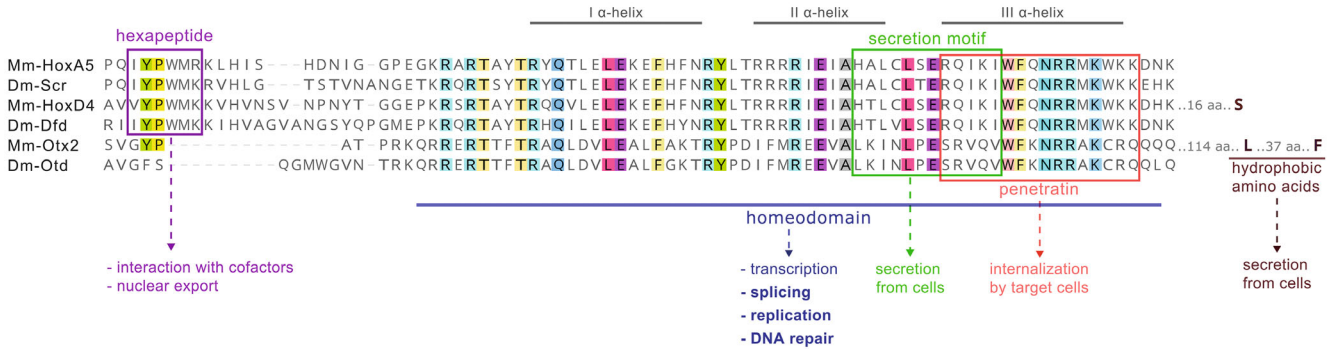

Fig. 2. Structural motifs of proteins from the ANTP class and their participation in the realization of the Hox protein functions. The scheme describes the functional significance of the main domains of Hox factors. The homeodomain, the most conserved region of ANTP-class proteins, is required for Hox factors to realize both canonical (transcription) and non-canonical functions. In addition to the homeodomain, a high degree of conservatism is also characteristic of the short hexapeptide motif by which Hox proteins interact with cofactors [102]. Surprisingly, the transport capacity of the Hox factors is also mediated by these domains: the homeodomain contains secretion and internalizing (penetratin) motifs, and the conserved tryptophan residue in the hexapeptide is required for Hox proteins to be exported from the nucleus [103]. For some Hox factors, it has been shown that their secretion could depend on individual hydrophobic amino acids localized at less conserved sites on the protein [97]. Colored letters in the sequences indicate conserved positions, colored boxes surround motifs, non-canonical functions are in bold; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; aa, amino acid residues.

in all three cell cultures, while HOXA5 and OTX2 were secreted in only two. Ten proteins, including only one Hox (HOXA10), were not secreted at all, which, however, does not exclude this possibility in other cell types. All tested proteins (including non-secreted ones) turned out to be capable of internalization. This means that secretion of the homeodomain proteins is the crucial stage of transduction at which cells control this process.

Homeodomain proteins can diffuse over two or three-cell diameters like paracrine signaling factors, but there are examples of their extensive diffusion. In the mouse, the Otx2 protein is synthesized in the vascular plexus, secreted into the cerebrospinal fluid, and accumulates throughout the cerebral cortex [104].

Importantly, the endogenous Hox proteins preferentially function as transcription factors. Immediately after translation, they are taken up by karyopherins, which recognize the nuclear localization signal, and transport Hox proteins to the nucleus. The exogenous Hox proteins exhibit a broader range of functions. It has been shown that the secreted mouse Otx2 moves into mitochondria, where it binds to mitochondrial ATP synthases and enhances ATP synthesis [105]. Earlier it was reported that the exogenous En2 in *Xenopus* accumulates in the growth cones of neurons, controls their axonal targeting, and indirectly enhances translation [106].

This does not exclude penetration of the exogenous proteins into the nuclei, where they trigger transcription of their mRNAs and other specific targets. A classic example of this type is vertical signaling during gastrulation in Xenopus. It was shown that the Hox proteins from presomitic mesoderm sequentially switch on the expression of their own Hox genes in the gastrula neuroectoderm, i.e., copying of positional information from one germ layer to another occurs in this case [104]. Direct exchange of transcription factors between the cell layers coordinates the operation of developmental programs without the mediation of morphogens and signaling cascades.

Remarkably, the signaling and regulatory functions in general cases are mediated by the same evolutionarily ancient and conserved motif – homeodomain. The ability to solve two tasks with a single tool could have been used to coordinate growth and development by the first Metazoa, even before the emergence of modern relationships between the long-range signaling ligands, their messengers, and targets [104].

HOW DID NON-CANONICAL FUNCTIONS BRING HOX CLUSTER ACTIVITY TO A CANONICAL STATE?

The coherent operation of Hox genes required for canonical function remains a great evolutionary mystery because it is a multi-event process. It consists of:

– epigenetic tuning of Hox loci, including through the regulatory RNAs encoded in the Hox clusters;

– establishment of the topology-associated domains (TADs), which are stabilized according to the position of cells along the anteroposterior axis;

– responses to multidirectional signals from morphogens (retinoic acid, Wnt, Fgf, Bmp) in three-dimensional coordinates of the embryo;

– coordinated expression in cells of different germ layers due to the mechanism unique for homeodomain proteins – vertical signaling;

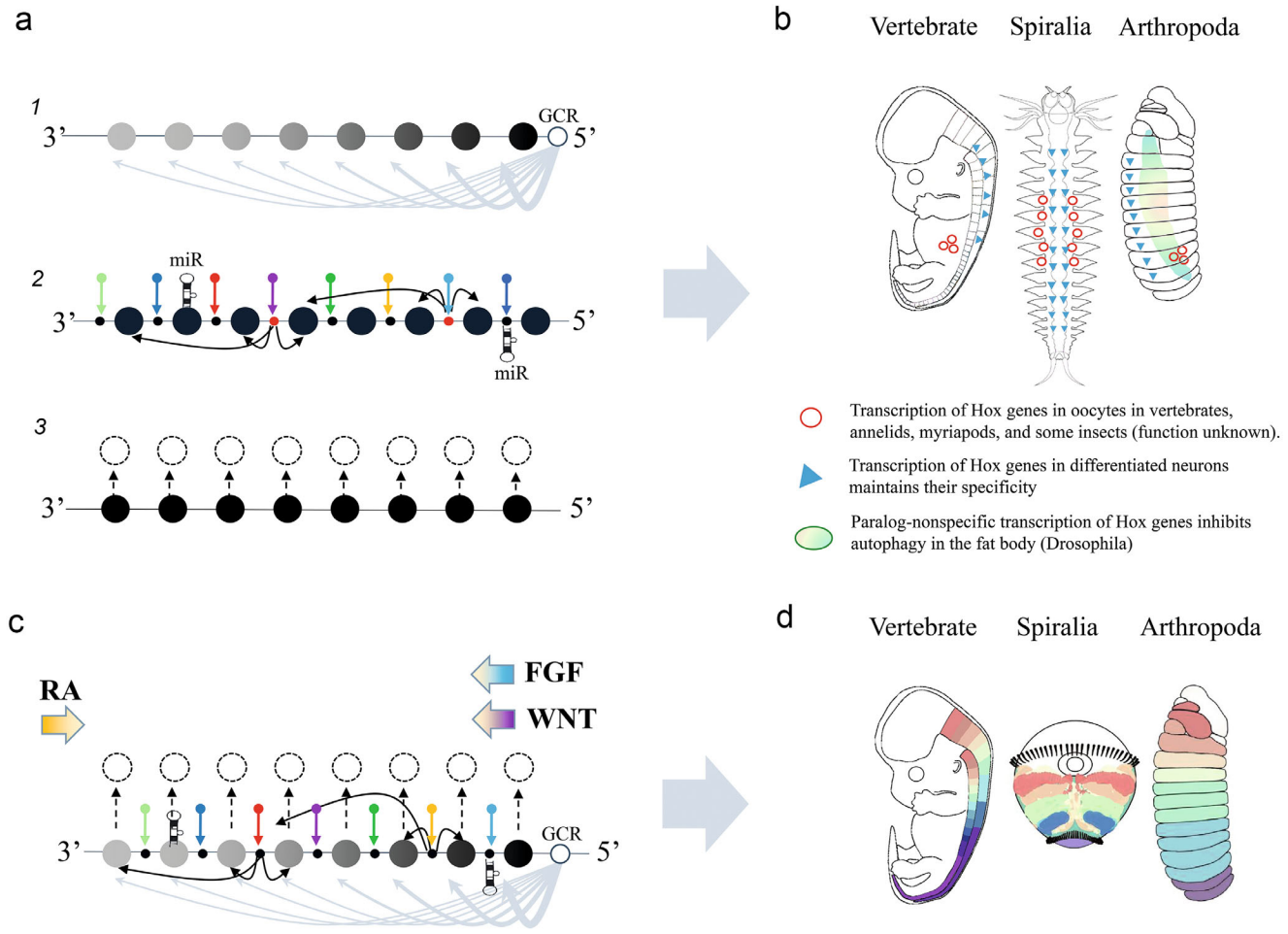

Fig. 3. Control of Hox cluster transcription. a) Some control mechanisms of the Hox cluster in bilateral animals. *1*) GCR, Global controlling element. Regulatory elements of this type have been described in vertebrates; *2*) local *cis*-regulatory modules (individual and shared), site-specific transcription factors, and microRNAs (miR) encoded in Hox cluster sequences. These modes of regulation are present in protostomes and deuterostomes; *3*) vertical signaling has been described in vertebrates. b) Non-canonical functions of Hox genes that could be realized by separate elements of the controls; c) Simplified scheme of Hox cluster regulation during realization of the canonical function of axial patterning (d). Not all mechanisms are represented in the scheme, and not all of those represented are universal. GCR and vertical signaling are not found in the model arthropods. Spiral animals are generally poorly studied at the level of Hox cluster activation mechanisms, but among them, there are organisms with temporal colinearity and early mesodermal transcription. RA (Retinoic Acid), FGF, and WNT are gradients of morphogens.

– response to individual signals from upstream regulatory proteins, which can turn on/off individual genes;

– reciprocal interactions of Hox genes (posterior suppression and beyond).

Some of the regulatory mechanisms are shown in Fig. 3. How could this very complex picture emerge from non-canonical functions? Perhaps the different modes of Hox cluster regulation needed to perform separate tasks were co-opted into a new program. It seems intuitively correct to assume that this new program is gastrulation in its "bilateral version." This seems to be suggested by the temporal conjugation of the Hox cluster activation and gastrulation processes in deuterostomes. Moreover, there is evidence for pre-adaptation – two Hox genes of the cnidarian *Nematostella vectensis* (anterior *NvAx6* and mid-posterior *NvAx1*) are important for gastrulation and specification of the oral-aboral axis. Their expression sites label the oral and aboral poles, and the morpholino knockdown suppresses gastrulation [107]. However, these genes do not form a cluster and operate in different germ layers. In *Drosophila*, the axial pattern of Hox genes is established before the onset of gastrulation, and there are no studies to reliably confirm or refute the involvement of the Hox genes in arthropods in gastrulation. In some spiral animals (annelids, brachiopods, mollusks), early activation of the Hox genes coincides with the onset or continuation of gastrulation, but functional tests are still lacking [26, 108, 109].

With a high degree of confidence, it can be stated that the last common ancestor of bilateral animals

had a single Hox cluster. This cluster could have been used for different tasks, such as the specification of motor neurons, the establishment of neuromuscular junctions, and a function requiring quantitative changes in transcripts in response to a stimulus (probably a morphogen concentration). Such an ancient function may have been related to autophagy, control of proliferation, or gametogenesis. Importantly, this function was enabled by the gradient distribution of Hox proteins along the axis and kept the Hox genes in the cluster. The Nephrozoa ancestor could use the whole cluster or some of its genes to control gastrulation, but this is not the case in the lineage of modern Acoelomorpha (sister branch of Nephrozoa) because all Hox genes of *Convolutriloba* are turned on after gastrulation [53].

Different ways of controlling the same cluster could lead to errors in its regulation, and some of the resulting aberrant variants were preserved by natural selection. Two important events could have occurred in the evolution of bilateral animals from the Nephrozoa lineage. Firstly, several mechanisms controlling the Hox cluster could have united to control a single morphogenetic program. Secondly, there might have been a heterochronic shift in the activation of this program towards earlier development. The least catastrophic variant suggests a series of heterochronic shifts of colinear expression of the Hox genes in internal, mesodermal in origin, structures up to the gastrula stage. Then, through the vertical signaling, the Hox genes began to turn on colinearly in the adjacent ectoderm (future neuroectoderm), providing animals with a new powerful tool for controlling early development. This tool could be easily scaled by the gradients of morphogens, it coordinated the development of ectodermal and mesodermal tissues, and it was evolutionarily plastic due to many controls coming from the older programs. Perhaps it was this new molecular mechanism that "detonated" and triggered the "Cambrian Explosion" because of its ability to rapidly alter early development.

CONCLUSION

The diversity of non-canonical functions of Hox genes is determined by the structure of the homeodomain protein itself, which can work not only as a transcription factor, but also as a regulator of general biological processes, such as DNA repair, replication, translation, and RNA splicing.

The "hourglass" model, while illustrative, leaves non-canonical functions of the Hox genes invisible. According to the inverse hourglass model, which is valid for Metazoa as a whole [110], there is a fundamental similarity in gene functioning at the earliest stages of development (pluripotent state of cells, cleavage) and at later stages (differentiation, organogenesis). However, animals from different phyla will vary significantly in the ensembles of regulatory genes and nature of their involvement in morphogenesis in the middle of development, just between cleavage and committed differentiation [110]. These are the differences that define fundamental distinction between the phyla within Metazoa. In other words, it is possible to identify distinct sets of signaling pathways and transcription factors that interact during the establishment of Metazoa organization plans. Their specific combination defines the appearance of each phylum. It turned out that homeobox genes in general and Hox genes in particular do not fall into the category of such "phylum-specific" regulators because their functions are broader and more conserved during the divergent period of development.

We assume that the proto-Hox gene initially possessed a wide repertoire of functions, some of which relied on the signaling nature of its protein. Animals from the ParaHoxozoa branch turned out to be heirs of this regulatory complexity. They further enhanced it through cooperation between developmental programs that used different functional capabilities of Hox proteins. These programs emerged at various stages of evolution, and their traces are preserved in modern animals in the form of distinct paralog-nonspecific and dose-dependent functions.

Acknowledgments. In the study, Geneious® 2023.2.1 software was used for sequence analysis, with access provided by the research resource center "Chromas" of Saint Petersburg State University.

Contributions. M.A.K. developed the study concept, supervised the study, and prepared and edited the manuscript; G.P.M. prepared and edited the manuscript; L.O.P. prepared and edited the manuscript.

Funding. This work was financially supported by the Russian Science Foundation (project no. 23-24-00426).

Ethics declarations. This work does not contain any studies involving human and animal subjects. The authors of this work declare that they have no conflicts of interest.

REFERENCES

1. Degnan, B. M., Vervoort, M., Larroux, C., and Richards, G. S. (2009) Early evolution of metazoan transcription factors, *Curr. Opin. Genet. Dev.*, **19**, 591-599, doi: 10.1016/j.gde.2009.09.008.
2. Ryan, J. F., Pang, K., Mullikin, J. C., Martindale, M. Q., and Baxevanis, A. D. (2010) The homeodomain complement of the ctenophore *Mnemiopsis leidyi* suggests that Ctenophora and Porifera diverged prior to the ParaHoxozoa, *Evodevo*, **1**, 9, doi: 10.1186/2041-9139-1-9.
3. Pang, K., and Martindale, M. Q. (2008) Developmental expression of homeobox genes in the ctenophore *Mnemiopsis leidyi*, *Dev. Genes Evol.*, **218**, 307-319, doi: 10.1007/s00427-008-0222-3.
4. Srivastava, M., Begovic, E., Chapman, J., Putnam, N. H., Hellsten, U., Kawashima, T., Kuo, A., Mitros, T., Salamov, A., and Carpenter, M. L. (2008) The Trichoplax genome and the nature of placozoans, *Nature*, **454**, 955-960, doi: 10.1038/nature07191.
5. Pastrana, C. C., DeBiasse, M. B., and Ryan, J. F. (2019) Sponges lack ParaHox genes, *Genome Biol. Evol.*, **11**, 1250-1257, doi: 10.1093/gbe/evz052.
6. Lewis, E. B. (1978) A gene complex controlling segmentation in Drosophila, *Nature*, **276**, 565-570, doi: 10.1038/276565a0.
7. Korchagina, N. M., Bakalenko, N. I., and Kulakova, M. A. (2010) Hox-cluster and evolution of morphogeneses, *Russ. J. Dev. Biol.*, **41**, 353-363.
8. Hubert, K. A., and Wellik, D. M. (2023) Hox genes in development and beyond, *Development*, **150**, dev192476, doi: 10.1242/dev.192476.
9. De Rosa, R., Grenier, J. K., Andreeva, T., Cook, C. E., Adoutte, A., Akam, M., Carroll, S. B., and Balavoine, G. (1999) Hox genes in brachiopods and priapulids and protostome evolution, *Nature*, **399**, 772-776, doi: 10.1038/21631.
10. Balavoine, G., de Rosa, R., and Adoutte, A. (2002) Hox clusters and bilaterian phylogeny, *Mol. Phylogenet. Evol.*, **24**, 366-373, doi: 10.1016/s1055-7903(02)00237-3.
11. Dressler, G. R., and Gruss, P. (1989) Anterior boundaries of Hox gene expression in mesoderm-derived structures correlate with the linear gene order along the chromosome, *Differentiation*, **41**, 193-201, doi: 10.1111/j.1432-0436.1989.tb00747.x.
12. Duboule, D., and Dolle, P. (1989) The structural and functional organization of the murine HOX gene family resembles that of Drosophila homeotic genes, *EMBO J.*, **8**, 1497-1505, doi: 10.1002/j.1460-2075.1989.tb03534.x.
13. Duboule, D. (1994) Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony, *Dev. Suppl.*, doi: 10.1242/dev.1994.Supplement.135.
14. Larroux, C., Fahey, B., Degnan, S. M., Adamski, M., Rokhsar, D. S., and Degnan, B. M. (2007) The NK homeobox gene cluster predates the origin of Hox genes, *Curr. Biol.*, **17**, 706-710, doi: 10.1016/j.cub.2007.03.008.
15. Copley, R. R. (2023) The ancestry of Antennapedia-like homeobox genes, *bioRxiv*, doi: 10.1101/2023.03.14.532566.
16. DuBuc, T. Q., Ryan, J. F., Shinzato, C., Satoh, N., and Martindale, M. Q. (2012) Coral comparative genomics reveal expanded Hox cluster in the cnidarian-bilaterian ancestor, *Integr. Comp. Biol.*, **52**, 835-841, doi: 10.1093/icb/ics098.
17. Lynch, V. J., Roth, J. J., and Wagner, G. P. (2006) Adaptive evolution of Hox-gene homeodomains after cluster duplications, *BMC Evol. Biol.*, **6**, 86, doi: 10.1186/1471-2148-6-86.
18. Duboule, D. (2007) The rise and fall of Hox gene clusters, *Development*, **134**, 2549-2560, doi: 10.1242/dev.001065.
19. Seo, H. C., Edvardsen, R. B., Maeland, A. D., Bjordal, M., Jensen, M. F., Hansen, A., Flaat, M., Weissenbach, J., Lehrach, H., Wincker, P., Reinhardt, R., and Chourrout, D. (2004) Hox cluster disintegration with persistent anteroposterior order of expression in *Oikopleura dioica*, *Nature*, **431**, 67-71, doi: 10.1038/nature02709.
20. Fröbius, A. C., and Funch, P. (2017) Rotiferan Hox genes give new insights into the evolution of metazoan bodyplans, *Nat. Commun.*, **8**, 9, doi: 10.1038/s41467-017-00020-w.
21. Lee, P. N., Callaerts, P., De Couet, H. G., and Martindale, M. Q. (2003) Cephalopod Hox genes and the origin of morphological novelties, *Nature*, **424**, 1061-1065, doi: 10.1038/nature01872.
22. Monteiro, A. S., and Ferrier, D. E. (2006) Hox genes are not always Colinear, *Int. J. Biol. Sci.*, **2**, 95-103, doi: 10.7150/ijbs.2.95.
23. Stern, D. L. (1998) A role of Ultrabithorax in morphological differences between Drosophila species, *Nature*, **396**, 463-466, doi: 10.1038/24863.
24. Slack, J. M., Holland, P. W., and Graham, C. F. (1993) The zootype and the phylotypic stage, *Nature*, **361**, 490-492, doi: 10.1038/361490a0.
25. Lemaire, L., and Kessel, M. (1997) Gastrulation and homeobox genes in chick embryos, *Mech. Dev.*, **67**, 3-16, doi: 10.1016/s0925-4773(97)00102-0.
26. Kulakova, M., Bakalenko, N., Novikova, E., Cook, C. E., Eliseeva, E., Steinmetz, P. R., Kostyuchenko, R. P., Dondua, A., Arendt, D., Akam, M., and Andreeva, T. (2007) Hox gene expression in larval development of the polychaetes *Nereis virens* and *Platynereis dumerilii*

(Annelida, Lophotrochozoa), *Dev. Genes Evol.*, **217**, 39-54, doi: 10.1007/s00427-006-0119-y.
27. Michaut, L., Jansen, H. J., Bardine, N., Durston, A. J., and Gehring, W. J. (2011) Analyzing the function of a hox gene: an evolutionary approach, *Dev. Growth Differ.*, **53**, 982-993, doi: 10.1111/j.1440-169X.2011.01307.x.
28. Fröbius, A. C., Matus, D. Q., and Seaver, E. C. (2008) Genomic organization and expression demonstrate spatial and temporal *Hox* gene colinearity in the lophotrochozoan *Capitella* sp. I, *PLoS One*, **3**, e4004, doi: 10.1371/journal.pone.0004004.
29. Garcia-Fernàndez, J. (2005) The genesis and evolution of homeobox gene clusters, *Nat. Rev. Genet.*, **6**, 881-892, doi: 10.1038/nrg1723.
30. Philippidou, P., and Dasen, J. S. (2013) Hox genes: choreographers in neural development, architects of circuit organization, *Neuron*, **80**, 12-34, doi: 10.1016/j.neuron.2013.09.020.
31. Joshi, R., Sipani, R., and Bakshi, A. (2021) Roles of Drosophila Hox genes in the assembly of neuromuscular networks and behavior, *Front. Cell Dev. Biol.*, **9**, 786993, doi: 10.3389/fcell.2021.786993.
32. Feng, W., Li, Y., and Kratsios, P. (2021) Emerging roles for hox proteins in the last steps of neuronal development in worms, flies, and mice, *Front. Neurosci.*, **15**, 801791, doi: 10.3389/fnins.2021.801791.
33. Kourakis, M. J., Master, V. A., Lokhorst, D. K., Nardelli-Haefliger, D., Wedeen, C. J., Martindale, M. Q., and Shankland, M. (1997) Conserved anterior boundaries of *Hox* gene expression in the central nervous system of the leech Helobdella, *Dev. Biol.*, **190**, 284-300, doi: 10.1006/dbio.1997.8689.
34. Samadi, L., and Steiner, G. (2010) Expression of Hox genes during the larval development of the snail, *Gibbula varia* (L.)-further evidence of non-colinearity in molluscs, *Dev. Genes Evol.*, **220**, 161-172, doi: 10.1007/s00427-010-0338-0.
35. Hinman, V. F., O'Brien, E. K., Richards, G. S., and Degnan, B. M. (2003) Expression of anterior Hox genes during larval development of the gastropod *Haliotis asinina*, *Evol. Dev.*, **5**, 508-521, doi: 10.1046/j.1525-142x.2003.03056.x.
36. Barrera Grijalba, C. C., Rodríguez Monje, S. V., Gestal, C., and Wollesen, T. (2023) Octopod Hox genes and cephalopod plesiomorphies, *Sci. Rep.*, **13**, 15492, doi: 10.1038/s41598-023-42435-0.
37. Kratsios, P., Kerk, S. Y., Catela, C., Liang, J., Vidal, B., Bayer, E. A., Feng, W., De La Cruz, E. D., Croci, L., Consalez, G. G., Mizumoto, K., and Hobert, O. (2017) An intersectional gene regulatory strategy defines subclass diversity of *C. elegans* motor neurons, *Elife*, **6**, e25751, doi: 10.7554/eLife.25751.
38. Feng, W., Li, Y., Dao, P., Aburas, J., Islam, P., Elbaz, B., Kolarzyk, A., Brown, A. E., and Kratsios, P. (2020) A terminal selector prevents a Hox transcriptional switch to safeguard motor neuron identity throughout life, *Elife*, **9**, e50065, doi: 10.7554/eLife.50065.
39. Li, Y., Osuma, A., Correa, E., Okebalama, M. A., Dao, P., Gaylord, O., Aburas, J., Islam, P., Brown, A. E., and Kratsios, P. (2020) Establishment and maintenance of motor neuron identity via temporal modularity in terminal selector function, *Elife*, **9**, e59464, doi: 10.7554/eLife.59464.
40. White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1986) The structure of the nervous system of the nematode *Caenorhabditis elegans*, *Philos. Trans. R Soc. Lond. B Biol. Sci.*, **314**, 1-340, doi: 10.1098/rstb.1986.0056.
41. Raji, J. I., and Potter, C. J. (2021) The number of neurons in Drosophila and mosquito brains, *PLoS One*, **16**, e0250381, doi: 10.1371/journal.pone.0250381.
42. Estacio-Gómez, A., and Díaz-Benjumea, F. J. (2014) Roles of Hox genes in the patterning of the central nervous system of Drosophila, *Fly (Austin)*, **8**, 26-32, doi: 10.4161/fly.27424.
43. Friedrich, J., Sorge, S., Bujupi, F., Eichenlaub, M. P., Schulz, N. G., Wittbrodt, J., and Lohmann, I. (2016) Hox function is required for the development and maintenance of the Drosophila feeding motor unit, *Cell Rep.*, **14**, 850-860, doi: 10.1016/j.celrep.2015.12.077.
44. Estacio-Gómez, A., Moris-Sanz, M., Schäfer, A. K., Perea, D., Herrero, P., and Díaz-Benjumea, F. J. (2013) Bithorax-complex genes sculpt the pattern of leucokinergic neurons in the Drosophila central nervous system, *Development*, **140**, 2139-2148, doi: 10.1242/dev.090423.
45. Catela, C., Chen, Y., Weng, Y., Wen, K., and Kratsios, P. (2022) Control of spinal motor neuron terminal differentiation through sustained *Hoxc8* gene activity, *Elife*, **11**, e70766, doi: 10.7554/eLife.70766.
46. Hutlet, B., Theys, N., Coste, C., Ahn, M. T., Doshishti-Agolli, K., Lizen, B., and Gofflot, F. (2016) Systematic expression analysis of *Hox* genes at adulthood reveals novel patterns in the central nervous system, *Brain Struct. Funct.*, **221**, 1223-1243, doi: 10.1007/s00429-014-0965-8.
47. Reilly, M. B., Cros, C., Varol, E., Yemini, E., and Hobert, O. (2020) Unique homeobox codes delineate all the neuron classes of *C. elegans*, *Nature*, **584**, 595-601, doi: 10.1038/s41586-020-2618-9.
48. Hobert, O. (2021) Homeobox genes and the specification of neuronal identity, *Nat. Rev. Neurosci.*, **22**, 627-636, doi: 10.1038/s41583-021-00497-x.
49. Sheng, G., Thouvenot, E., Schmucker, D., Wilson, D. S., and Desplan, C. (1997) Direct regulation of rhodopsin 1 by Pax-6/eyeless in Drosophila: evidence for a conserved function in photoreceptors, *Genes Dev.*, **11**, 1122-1131, doi: 10.1101/gad.11.9.1122.

50. Gehring, W. J., and Ikeo, K. (1999) Pax 6: mastering eye morphogenesis and eye evolution, *Trends Genet.*, **15**, 371-377, doi: 10.1016/s0168-9525(99)01776-x.
51. Gehring, W. J. (2005) New perspectives on eye development and the evolution of eyes and photoreceptors, *J. Hered.*, **96**, 171-184, doi: 10.1093/jhered/esi027.
52. Merabet, S., and Mann, R. S. (2016) To be specific or not: the critical relationship between Hox and TALE proteins, *Trends Genet.*, **32**, 334-347, doi: 10.1016/j.tig.2016.03.004.
53. Hejnol, A., and Martindale, M. Q. (2009) Coordinated spatial and temporal expression of Hox genes during embryogenesis in the acoel *Convolutriloba longifissura*, *BMC Biol.*, 7, 65, doi: 10.1186/1741-7007-7-65.
54. Moreno, E., Nadal, M., Baguñà, J., and Martínez, P. (2009) Tracking the origins of the bilaterian Hox patterning system: insights from the acoel flatworm *Symsagittifera roscoffensis*, *Evol. Dev.*, **11**, 574-581, doi: 10.1111/j.1525-142X.2009.00363.x.
55. Moreno, E., De Mulder, K., Salvenmoser, W., Ladurner, P., and Martínez, P. (2010) Inferring the ancestral function of the posterior *Hox* gene within the bilateria: controlling the maintenance of reproductive structures, the musculature and the nervous system in the acoel flatworm *Isodiametra pulchra*, *Evol. Dev.*, **12**, 258-266, doi: 10.1111/j.1525-142X.2010.00411.x.
56. Moreno, E., Permanyer, J., and Martinez, P. (2011) The origin of patterning systems in bilateria-insights from the Hox and ParaHox genes in Acoelomorpha, *Genom. Proteom. Bioinform.*, **9**, 65-76, doi: 10.1016/s1672-0229(11)60010-7.
57. Finnerty, J. R., and Martindale, M. Q. (1999) Ancient origins of axial patterning genes: *Hox* genes and *ParaHox* genes in the Cnidaria, *Evol. Dev.*, **1**, 16-23, doi: 10.1046/j.1525-142x.1999.99010.x.
58. Chiori, R., Jager, M., Denker, E., Wincker, P., Da Silva, C., Le Guyader, H., Manuel, M., and Quéinnec, E. (2009) Are Hox genes ancestrally involved in axial patterning? Evidence from the hydrozoan *Clytia hemisphaerica* (Cnidaria), *PLoS One*, **4**, e4231, doi: 10.1371/journal.pone.0004231.
59. Nong, W., Cao, J., Li, Y., Qu, Z., Sun, J., Swale, T., Yip, H. Y., Qian, P. Y., Qiu, J. W., Kwan, H. S., Bendena, W., Tobe, S., Chan, T. F., Yip, K. Y., Chu, K. H., Ngai, S. M., Tsim, K. Y., Holland, P. W. H., and Hui, J. H. L. (2020) Jellyfish genomes reveal distinct homeobox gene clusters and conservation of small RNA processing, *Nat. Commun.*, **11**, 3051, doi: 10.1038/s41467-020-16801-9.
60. Galliot, B., Quiquand, M., Ghila, L., de Rosa, R., Miljkovic-Licina, M., and Chera, S. (2009) Origins of neurogenesis, a cnidarian view, *Dev. Biol.*, **332**, 2-24, doi: 10.1016/j.ydbio.2009.05.563.
61. Faltine-Gonzalez, D., Havrilak, J., and Layden, M. J. (2023) The brain regulatory program predates central nervous system evolution, *Sci. Rep.*, **13**, 8626, doi: 10.1038/s41598-023-35721-4.
62. Oren, M., Brickner, I., Appelbaum, L., and Levy, O. (2014) Fast neurotransmission related genes are expressed in non nervous endoderm in the sea anemone *Nematostella vectensis*, *PLoS One*, **9**, e93832, doi: 10.1371/journal.pone.0093832.
63. Steinworth, B. M., Martindale, M. Q., and Ryan, J. F. (2023) Gene Loss may have shaped the Cnidarian and Bilaterian Hox and ParaHox complement, *Genome Biol. Evol.*, **15**, evac172, doi: 10.1093/gbe/evac172.
64. Reggiori, F., and Klionsky, D. J. (2002) Autophagy in the eukaryotic cell, *Eukaryot. Cell*, **1**, 11-21, doi: 10.1128/ec.01.1.11-21.2002.
65. Wada, Y., Sun-Wada, G. H., Kawamura, N., and Aoyama, M. (2014) Role of autophagy in embryogenesis, *Curr. Opin. Genet. Dev.*, **27**, 60-66, doi: 10.1016/j.gde.2014.03.010.
66. Tsukamoto, S., Kuma, A., and Mizushima, N. (2008) The role of autophagy during the oocyte-to-embryo transition, *Autophagy*, **4**, 1076-1078, doi: 10.4161/auto.7065.
67. Banreti, A., Hudry, B., Sass, M., Saurin, A. J., and Graba, Y. (2014) Hox proteins mediate developmental and environmental control of autophagy, *Dev. Cell*, **28**, 56-69, doi: 10.1016/j.devcel.2013.11.024.
68. Sachs, M., Onodera, C., Blaschke, K., Ebata, K. T., Song, J. S., and Ramalho-Santos, M. (2013) Bivalent chromatin marks developmental regulatory genes in the mouse embryonic germline *in vivo*, *Cell Rep.*, **3**, 1777-1784, doi: 10.1016/j.celrep.2013.04.032.
69. Paul, D., Bridoux, L., Rezsöhazy, R., and Donnay, I. (2011) HOX genes are expressed in bovine and mouse oocytes and early embryos, *Mol. Reprod. Dev.*, **78**, 436-449, doi: 10.1002/mrd.21321.
70. Kondo, M., Yamamoto, T., Takahashi, S., and Taira, M. (2017) Comprehensive analyses of hox gene expression in *Xenopus laevis* embryos and adult tissues, *Dev. Growth Differ.*, **59**, 526-539, doi: 10.1111/dgd.12382.
71. Maslakov, G. P., Kulishkin, N. S., Surkova, A. A., and Kulakova, M. A. (2021) Maternal transcripts of Hox genes are found in oocytes of *Platynereis dumerilii* (Annelida, Nereididae), *J. Dev. Biol.*, **9**, 37, doi: 10.3390/jdb9030037.
72. Chipman, A. D., Ferrier, D. E., Brena, C., Qu, J., Hughes, D. S., Schröder, R., Torres-Oliva, M., Znassi, N., Jiang, H., Almeida, F. C., Alonso, C. R., Apostolou, Z., Aqrawi, P., Arthur, W., Barna, J. C., Blankenburg, K. P., Brites, D., Capella-Gutiérrez, S., Coyle, M., Dearden, P. K., et al. (2014) The first myriapod genome sequence reveals conservative arthropod gene content and genome organisation in the centipede *Strigamia maritima*, *PLoS Biol.*, **12**, e1002005, doi: 10.1371/journal.pbio.1002005.
73. Qu, Z., Nong, W., So, W. L., Barton-Owen, T., Li, Y., Leung, T. C. N., Li, C., Baril, T., Wong, A. Y. P., Swale, T., Chan, T. F., Hayward, A., Ngai, S. M., and Hui, J. H. L. (2020) Millipede genomes reveal unique adaptations during myriapod evolution, *PLoS Biol.*, **18**, e3000636, doi: 10.1371/journal.pbio.3000636.

74. Rafiqi, A. M., Rajakumar, A., and Abouheif, E. (2020) Origin and elaboration of a major evolutionary transition in individuality, *Nature*, **585**, 239-244, doi: 10.1038/s41586-020-2653-6.
75. Jaramillo, M. L., Ammar, D., Quispe, R. L., Bonatto Paese, C. L., Gruendling, A. P., Müller, Y. M., and Nazari, E. M. (2022) Identification of Hox genes and their expression profiles during embryonic development of the emerging model organism, *Macrobrachium olfersii*, *J. Exp. Zool. B Mol. Dev. Evol.*, **338**, 292-300, doi: 10.1002/jez.b.23118.
76. Ferreira, E. M., Vireque, A. A., Adona, P. R., Meirelles, F. V., Ferriani, R. A., and Navarro, P. A. (2009) Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence, *Theriogenology*, **71**, 836-848, doi: 10.1016/j.theriogenology.2008.10.023.
77. Sauvegarde, C., Paul, D., Bridoux, L., Jouneau, A., Degrelle, S., Hue, I., Rezsohazy, R., and Donnay, I. (2016) Dynamic pattern of HOXB9 protein localization during oocyte maturation and early embryonic development in mammals, *PLoS One*, **11**, e0165898, doi: 10.1371/journal.pone.0165898.
78. Rinn, J. L., Kertesz, M., Wang, J. K., Squazzo, S. L., Xu, X., Brugmann, S. A., Goodnough, L. H., Helms, J. A., Farnham, P. J., Segal, E., and Chang, H. Y. (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs, *Cell*, **129**, 1311-1323, doi: 10.1016/j.cell.2007.05.022.
79. Iyyappan, R., Aleshkina, D., Zhu, L., Jiang, Z., Kinterova, V., and Susor, A. (2021) Oocyte specific lncRNA variant *Rose* influences oocyte and embryo development, *Noncoding RNA Res.*, **6**, 107-113, doi: 10.1016/j.ncrna.2021.06.001.
80. Kageyama, S., Gunji, W., Nakasato, M., Murakami, Y., Nagata, M., and Aoki, F. (2007) Analysis of transcription factor expression during oogenesis and preimplantation development in mice, *Zygote*, **15**, 117-128, doi: 10.1017/s096719940700411x.
81. Holland, P. W., Booth, H. A., and Bruford, E. A. (2007) Classification and nomenclature of all human homeobox genes, *BMC Biol.*, **5**, 47, doi: 10.1186/1741-7007-5-47.
82. Rajkovic, A., Pangas, S. A., Ballow, D., Suzumori, N., and Matzuk, M. M. (2004) NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression, *Science*, **305**, 1157-1159, doi: 10.1126/science.1099755.
83. Jukam, D., Shariati, S. A. M., and Skotheim, J. M. (2017) Zygotic genome activation in vertebrates, *Dev. Cell*, **42**, 316-332, doi: 10.1016/j.devcel.2017.07.026.
84. Carnesecchi, J., Boumpas, P., van Nierop, Y. S. P., Domsch, K., Pinto, H. D., Borges Pinto, P., and Lohmann, I. (2022) The Hox transcription factor Ultrabithorax binds RNA and regulates co-transcriptional splicing through an interplay with RNA polymerase II, *Nucleic Acids Res.*, **50**, 763-783, doi: 10.1093/nar/gkab1250.
85. Rubin, E., Wu, X., Zhu, T., Cheung, J. C., Chen, H., Lorincz, A., Pandita, R. K., Sharma, G. G., Ha, H. C., Gasson, J., Hanakahi, L. A., Pandita, T. K., and Sukumar, S. (2007) A role for the HOXB7 homeodomain protein in DNA repair, *Cancer Res.*, **67**, 1527-1535, doi: 10.1158/0008-5472.Can-06-4283.
86. Salsi, V., Ferrari, S., Ferraresi, R., Cossarizza, A., Grande, A., and Zappavigna, V. (2009) HOXD13 binds DNA replication origins to promote origin licensing and is inhibited by geminin, *Mol. Cell Biol.*, **29**, 5775-5788, doi: 10.1128/MCB.00509-09.
87. Primon, M., Hunter, K. D., Pandha, H. S., and Morgan, R. (2019) Kinase regulation of HOX transcription factors, *Cancers (Basel)*, **11**, 508, doi: 10.3390/cancers11040508.
88. Lavin, M. F., and Kozlov, S. (2007) ATM activation and DNA damage response, *Cell Cycle*, **6**, 931-942, doi: 10.4161/cc.6.8.4180.
89. Comelli, L., Marchetti, L., Arosio, D., Riva, S., Abdurashidova, G., Beltram, F., and Falaschi, A. (2009) The homeotic protein HOXC13 is a member of human DNA replication complexes, *Cell Cycle*, **8**, 454-459, doi: 10.4161/cc.8.3.7649.
90. Marchetti, L., Comelli, L., D'Innocenzo, B., Puzzi, L., Luin, S., Arosio, D., Calvello, M., Mendoza-Maldonado, R., Peverali, F., Trovato, F., Riva, S., Biamonti, G., Abdurashidova, G., Beltram, F., and Falaschi, A. (2010) Homeotic proteins participate in the function of human-DNA replication origins, *Nucleic Acids Res.*, **38**, 8105-8119, doi: 10.1093/nar/gkq688.
91. Vieux, K. F., and Clarke, H. J. (2018) CNOT6 regulates a novel pattern of mRNA deadenylation during oocyte meiotic maturation, *Sci. Rep.*, **8**, 6812, doi: 10.1038/s41598-018-25187-0.
92. Merabet, S., and Carnesecchi, J. (2024) Hox dosage and morphological diversification during development and evolution, *Semin. Cell Dev. Biol.*, **152-153**, 70-75, doi: 10.1016/j.semcdb.2022.11.009.
93. Horan, G. S., Ramírez-Solis, R., Featherstone, M. S., Wolgemuth, D. J., Bradley, A., and Behringer, R. R. (1995) Compound mutants for the paralogous hoxa-4, hoxb-4, and hoxd-4 genes show more complete homeotic transformations and a dose-dependent increase in the number of vertebrae transformed, *Genes Dev.*, **9**, 1667-1677, doi: 10.1101/gad.9.13.1667.
94. Zákány, J., Fromental-Ramain, C., Warot, X., and Duboule, D. (1997) Regulation of number and size of digits by posterior Hox genes: a dose-dependent mechanism with potential evolutionary implications, *Proc. Natl. Acad. Sci. USA*, **94**, 13695-13700, doi: 10.1073/pnas.94.25.13695.
95. Paul, R., Giraud, G., Domsch, K., Duffraisse, M., Marmigère, F., Khan, S., Vanderperre, S., Lohmann, I.,

Stoks, R., Shashidhara, L. S., and Merabet, S. (2021) Hox dosage contributes to flight appendage morphology in Drosophila, *Nat. Commun.*, **12**, 2892, doi: 10.1038/s41467-021-23293-8.

96. Joliot, A., Pernelle, C., Deagostini-Bazin, H., and Prochiantz, A. (1991) Antennapedia homeobox peptide regulates neural morphogenesis, *Proc. Natl. Acad. Sci. USA*, **88**, 1864-1868, doi: 10.1073/pnas.88.5.1864.
97. Lee, E. J., Kim, N., Park, J. W., Kang, K. H., Kim, W. I., Sim, N. S., Jeong, C. S., Blackshaw, S., Vidal, M., Huh, S. O., Kim, D., Lee, J. H., and Kim, J. W. (2019) Global analysis of intercellular homeodomain protein transfer, *Cell Rep.*, **28**, 712-722.e713, doi: 10.1016/j.celrep.2019.06.056.
98. Lucas, W. J., Bouché-Pillon, S., Jackson, D. P., Nguyen, L., Baker, L., Ding, B., and Hake, S. (1995) Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata, *Science*, **270**, 1980-1983, doi: 10.1126/science.270.5244.1980.
99. Prochiantz, A., and Di Nardo, A. A. (2015) Homeoprotein signaling in the developing and adult nervous system, *Neuron*, **85**, 911-925, doi: 10.1016/j.neuron.2015.01.019.
100. Spatazza, J., Di Lullo, E., Joliot, A., Dupont, E., Moya, K. L., and Prochiantz, A. (2013) Homeoprotein signaling in development, health, and disease: a shaking of dogmas offers challenges and promises from bench to bed, *Pharmacol. Rev.*, **65**, 90-104, doi: 10.1124/pr.112.006577.
101. Joliot, A. H., Triller, A., Volovitch, M., Pernelle, C., and Prochiantz, A. (1991) Alpha-2,8-polysialic acid is the neuronal surface receptor of antennapedia homeobox peptide, *New Biol.*, **3**, 1121-1134.
102. Merabet, S., Kambris, Z., Capovilla, M., Bérenger, H., Pradel, J., and Graba, Y. (2003) The hexapeptide and linker regions of the AbdA Hox protein regulate its activating and repressive functions, *Dev. Cell*, **4**, 761-768, doi: 10.1016/s1534-5807(03)00126-6.
103. Duffraisse, M., Paul, R., Carnesecchi, J., Hudry, B., Banreti, A., Reboulet, J., Ajuria, L., Lohmann, I., and Merabet, S. (2020) Role of a versatile peptide motif controlling Hox nuclear export and autophagy in the Drosophila fat body, *J. Cell Sci.*, **133**, jcs241943, doi: 10.1242/jcs.241943.
104. Bardine, N., Lamers, G., Wacker, S., Donow, C., Knoechel, W., and Durston, A. (2014) Vertical signalling involves transmission of Hox information from gastrula mesoderm to neurectoderm, *PLoS One*, **9**, e115208, doi: 10.1371/journal.pone.0115208.
105. Kim, H. T., Kim, S. J., Sohn, Y. I., Paik, S. S., Caplette, R., Simonutti, M., Moon, K. H., Lee, E. J., Min, K. W., Kim, M. J., Lee, D. G., Simeone, A., Lamonerie, T., Furukawa, T., Choi, J. S., Kweon, H. S., Picaud, S., Kim, I. B., Shong, M., and Kim, J. W. (2015) Mitochondrial protection by exogenous Otx2 in mouse retinal neurons, *Cell Rep.*, **13**, 990-1002, doi: 10.1016/j.celrep.2015.09.075.
106. Brunet, I., Weinl, C., Piper, M., Trembleau, A., Volovitch, M., Harris, W., Prochiantz, A., and Holt, C. (2005) The transcription factor Engrailed-2 guides retinal axons, *Nature*, **438**, 94-98, doi: 10.1038/nature04110.
107. DuBuc, T. Q., Stephenson, T. B., Rock, A. Q., and Martindale, M. Q. (2018) Hox and Wnt pattern the primary body axis of an anthozoan cnidarian before gastrulation, *Nat. Commun.*, **22**, 2007, doi: 10.1038/s41467-018-04184-x.
108. Schiemann, S. M., Martín-Durán, J. M., Børve, A., Vellutini, B. C., Passamaneck, Y. J., and Hejnol, A. (2017) Clustered brachiopod Hox genes are not expressed collinearly and are associated with lophotrochozoan novelties, *Proc. Natl. Acad. Sci. USA*, **114**, 1913-1922, doi: 10.1073/pnas.1614501114.
109. Salamanca-Díaz, D. A., Calcino, A. D., de Oliveira, A. L., and Wanninger, A. (2021) Non-collinear Hox gene expression in bivalves and the evolution of morphological novelties in mollusks, *Sci. Rep.*, **11**, 3575, doi: 10.1038/s41598-021-82122-6.
110. Levin, M., Anavy, L., Cole, A. G., Winter, E., Mostov, N., Khair, S., Senderovich, N., Kovalev, E., Silver, D. H., Feder, M., Fernandez-Valverde, S. L., Nakanishi, N., Simmons, D., Simakov, O., Larsson, T., Liu, S. Y., Jerafi-Vider, A., Yaniv, K., Ryan, J. F., Martindale, M. Q., et al. (2016) The mid-developmental transition and the evolution of animal body plans, *Nature*, **531**, 637-641, doi: 10.1038/nature16994.