CHAPTER 4

Evolution of Hox Gene Clusters

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Introduction

The Hox gene clusters have been one of the most prominent paradigms within Developmental Biology. This stems from the great excitement that surrounded the discovery that the genes all contained the conserved homeobox motif and that the homologous genes were operating in broadly homologous ways in the development of organisms as phylogenetically widespread as flies and vertebrates. The sequence similarity between the genes based on the homeobox, and their specific genomic organization in both flies and vertebrates, immediately implied a particular mode of evolution of the Hox gene cluster by tandem duplication and, more intriguingly, a functional constraint on the organization of the cluster to conserve colinearity. This general picture still holds true, but our understanding of the nature and extent of the constraints on cluster organization have been modified in recent years as data has become available from a much wider selection of animal phyla.

Colinearity, whereby the order of the genes along the chromosome corresponds to (or is colinear with) the domains of action of the genes along the anterior-posterior axis, has been one of the most intriguing and mysterious phenomena associated with the Hox cluster. Despite intense effort we do not have a clear idea about its mechanistic basis. It has even been argued that there may not be a universal underlying mechanism.[1] Whilst this probable lack of a universal mechanism operating across the various different instances of colinearity in an animal like the mouse is becoming clearer, whether this 'complicated' view can be extended to a lack of a universal, ancestral mechanism of colinearity (with add-on, lineage-specific mechanisms elaborating the picture) is unresolved. We must adopt a broad, phylogenetically informed, comparative approach (incorporating genomics, phylogenetics and developmental biology) to have a chance of finding an ancestral mechanism and finally unraveling the mystery of colinearity.

Origin of the Cluster: Unraveling Patterns of Gene Duplication and Duplicate Evolution

Homeobox Phylogenies, Tandem Duplication and the ProtoHox Cluster

Through the intricate and insightful genetic work of Ed Lewis the Bithorax Complex (BX-C) portion of the *Drosophila melanogaster* Hox cluster was elucidated. Lewis developed the idea that the gene complex consisted of a series of duplicated elements, or pseudoalleles as he first called them.[2-4] In time the BX-C was found to consist of three genes,[5] and the homeotic complex for the anterior end of the fly, the Antennapedia Complex (ANT-C), was found to contain 5 homeotic genes with a handful of interspersed nonhomeotic loci.[6] In the 1980's the homeobox was discovered in the genes of these complexes,[7] and this 180bp motif rapidly

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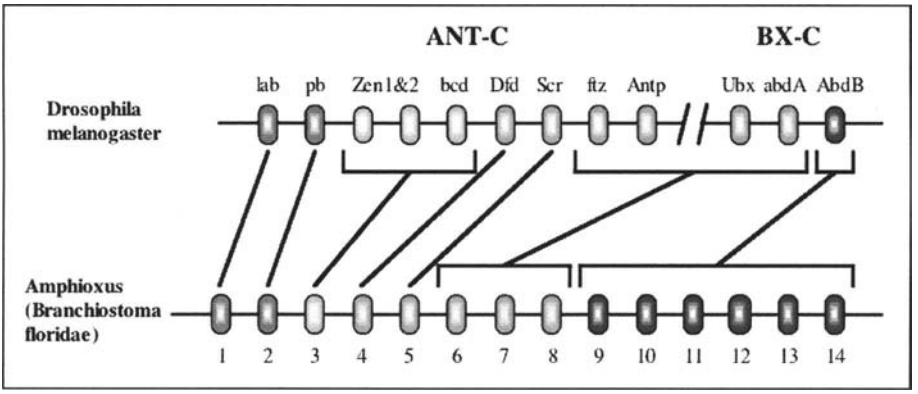

Figure 1. The homeobox gene content of the Hox clusters of *Drosophila melanogaster* and amphioxus. The fly cluster is broken into two, the ANT-C and BX-C. The ANT-C contains some homeobox genes that are derived from Hox genes but have evolved new, nonHox functions (*zen 1, zen2, bcd* and *ftz*). *Zen 1, zen2* and *bcd* evolved from duplications of an ancestral *Hox3* gene.[83,84] Several of the Central (green) genes cannot be distinguished as direct orthologues between insects and chordates, and at least some of them may have evolved from independent sets of tandem duplications. The single Posterior Hox gene (dark blue), *AbdB*, in flies is orthologous to the multiple Posterior genes of chordates, *Hox9-14*. The amphioxus cluster is prototypical for the multiple clusters of vertebrates (e.g., the four mammalian Hox clusters HoxA, B, C and D), which arose at the origin of the vertebrates, with subsequent gene loss occurring within each vertebrate cluster. A color version of this figure is available online at www.Eurekah.com.

found in the genomes of other animals, including ourselves. Together the ANT-C and BX-C of flies are homologous to the Hox clusters of chordates (Fig. 1).

The widespread conservation of the homeobox and its presence in a large family of transcription factor-encoding genes enabled the rapid isolation of multitudes of homeobox genes from a diverse array of phyla. Construction of phylogenetic trees of homeodomain sequences has been vital in understanding their modes of evolution and their classification.[8] From such trees it is evident that the Hox genes are closely related to each other, that is, the cluster is not an amalgamation of disparate, unrelated genes. Clearly this is consistent with the Hox cluster having formed via tandem duplication (Fig. 2). The specific nature of these tandem duplications has been debated. Some have proposed that the 'multiplication' of the genes in the cluster has occurred via unequal cross-overs, such that the genes inside the cluster are 'chimaeras' of the flanking genes.[9] Alternatively tandem duplication without chimaeric gene formation is also clearly possible. Indeed there are instances of this having occurred in the evolution of some present-day Hox clusters. For example the *Zerknüllt* (*zen*) genes of *Drosophila* are duplicated and are clearly related to each other rather than being chimaeras of flanking genes.

In addition to the homeobox phylogenies revealing the close relationship of the Hox genes to each other, it is also clear that several other genes that are not members of Hox clusters are interspersed amongst the Hox genes themselves in these trees. It was supposed that these represented dispersed or orphan Hox genes that had evolved by trans duplication of particular Hox genes deep in animal evolution, so that some Hox-related genes became scattered around the genome. This view was transformed when the ParaHox cluster was discovered in the cephalochordate amphioxus (*Branchiostoma floridae*).[10] The three genes of the ParaHox cluster are Gsx, Xlox and Cdx (in humans GSH1, IPF1 and CDX2). In homeobox phylogenies Gsx groups with the anterior Hox genes of groups 1 and 2, Xlox groups with Hox3 genes, and Cdx groups with the Posterior Hox genes. These gene relationships and the order of Gsx, Xlox and Cdx along the chromosome are consistent with a model in which the Hox and ParaHox clusters arose from a common ancestral homeobox cluster by duplication (the ProtoHox hypothesis).[10-14]

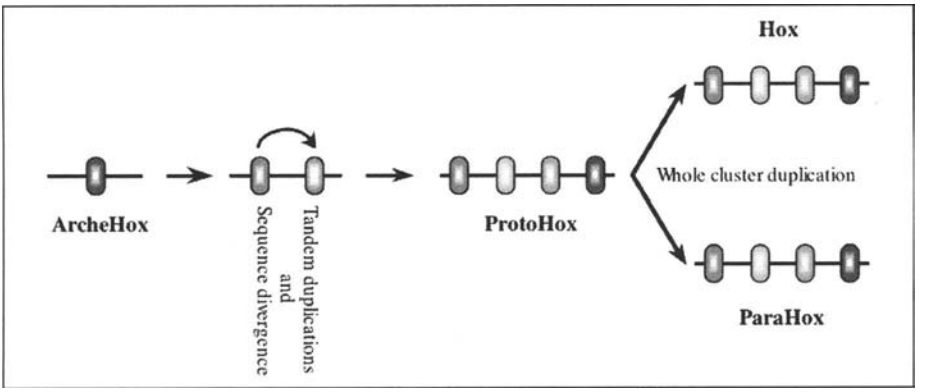

Figure 2. The origin of the Hox genes. An ancestral Hox-like gene, ArcheHox, underwent a series of tandem duplications and the duplicated genes diverged to establish the precursors of each of the different classes of Hox gene, Anterior (red), Group 3 (pale blue), Central (green) and Posterior (dark blue), in the ProtoHox cluster. The entire ProtoHox cluster duplicated, and gave rise to the Hox cluster and its evolutionary sister the ParaHox cluster. The precise timing of these duplication events is uncertain, as is whether the ProtoHox cluster contained a precursor of each of the 4 main groups of Hox gene or not (see the text). A color version of this figure is available online at www Eurekah.com.

Furthermore the expression of the ParaHox genes also exhibits Colinearity, with Gsx being expressed from the anterior of the embryo/larva, Xlox in mid-body regions and Cdx at the posterior end of the organism.[10] The ParaHox cluster is thus the evolutionary sister, or paralogue, of the Hox cluster (Fig. 2).

Basal Animals and the "Trox2" Model of Duplicate Evolution

When did this transition from a ProtoHox cluster to the Hox and ParaHox clusters occur? The answer is that it was clearly very early in the evolution of animals, but the precise time relative to the origin of particular animal phyla is still to be resolved. When the ParaHox cluster was first discovered it was thought that the ProtoHox duplication may have occurred at the transition between the radially symmetrical, diploblastic animals and the triploblastic bilaterians.[10] However as more sequences have become available from diploblasts, such as the cnidarians, it has become clear that both Hox and ParaHox genes are present in this diploblastic phylum.[11-13] So we must look deeper into the animal phylogeny to find the transition. However this becomes problematic due to the lack of clear consensus on the relationships of these basal animal phyla (Porifera, Placozoa, Cnidaria and Ctenophora).[15-17] Further work on the phylogeny of these diploblast phyla is required to clearly resolve their relationships and the ordering of their divergences from the lineage leading to the Bilateria, so that we can relate the homeobox content of the phyla to models of cluster evolution.

The Porifera, or sponges, are one candidate for the most basal animal group.[15,16] No clear Hox-like or ProtoHox genes have been found so far, although the search continues and will be aided by whole genome sequences of sponges. The enigmatic diploblast phylum Placozoa is an alternative candidate as the most basal animal phylum[17] (Fig. 3), and so may also provide us with a crucial data point in understanding the origin of the Hox cluster.

Trox2 is the only Hox-like gene to have been found in *Trichoplax adhaerens*, a placozoan. This gene however raises as many issues as it answers. Thorough searches have been performed for ANTP-class genes in *Trichoplax* (A.S. Monteiro et al in press), and no other Hox-like genes besides *Trox2* have been found. So potentially *Trox2* could be directly descended from an ancestral ProtoHox gene, or even the ArcheHox gene. However the sequence of Trox2 does not behave as expected for a ProtoHox protein in phylogenetic trees. Trox2 groups robustly with

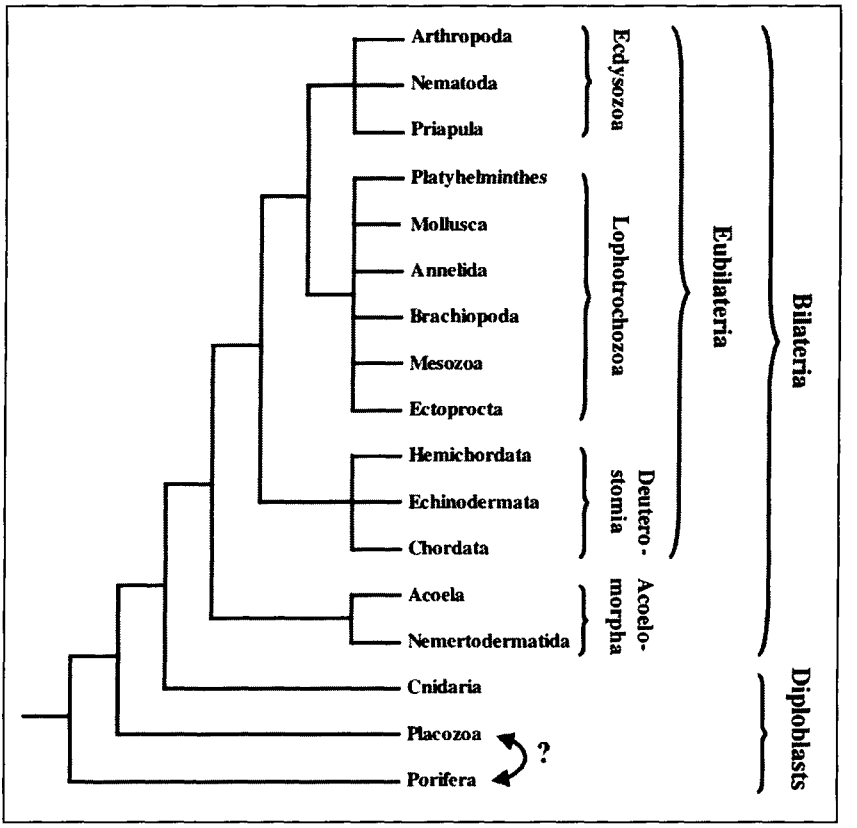

Figure 3. A phylogeny of the animal kingdom. The tree represents a consensus of references 21, 29, 32, 35, 36 and 85. The most basal animal lineage is not well resolved.[15-17]

the ParaHox protein Gsx.[18-20] So is *Trox2* really a descendent of a ProtoHox gene, or is it a Gsx gene and other Hox and ParaHox genes have been lost in the *Trichoplax* lineage? It is very difficult to distinguish between these two possibilities. However by building phylogenies of other *Trichoplax* genes and seeing how they relate to their bilaterian homologues we can see if many other *Trichoplax* genes behave as does *Trox2*, i.e., grouping with particular bilaterian genes within the bilaterian gene families rather than corroborating the a priori expectation that the *Trichoplax* gene would be basal or sister to the entire bilaterian gene family (Fig. 4A). If further *Trichoplax* genes behave like *Trox2* in phylogenetic trees then this could be taken as evidence for extensive gene loss in placozoans, which has hit the homeobox genes just as much as other gene families.

Alternatively *Trichoplax* genes are descended from ancestral 'Proto' genes and our models and assumptions about sequence evolution following gene duplication need to be modified. If the *Trichoplax* genes reflect the ancestral condition, and *Trox2* is a direct descendent from an ArcheHox/ProtoHox gene without duplication for example, then rather than both daughters of a gene duplication event diverging from the ancestral, preduplicate sequence, only one daughter diverges whilst the other retains the characteristics of the ancestral sequence (Fig. 4B). This revolutionary view of gene evolution may seem reasonable if the ancestral Proto gene is embedded in the developmental networks of the animal, with all of the consequent constraints on its function and hence sequence. After the duplication event all of these constraints are still present, and they are all still focused on one of the daughters, if for

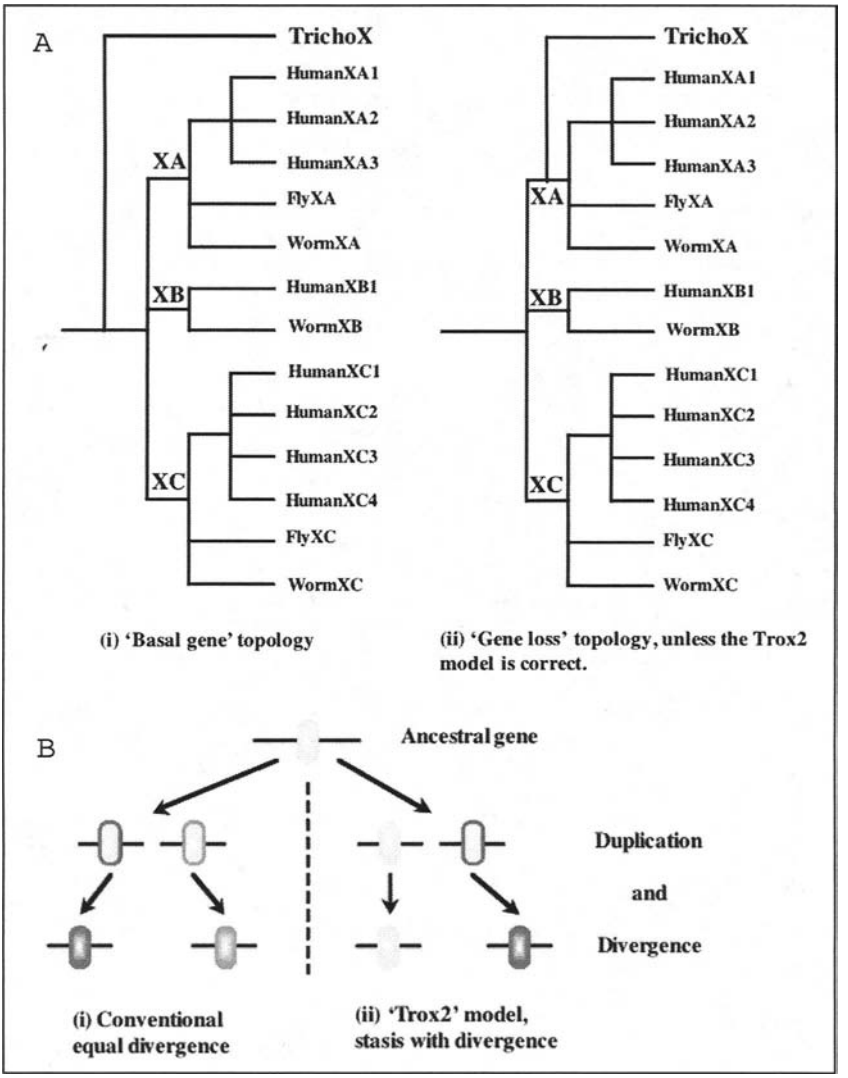

Figure 4. A) Inference of ancestral gene content from molecular phylogeny topologies. A gene family (X) has a diversity of members in the Bilateria (XA, XB and XC), whilst the placozoan *Trichoplax* has a single X gene (TrichoX). (i) If the Trichoplax gene is a direct, unduplicated descendent of the gene ancestral to XA, XB and XC, then TrichoX should diverge from a node more basal than the separations of the XA, XB and XC groups. (ii) If TrichoX groups with a particular bilaterian group such as XA with a node more crownward than the separation of XA, XB and XC, then we can infer that TrichoX is an XA gene and Trichoplax has lost its XB and XC genes. If in fact TrichoX really is a direct, unduplicated descendent of the ancestral X gene but still groups robustly with a particular clade such as XA, then an unconventional mode of gene evolution, the 'Trox2' model, has occurred. B) The Trox2 model of gene duplicate evolution. (i) Conventionally it is assumed that after a gene duplication event both daughter genes will diverge from the ancestral sequence. (ii) An alternative pattern of sequence evolution is the one that has been proposed to have occurred for Trox2,[86] whereby one of the duplication daughters diverges from the ancestral sequence, whilst the other daughter remains largely unchanged. A color version of this figure is available online at www.Eurekah.com.

example only one of the daughters has retained all of the regulatory elements and hence the full expression pattern of the ancestral gene, whilst the other daughter consists of the full coding sequence but not all of the regulatory 'baggage'. The result is that the second daughter is less constrained, diverges from the ancestral sequence and then settles into a new developmental network before it becomes a nonfunctional pseudogene. This stabilises its sequence so that it is conserved and recognisable across subsequently evolving phyla, and is recognised as a new, distinct gene class.

Perhaps whole genome sequences from basal animal groups such as Porifera, Placozoa and Cnidaria will help to resolve the issue of ancestral Hox-like gene content, and hopefully organization, and whether this rather revolutionary view of duplicated gene evolution is real or not. However due to the extensive time that has elapsed, combined with lineage-specific genomic evolution (e.g., the cnidarian Hox-like genes may even be independent duplications from a ProtoHox condition relative to the bilaterian Hox/ParaHox genes) even whole genome sequences may well not help, as no extant animal may have retained enough of the ancestral condition for us to discover. An alternative approach that must be pursued in parallel is to sample more widespread bilaterian taxa to establish patterns of conservation, and extrapolate back into the animal phylogeny to reconstruct ancestral conditions.

ProtoHox Content and the Nature of its Duplication

This extrapolation approach was how the ProtoHox hypothesis was first constructed, with the Hox cluster originating from the duplication of a 4-gene ProtoHox cluster. Alternative models of 2- and 3-gene ProtoHox clusters have since also been proposed.[12-14] The 4-gene ProtoHox model stems from the fact that Gsx groups with Hox1-2, Xlox with Hox3 and Cdx with Posterior Hox genes. There is not a ParaHox gene in the chordate ParaHox clusters that is allied with the Central Hox genes (groups 4-8). The 4-gene model thus posits that this fourth ParaHox gene was lost after the duplication of the ProtoHox cluster. An equally parsimonious scenario is a 3-gene ProtoHox model in which the Central Hox genes evolved after the ProtoHox duplication, so that the ParaHox cluster only ever had Gsx, Xlox and Cdx.[12,13] More recently a 2-gene ProtoHox model has been developed,[14,19-22] which proposes that Xlox, in the ParaHox cluster, and Hox3, in the Hox cluster, did not have a common ancestral gene in the ProtoHox cluster, but evolved by independent duplication in their respective clusters followed by convergence of their sequences, so that their grouping in phylogenetic trees is no longer a true reflection of their ancestry. In this 2-gene, as with the 3-gene scenario, the Central Hox genes then evolved just in the Hox cluster well after the ProtoHox duplication (reviewed in refs. 14,22).

The genomic organization of the Hox cluster has been one of its most prominent features, given its link to the functioning of the genes to produce Colinearity. The genomic organization around the cluster has also been informative, not necessarily with relevance to Hox function, but more with regard to the mode of the evolution of the cluster. Evx is another homeobox gene within the Antp-superclass that lies within a group of genes called the Extended Hox class,[23,24] not only because of its location in phylogenetic trees, but also because in several organisms it is tightly linked to the Hox cluster. This linkage clearly reflects the ancestral condition, and is still found in chordates and cnidarians.[25-27] Gauchat et al[19] proposed that Evx and the ProtoHox/ArcheHox gene arose from a duplication event. Minguillón and Garcia-Fernàndez[24] on the other hand propose that it was not Evx that was produced by this first duplication that gave rise to the initial ProtoHox gene, but rather the ancestor of both Evx and Mox was produced. Mox is another member of the Extended Hox class, and is also linked to the Hox clusters of vertebrates.[23,24] Mox and Evx genes show a weak association in phylogenetic trees. Taken in concert with their linkage to the vertebrate Hox clusters, and nonhomeobox genes that neighbour the Hox and ParaHox clusters, it appears that the duplication of the ProtoHox cluster may have been a tandem event, and ancestrally the newly formed Hox and ParaHox clusters were adjacent to each other, only subsequently being separated to different regions of the genome by translocation.[24]

Evolution of Cluster Composition and the Impact of Hox on Animal Phylogeny

The Key Position of the Acoelomorpha

Although there is the uncertainty about the exact composition of the ProtoHox cluster and where gene losses or gains happened in relation to the origin of particular diploblast phyla, as outlined above, the pattern of four basic groups of Hox genes (anterior, group3, central, posterior) had been established by the origin of the Eubilateria. Whether the full complement of the four Hox groups was also present right at the origin of the basal Bilateria is not completely resolved at present (see Fig. 3 and Baguñà and Riutort[21] for a discussion of the distinction between Bilateria and Eubilateria), and to a certain extent is linked to which of the 3/4-gene models or the 2-gene model of the ProtoHox cluster is true.

A phylogeny of the animal kingdom is of course essential for our interpretations of Hox evolution. A present consensus would be something like (Fig. 3). The phylogeny of the diploblasts is uncertain at present, but in recent years major advances have been made in resolving the tree of the Bilateria. The Acoelomorpha (= Acoela + Nemertodermatida) are likely to be basal bilaterians, and as such can provide us with an important outgroup to the higher bilaterians (eubilaterians) (reviewed in ref. 21). The Acoela have Hox genes that fall into the Anterior, Central and Posterior groups, but no Hox3 group has yet been found.[28] In the other Acoelomorph group, the Nemertodermatida, a fragment of an Xlox ParaHox gene has been found (Eva Jimenez-Guri pers. comm.), in addition to posterior and central Hox genes. If the 3 or 4-gene models of the ProtoHox are true, then the presence of an Xlox in Nemertodermatids means that the Xlox/Hox3 group was present in the Last Common Bilaterian (LCB). If the 2-gene ProtoHox model reflects reality then we still do not know whether the Hox3 group was in the LCB, or only Xlox had originated before the LCB, and Hox3 did not appear until after the divergence of the Acoelomorpha lineage but before the Last Common Eubilaterian (LCE).

Characteristic Hox Content of the Ecdysozoa, Lophotrochozoa and Deuterostomia

The Eubilaterians are conventionally divided into the protostomes and deuterostomes, and the protostomes in turn split into some variant of the Ecdysozoa and Lophotrochozoa.[29-31] Distinctive patterns of Hox genes seem to be broadly representative for each of these major eubilaterian groups.[32] In particular the Central Hox genes seem to be characteristic for each group, possibly reflecting independent duplications and expansions of this region of the Hox cluster in each of the three eubilaterian groups.[33] The types of Posterior Hox genes are even more clearly distinctive for each eubilaterian group,[32] with Ecdysozoa having variants of AbdB, Lophotrochozoa having Post1 and Post2, and deuterostomes having multiple Posterior Hox genes that are given the names Hox9-Hox14[34] (although this deuterostome nomenclature encompasses some extensive sequence diversity (see below)).

Such distinctive patterns of Hox gene possession have been useful in determining the phylogenetic position of several enigmatic phyla: Mesozoa,[35] Bryozoa/Ectoprocta,[36] and Brachiopoda,[32] although using Hox genes in such a fashion must be done with care.[37] One enigmatic group that could well benefit from further investigations of its Hox gene content to resolve its phylogenetic position is the Chaetognatha. This phylum has been allied with all three of the eubilaterian groups at one time or another: deuterostomes due to their embryology, Ecdysozoa due to their 18S rDNA sequences, and most recently the Lophotrochozoa via their mitochondrial sequences (mtDNA).[38] The difficulty in locating the Chaetognatha is exemplified by the same form of data, the mtDNA, being analysed independently and producing slightly different answers. In both analyses of the mtDNA the Chaetognatha group with the protostomes, but in one analysis they are basal protostomes and in the other they are lophotrochozoans.[38-40] Some information is available on chaetognath Hox genes. Unfortunately there is insufficient sequence information

from the Central Hox genes of chaetognaths to determine which genes are present as yet (Lox5/Lox2/Lox4 or Antp/abdA/Ubx, or Hox6/7/8), and no Posterior sequences have been cloned so far. However one gene in chaetognaths, *SceMedPost*, is distinctive.[41]

SceMedPost was proposed to be a mosaic gene, sharing sequence characteristics between Medial/Central and Posterior Hox genes.[41] This was hypothesised to reflect an ancestral state prior to divergence into Medial and Posterior states, with the chaetognath lineage originating prior to the split of deuterostomes and protostomes. The latest molecular data suggests that this basal bilaterian position of the chaetognaths is not true, and that they are in fact with the protostomes. Also it is now clear that the distinction between Posterior and Central/Medial Hox genes had already occurred at the base of the bilaterians, from the Acoelomorph data.[28] *SceMedPost* may yet provide us with some phylogenetic clues however. Another Hox gene that has always proved difficult to classify as Medial or Posterior is the *egl-5* gene of nematodes. Originally *egl-5* was thought to be the Posterior Hox gene of *Caenorhabditis elegans* due to its position at the end of the nematode Hox cluster (although the use of the term 'cluster' can only be loosely applied to nematodes). Other, clearer Posterior Hox genes have since been found in nematodes,[42] and egl-5 never groups robustly with Posterior Hox proteins in phylogenetic trees (e.g., see ref. 32). Intriguingly some of the residues that are considered to provide 'Medial' characteristics to SceMedPost are also present in egl-5 (namely Q6, T7, I45 and E59). Might this then indicate a phylogenetic affinity of chaetognaths and nematodes? This would be consistent with some phylogenetic reconstructions,[16,43] and the possession of some Ecdysozoan characters by chaetognaths, such as a ventral nerve chord combined with radial cleavage. Importantly however chaetognaths are thought not to undergo ecdysis, which led Peterson and Eernisse[16] to speculate that chaetognaths may be basal Ecdysozoans. This loose similarity of SceMedPost and nematode egl5 is clearly weak evidence on its own, but sequences of Posterior Hox genes may well be much more revealing if chaetognaths are found to have AbdB/nob1/php3 sequences.

How Many Posterior Hox Genes Did the Ancestors Have?

The Posterior Hox genes are evidently extremely useful tools for resolution of the broad relationships of animal phyla, however our understanding of the evolution of these Hox genes themselves is far from complete. Even the basic question as to how many Posterior Hox genes did the bilaterian ancestor have is far from resolved. Historically the Hox community has usually assumed that there was a single Posterior Hox, and the multiple genes seen in vertebrates arose from a series of tandem duplications in the chordate or deuterostome lineage. Such a view was inevitably coloured by the first known Hox cluster being that of *Drosophila*, and flies only having a single Posterior Hox gene, *Abdominal-B* (*AbdB*). If we examine the Posterior Hox gene content of a broader range of phyla in the context of a phylogenetic tree, then we can immediately see that the possession of a single Posterior Hox gene in flies is rather unusual.[32] Even the most closely related phyla to the arthropods (i.e., other Ecdysozoans) for which we have some data (nematodes and priapulids) seem to have more than one Posterior Hox gene (*nob1* and *php3* in *C.elegans* and *Pca-AbdB* and *Pca-HB4* in *Priapulus caudatus*).[32,42] Elsewhere, in the Lophotrochozoa, there are at least two Posterior Hox genes (Post1 and Post2), and in deuterostomes there are a whole array of Posterior Hox genes. Chordates can have up to six (Hox9-Hox14),[34,44] whilst the full complement of more basal deuterostomes (echinoderms and hemichordates) is still to be determined. This lack of resolution of the basal deuterostome condition is present even though we have a completely cloned and sequenced Hox cluster from an echinoderm, the purple sea urchin *Strongylocentrotus purpuratus*.[45] Contrary to first impressions of the urchin Hox cluster[46] the organization of the genes is extremely derived and scrambled.[45] Gene loss has also evidently occurred relative to the ancestral echinoderm condition, as Hox4 has been lost in *S.purpuratus* but is present in asteroids,[47] and other echinoderms have a more extensive set of Posterior Hox genes than the purple sea urchin.[48,49]

The relationships between the Posterior Hox genes of chordates and those of Ambulacraria (echinoderms and hemichordates) is also difficult to resolve, which leaves uncertainty as to what the Posterior Hox content of the ancestral deuterostome actually was. Although vertebrates, amphioxus and urochordates are all said to have a Hox10 for example, the genes of this name from each of these three groups of chordates do not actually group together robustly in phylogenetic trees. This lack of resolution can be extended to the nonchordate deuterostomes such as the sea urchin, which has a so-called Hox9/10 gene, the name arising because of its loose association with the chordate Hox9 and Hox10 groups but its lack of clear resolution with either particular group. Such a lack of resolution of the orthology relationships amongst the deuterostome Posterior Hox genes is in stark contrast to the Posterior genes of protostomes, in which robust groupings of AbdB, Post1 and Post2 are formed even between phyla. This contrasting behaviour of the Posterior Hox genes in phylogenetic trees was given the name Deuterostome Posterior Flexibility.[44] It was hypothesised to be the result of higher rates of sequence evolution in the deuterostome Posterior Hox genes than in those of protostomes, which in turn leads to a lack of resolution of the deuterostome gene relationships when constructing trees. However since the original Deuterostome Posterior Flexibility hypothesis was proposed more gene sequences have been isolated. In particular some Posterior Hox genes have been cloned from a hemichordate, and a couple of these acorn worm sequences can clearly be identified as orthologues of some echinoderm counterparts.[50] This led to the suggestion that Posterior Flexibility is only a chordate phenomenon rather than a deuterostome-wide mode of evolution.[50] Several of the hemichordate Posterior Hox genes still do not resolve clearly with echinoderm counterparts however, and so one alternative scenario that has been proposed is that the Posterior Hox genes of chordates and Ambulacraria are the result of two independent sets of tandem duplications.[45] The patterns of the deuterostome Posterior Hox gene groupings in phylogenetic trees however do not show two independent sets of Posterior Hox genes, one in Ambulacraria and one in chordates. Consequently independent duplications do not seem to be a plausible sole explanation. Perhaps the deuterostome Posterior Hox genes have evolved via a mixture of the two phenomena, lineage-specific duplications and higher rates of evolution as outlined by the Deuterostome Posterior Flexibility hypothesis. Further deuterostome Posterior Hox sequences, accompanied by careful phylogenetic tree building, may help to resolve the issue. One intriguing possibility is that an extant basal deuterostome, *Xenoturbella*,[51] may be available to provide another perspective on deuterostome Posterior Hox gene evolution.

It is of course very difficult to confidently resolve such issues with a handful of gene fragments from a few distinct phyla dotted around the animal kingdom. In the not-too-distant future we should have greater sampling of a more extensive diversity of taxa, and entire Hox clusters cloned and sequenced. Such entire cluster sequences will greatly improve our understanding by providing positional information within a cluster, and more importantly providing us with the entire Hox gene complement for the relevant taxon. For example does the chaetognath MedPost gene exist alongside Posterior genes, specifically Post1 and Post2 or AbdB? Are the acoelomorph Hox genes clustered, and are there other genes that have been missed by screens so far (e.g., more Acoel Central genes and Hox3)? What is the basal Hox complement for the Ambulacraria? What Hox genes are present in *Xenoturbella*, and can this animal help us to determine what the basal deuterostome condition was with regards to Hox cluster composition?

So the Hox cluster is a veritable maelstrom of gene duplications and losses across the Bilateria, and there are many more examples than those outlined above. As we sample the Hox genes across a broader phylogenetic sample of taxa we will have a much clearer picture of the evolution of Hox cluster composition, and will also probably discover more Hox characters that can resolve debates about various issues in animal phylogenetics.

Molecular Mechanisms and Evolution of Hox Cluster Organization

The Importance of Temporal Colinearity

Colinearity in the Hox cluster can take different forms; spatial, temporal, or quantitative.[1] To a certain extent the three types of Colinearity are not always mechanistically independent. For example Temporal Colinearity may be a route to Spatial Colinearity in some circumstances.[52] However we can find instances of one form of Colinearity occurring in the absence of any others, e.g., the axial Spatial Colinearity of the *Drosophila* Hox gene expression without any obvious Temporal or Quantitative components.

Myself and others have hypothesised that it is Temporal Colinearity that is the key to understanding Hox cluster organization.[52-57] This hypothesis is mainly based upon the observation that Hox clusters that conform to our view of the ancestral condition, of an ordered cluster of genes with anterior expression beginning at one end followed by a gradual progression through to posterior expression at the other end of the intact, complete cluster, is found in animals and clusters exhibiting Temporal Colinearity. In those taxa which do not or cannot use a temporal component in the initiation of their Hox expression, then the Hox cluster tends to be broken, dispersed and rearranged. Spatial Colinearity does not require an intact cluster, as is evident from flies, nematodes and urochordates,[57] and so the mechanistic basis for Spatial Colinearity seems an unlikely means for understanding the maintenance and organization of the Hox cluster.

Broken, dispersed Hox clusters are found in taxa with rapid modes of development, which also often correlates with a relatively low number of cells in the embryo.[54] Such a mode of development is generally considered to be rather derived within each respective lineage, such as the insects or chordates, which ancestrally developed gradually by progressive elongation of the posterior end of the embryo.[58] A more recent example of another broken, derived Hox cluster is that of the Schistosome flatworm, *Schistosoma mansoni*.[59] Again this correlates with a derived mode of development and life-style within the lineage, as Schistosomes are extremely specialised parasites, with a highly specialised and derived life-cycle to match.

When the hypothesis that Temporal Colinearity is the main constraining force on Hox cluster organization was formulated[53,54] the echinoderm Hox cluster was apparently an exception to the rule, due to its intact, well-ordered nature but with a clear lack of Temporal Colinearity.[46,60] With the recent clarification of the organization of the *S.purpuratus* Hox cluster we now know that the urchin Hox cluster is highly derived after all,[45] which correlates with its extreme form of indirect development, with almost complete loss of embryonic structures at metamorphosis, and a highly derived adult form that cannot easily be compared to the morphology of other phyla. The echinoderm Hox genes are however still maintained as a cluster and not dispersed like the clusters of flies, nematodes, schistosomes and urochordates. Such cluster maintenance may be indicative of enhancer sharing amongst the echinoderm Hox genes, which is largely unaffected by reordering of the genes but does require them to remain in close proximity.

The clearest evidence for an importance of time in the control of Hox expression comes from work in mammals. Duboule has pointed out that colinearity is obeyed most rigorously at the time of initial expression.[61] Investigations of enhancers of Hox genes, using LacZ reporters, show that those that seemingly reproduce the complete expression pattern of the relevant Hox gene still have one aberration from the gene itself. The timing of their initiation is later than the endogenous gene.[1,62] Also mutant phenotypes are more severe in animals that have lost only a subset of the genes from a cluster, compared to animals that have lost an entire cluster; deletion of the entire HoxC cluster of mice does not produce pronounced homeotic mutants.[63] Crawford[55] proposes that the deletion of a subset of Hox genes alters the timing and progress of gene initiation through the Hox cluster, leading to more severe homeotic phenotypes than whole cluster deletion. In a sense the experiment has also been done naturally. Teleosts have undergone an extra genome duplication relative to the tetrapods, but only have seven Hox clusters rather than the expected eight, due to the loss of an entire HoxD or HoxC cluster.[64] That the mammalian Hox clusters are gradually unwound and de-repressed during the earliest

stages of development is now clear.[65,66] The trigger for this process, and how it links to the activating enhancers of the Hox genes will be an important revelation. There is a caveat to this scenario so far however. Translocation of an Anterior Hox gene to a location near HoxD13 in mice does not cause the Anterior Hox gene to now be expressed at the time of a Posterior gene. It is still activated early, at least in the mesoderm.[1,67] It would be intriguing to know how the chromatin organization changes during the activation of this modified Hox cluster.

Alternatively Bilaterian Hox Colinearity Mechanisms Are not Homologous

A flip-side to these considerations of the mechanistic basis for Hox cluster integrity is to question whether the cluster really is as constrained as we thought it was? Could clustering simply be an indication of evolutionary history by tandem duplications of genes, followed by subsequent different break-ups in divergent lineages? An immediate riposte would be, why have linkages been conserved for so long? Perhaps different constraints were added on in different lineages after the initial gene origins by tandem duplications, i.e., is Colinearity in protostomes mechanistically comparable to Colinearity in deuterostomes, or even can comparisons be made within deuterostomes themselves? Duboule and coworkers have shown that in vertebrates there are several mechanisms contributing to Hox Colinearity, depending on the context. Thus there is no single universal mechanism of Colinearity in vertebrate Hox clusters. But this diversity of mechanisms must have been imposed on vertebrate Hox clusters, evolving from an ancestral cluster that presumably already exhibited Colinearity, perhaps resembling the Colinearity seen in the amphioxus Hox cluster.[68] Can we determine what the mechanistic basis for this ancestral Colinearity was? Is it a mechanism that is still used in vertebrates, presumably in a context that was also present in the ancestor, e.g., body axis or CNS patterning, rather than a vertebrate innovation such as limb patterning, and is this mechanism present outside of the deuterostomes?

The alternative hypothetical scenario is thus that the genes originated by tandem duplication, they use shared enhancers due to these tandem duplications, and this enhancer-sharing reduces the opportunity for viable genomic rearrangements of the cluster so that it is conserved for longer than would be a cluster of genes without enhancer-sharing. This cluster maintenance perhaps only needs to be kept for a relatively short time (in geological terms), maybe between the late Vendian Ediacaran animals and the explosive divergence of the bilaterian lineages in the Cambrian. At this point different Colinearity mechanisms then evolve along some Hox clusters of different animal lineages, whilst in other lineages the cluster finally disperses as rare, viable genomic rearrangements (with the appropriate enhancers) slowly accumulate (Fig. 5). Sharing of enhancers between more than one Hox gene occurs in mice and flies, and enhancers are widespread and densely packed throughout the Hox clusters.[69-71] This would fit with the above scenario, whereby recombination within the cluster would be deleterious more often than not, and so slows cluster disintegration considerably. A test of this hypothesis requires comparison of the organization of Hox clusters in more taxa, to see how often Hox clusters have disintegrated, followed by the characterization of the regulatory mechanisms to find the degree of mechanistic conservation across phyla.

Furthermore we can form an estimate of the time that it might take for cluster break-up to occur when there is enhancer-sharing but no global Colinearity mechanism. Importantly this time is longer than the Ediacara-Cambrian explosion period. The flies seem to provide us with a group of organisms that do not have a global Colinearity mechanism operating across their Hox cluster, since the cluster is breaking up in the Drosophilids. The Hox clusters of several species of *Drosophila* have been sequenced, and breaks have occurred in different locations within the cluster.[72-75] These rearrangements can be linked to the estimates for divergence times amongst *Drosophila* lineages, and it can be seen that only three different cluster breaks have occurred along lineages that have been separated for 30-60 Million Years.[76] The presence of broken Hox clusters also extends more deeply into the insects,[77] and so potentially the release from the constraints on clustering is even more ancient than the origin of the Drosophilids, and our estimate of three breaks in three lineages of 30-60 million years is rather conservative.

If the fossil record is to be believed, and the Ediacaran faunas represent the early stages of animal evolution with the diversification of the bilaterian lineages not happening until the Cambrian, then a period of less than 20 Million Years covers the origin of the ArcheHox gene (sometime after the origin of animals), the expansion to the ProtoHox cluster, the duplication into the Hox and ParaHox clusters, and then finally the origin of the bilaterians and their subsequent explosive radiation. Consequently the time that elapsed between the origin of the Hox cluster and the divergence of the bilaterian lineages may well be only a few million years, and a shorter period of time than has elapsed since the divergence of the various *Drosophila* lineages, in which no global Colinearity mechanism exists and yet still only a few viable Hox cluster breaks have evolved. It may thus be perfectly plausible that the ancestral Hox cluster was conserved as a cluster, by enhancer-sharing for example, without the constraint of a global Colinearity mechanism until the Bilateria diverged, and then lineage-specific Hox Colinearity mechanisms evolved after the bilaterian divergence (Fig. 5ii).

It has been postulated that two of the best-studied model organisms with regards to Hox gene function (namely *Drosophila* and mice) control and use their Hox genes in fundamentally different ways—flies are 'qualitative' whilst mice tend to be 'quantitative'.[1] Perhaps we cannot see the mechanistic connections and commonalities because we are dealing with the two extremes of a continuum. Other taxa must be studied, with less specialised, derived modes of development than the fly, and less redundancy than the mouse (i.e., a single cluster instead of four). This may well help us to penetrate the evolutionary fog accumulated around mice and flies. Despite the extreme situation with flies and mice we do still have some mechanistic starting points from fly-mouse comparisons. Both use Polycomb/Trithorax group genes to regulate their Hox genes,[78] and both have boundary or insulator elements in their Hox clusters,[79,80] as well as microRNAs.[81] Do other taxa? Are these Hox-specific mechanisms, or tools used by other clusters of genes (Polycomb group complexes are widespread across the chromosomes,[78] and interactions of protein-coding genes with microRNAs are common)?[82] Are they integral to the (ancestral) mechanisms of Colinearity? There are many open questions, and we are at a very early stage in understanding Hox Colinearity.

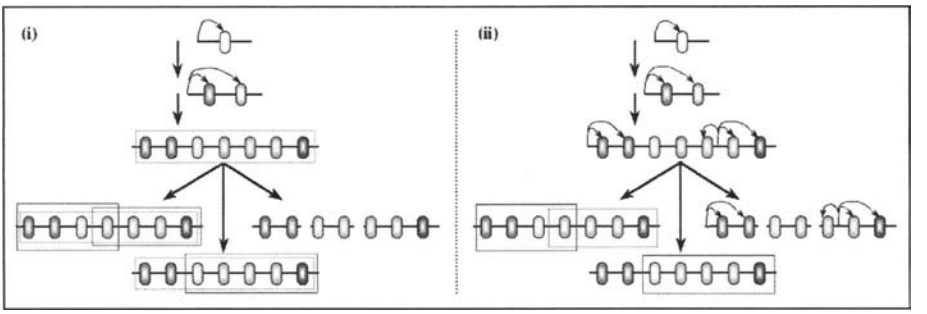

Figure 5. Is there a homologous mechanism of Colinearity amongst different bilaterian lineages? The Hox cluster evolved by tandem duplications from an ancestral ArcheHox gene (yellow). Evolution by tandem duplication could well have resulted in enhancer-sharing by multiple genes (curved arrows). In scenario (i) a Colinear mechanism (orange box) evolved before the divergence of the different bilaterian lineages. In the separate lineages further lineage-specific Colinear mechanisms evolved (blue, purple and brown boxes), and in some lineages the ancestral Colinear mechanism is lost and the cluster subsequently broken after the removal of this evolutionary constraint (discontinuous horizontal lines). In scenario (ii) the prevalence of enhancer-sharing in the ancestral Hox cluster results in viable breaks in the cluster being very unlikely. Consequently the cluster is maintained until after the divergence of the bilaterian lineages. Subsequently lineage-specific Colinearity mechanisms evolve in some lineages, whilst in others sufficient time eventually elapses for viable cluster breaks to evolve. A color version of this figure is available online at www.Eurekah.com.

Conclusion

From the earliest molecular days of the Hox cluster some of the fundamental elements of the evolution of the cluster were known, e.g., tandem duplication and Colinearity. But in the intervening years significant shifts have occurred: origin from a ProtoHox cluster, extensive cluster disintegration, distinct patterns of gene evolution across the cluster (such as Posterior versus Anterior), and between groups of animals, e.g., Ecdysozoa, Lophotrochozoa, Deuterostomia. Looking to the future many fundamental questions remain, such as the ancestral composition of the cluster (for animals as a whole, for basal bilaterians and eubilaterians, and for each of the major clades of Ecdysozoa, Lophotrochozoa and Deuterostomia), when did the ProtoHox to Hox/ParaHox transition occur, are there general cluster-wide mechanisms constraining the cluster across the animals, or is the conservation of clusters mechanistically different in separate lineages (if so was there an ancestral mechanism or does the cluster simply reflect the mode of gene evolution by tandem duplication)? Hox cluster sequencing from a greater diversity of taxa, combined with gene expression work in the light of the organization of the relevant clusters, and ultimately elucidation of gene regulation mechanisms in a diversity of taxa, will hopefully one day show us how and why the Hox cluster exists.

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