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Hox Genes: Master Regulators of the Animal Bodyplan

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1. Introduction

Typical vertebrates- like dogs and cats and fish - usually have their head-tail body axis parallel to the ground. The head is at the front end and the tail at the back. All limbs (legs or fins) are used for locomotion. In this configuration, we know the head- tail axis as the anteroposterior (main) axis. The upper side of the animal is called its dorsal side and the lower side its ventral side. In humans, the anteroposterior axis is held upright. Only the hind limbs are used for walking. Your front is your ventral side and your back your dorsal side. We use the terminology for a typical vertebrate in the sections that follow.

During embryonic development, a developing animal is built by a hierarchy of genes. These include effector genes, encoding building blocks of the embryo- like muscle actin and keratin. They also include developmental control genes, which control the expression or action of other genes. These can be genes encoding proteins controlling the genesis, secretion or transduction of intercellular signals or genes encoding proteins controlling transcription or translation or protein action. Such developmental control genes regulate each other and may be organised in very large hierarchies. Hox genes are developmental control genes.

2. Discovery and cloning of the *Hox* genes, their role and regulation in *Drosophila*

Hox genes were first discovered as homeotic genes in the fruitfly *Drosophila*. They are sometimes referred to as: homeotic selector genes or: *HOMC* genes. They are characterised by the fact that a gain or loss of function mutation in a typical *Hox* gene can result in conversion of one large or small part of the main body axis to another. These are clearly developmental control genes acting high up in the hierarchy. In the case of the *Hox* genes, the conversions take place between different parts of the anteroposterior axis. One famous example is: *Bithorax,* discovered by Nobel prize winner Ed Lewis, which makes a four winged fly in its loss of function format. *Drosophila* normally has only two wings, on the mid thorax. The posterior thorax has vestigial 'halteres' . *Bithorax* is a gene for posterior thorax which converts this to mid thorax by loss of function (Lewis, 1978, 1995). In another equally famous example, discovered by Walter Gehring, *Antennapedia*, a gene for mid thorax converts part of the fly's head to mid thorax and therefore antennae to legs by misregulated gain of function (Carrasco

et al., 1984) Vertebrate *Hox* genes similarly have drastic phenotypes but loss of function phenotypes are more difficult to visualise because each vertebrate *Hox* function is mediated by multiple *Hox* genes and these must all be knocked out. See Fig. 1.

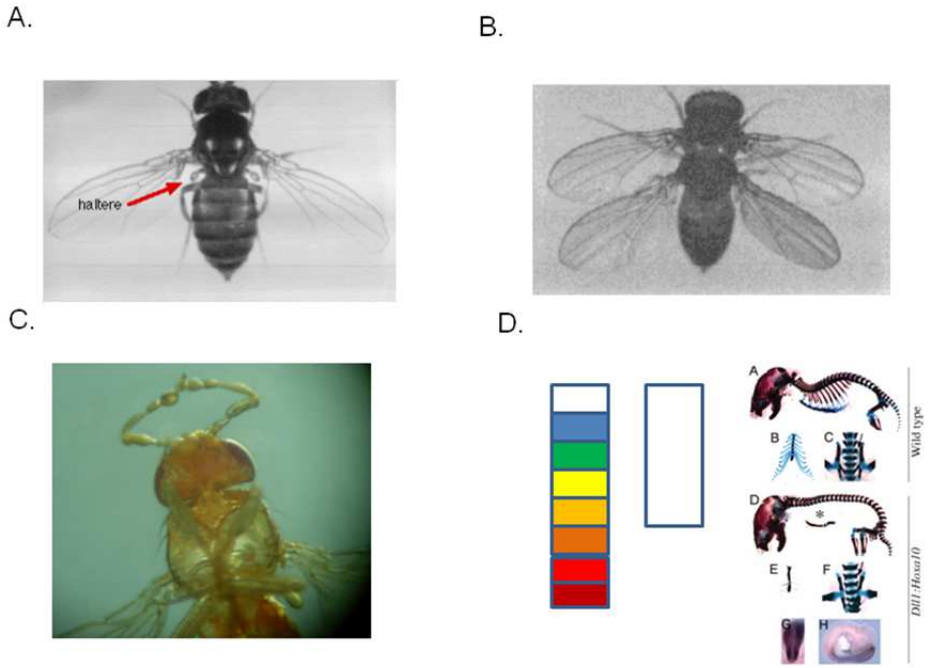

Fig. 1. **Hox gene phenotypes**
The function of Hox genes is indicated by gain and loss of function phenotypes. The figure shows this in Drosophila and vertebrates. A. A wild type Drosophila fly This has two wings on the anterior thorax and two halteres (red arrow) on the posterior thorax. B. A four winged fly, caused by a loss of function mutation in ultrabithorax, a gene for posterior thorax (Lewis, 1995). The halteres are transformed to wings. C Antennapedia mutation: anterior thoracic legs replace antennae on the head, due to a misregulated gain of function mutation for the gene Antennapedia (a gene for anterior thorax), leading to its expression in the head segments (Gehring, 1987). D. In vertebrates, mouse genetics has been bedevilled by the fact that there are 4 Hox clusters, with parallel functions. This once led to the erroneous idea that vertebrate Hox loss of function mutations have mild phenotypes. In fact, if you knock out all of the paralogues of a particular Hox paralogue group (pg), or ectopically express a Hox gene this can give a dramatic phenotype. Left: wild type Xenopus hindbrain. This has 8 segments (rhombomeres) 2-8 each express a different combination of Hox genes and so have different identities, indicated by the different colours. 1 (white) expresses no Hox genes. Its identity is determined by the gene Gbx2. Middle: hindbrain in Xenopus where Hox pg1 has been knocked down using morpholinos. The hindbrain is drastically anteriorised to the identity of r1. It is also shorter (redrawn from McNulty et al., 2005). Right: Skeletons of two mice. Above: wild type. Below, a mouse ectopically expressing HoxC10. The HoxC10 mouse is drastically different. For example, it lacks ribs (Carapuco et al., 2005). The thoracic vertebrae are posteriorised to abdominal identity. This is because Hox pg10 controls the transition from tthorax to abdomen, in the vertebral column.

These genes typically determine the identity of individual *Drosophila* body segments or groups of adjacent segments. In the early 80's strategies were developed for cloning developmental control genes. The first genes cloned were the *hox* genes *Bithorax-* by Hogness and his colleagues (Bender et al., 1983) (and *Antennapedia-* by the Gehring group (Carrasco et al., 1984). This was possible because these transcription factor genes contain a large highly conserved region- the homeobox- which encodes a 60 amino acid DNA binding domain and can be picked up by homology screening. It has, in fact emerged that *Hox* genes encode a subfamily of transcription factors and that the homeobox and another conserved region, the haxapeptide, are important in determining their specificity.

3. *Hox* clustering and colinearity: The key property

A key property of *Hox* genes is that they are often clustered in complexes. *Hox* complexes are among the most remarkable regions of the genome.. A *Hox* complex usually consists of up to 9-13 closely related Hox genes arranged in tandem . These genes specify patterning along body axes in all bilateria (Gehring *et al*., 2009, Duboule, 2007). Invertebrates have a single *Hox* complex, or dispersed *Hox* genes, but tetrapod vertebrates typically possess four similar *Hox* complexes (*HoxA–D*), located on different chromosomes (Duboule, 2007). (Fig. 2) The *Hox* complexes also contain 5 micro RNA (*miRNA*) genes intercalated at homologous positions (Pearson et al., 2005; Yekta *et al*., 2004, 2008; Woltering and Durston, 2007; Ronshaugen et al., 2005).

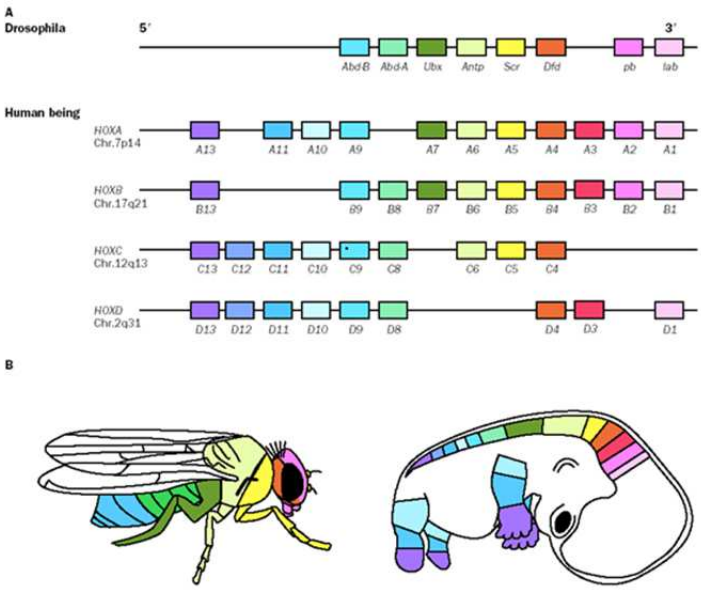

Fig. 2. **Hox Spatial and Functional Collinearity**
The four human and one Drosophila Hox complexes are homologues. The colour coding in Panels A and B shows the correspondence between the genomic order of Hox genes in the Hox complexes (A) and their spatial sequence of expression and action zones along the main body axis in Drosophila and human (B). From Goodman, 2003.

The 3′ to 5′ order of *Hox* genes along a chromosome corresponds to the order in which they act along body axes; this collinear property links clustering to function, emphasizing that *Hox* complexes are functional units or meta genes No one *Hox* gene can pattern an axis but a whole *Hox* cluster can. (Mainguy *et al*., 2007, Duboule 2007). Hox collinearity is crucial in embryogenesis and includes 3 important and interrelated properties: functional colinearity describes the spatial order in which *Hox* genes act along a body axis; spatial colinearity refers to the spatial order in which the *Hox* genes are expressed, and temporal colinearity is the time sequence in which they are expressed (**Text Box 1**). The organization of *Hox* complexes is highly conserved, and *Hox* and *mir* genes not only have remained clustered through bilaterian evolution but are also in close proximity to each other despite their very complex and dynamic expression patterns. Individual *Hox* genes are also very highly conserved in Evolution.

Text box 1: ***Collinearity***

Collinearity describes the sequential expression of a genomic cluster of Hox genes along an embryonic axis and associated properties.

There are three important forms of collinearity: Spatial collinearity is the sequential 3′ to 5′ expression of Hox genes along a body axis. This occurs from anterior to posterior along the main body axis and also in other axes, for example from proximal to distal in developing limbs. Spatial colinearity can be associated with time dependence. The most 3′ gene is expressed first and more 5′ genes are expressed sequentially later. This is defined as temporal collinearity and, in early vertebrate development, spatial collinearity is generated from pre-existing temporal collinearity by time space translation. The gastrula's organiser interacts with Hox expressing non organiser mesoderm to translate a temporal sequence of Hox codes to a spatially collinear pattern. We also define a third property, functional collinearity- – which is the capacity of Hox genes to collinearly define region-specific structures along an axis.

Hox collinearity and the organisation of the *Hox* complexes are phenomena that have long fascinated developmental, molecular and evolutionary biologists. These phenomena represent an important example of genomic regulation. Understanding the structure and function of *Hox* genes is crucially important, because they are implicated in a growing number of diseases, including important cancers (Grier *et al*., 2005). See also below.

Research and thinking on *Hox* collinearity has concentrated on three aspects. First, there is the question of how collinearity evolved, which is clearly one of the keys to understanding this phenomenon. Second, there are three mechanistic ideas. The first is that Hox spatial colinearity is secondary and due to an upstream hierarchy of spatially ordered genes. Hox collinearity is thus not utilised. The second is that collinearity is based on transcriptional regulation, and specifically that it is limited by the progressive 3′ to 5′ opening of *Hox* cluster chromatin and/or mediated by global control regions. The third model is that collinearity depends on interactions among the *Hox* genes themselves. These interactions include 'posterior prevalence', - a negative interaction among Hox proteins that clearly relates to functional collinearity in *Drosophila* (and possibly also to spatial and temporal collinearity; see **Text Box 1**).

In this article, we review the basis of *Hox* evolution and of the three longstanding mechanistic hypotheses to explain *Hox* gene collinearity. But we also propose a new

explanation. Based on evidence from *Amphibian* and other vertebrate embryos, we reason that synchronised temporally collinear expression of the *Hox* complexes in early vertebrate embryos involves *trans*-acting factors and intercellular interactions. We review data implicating activating as well as repressive interactions among the *Hox* genes themselves, and timed signals from the somitogensis clock. This model provides a mechanistic link between the different aspects of collinearity. A review of potential collinearity mechanisms is now opportune because new data that have never been reviewed in the literature are now available and because the existing, entrenched models are limiting in the sense that they direct research in the same direction- that of chromatin opening and transcriptional control- and that they do not explain all of the facts (below). This has spurred us to interpret the data in a different light. The field gains a new perspective from this new synthesis of the data.

4. The evolution of *Hox* genes in different taxa, including vertebrates

Hox genes are available in all metazoans that have been studied. In all bilateria where there is information, they are concerned with patterning the main body axis. Invertebrates have one *Hox* gene complex: vertebrates have 4 or 8. The 4 *Hox* gene complexes typically present in most tetrapod vertebrates arose through 2 rounds of genome duplication during evolution. *Xenopus laevis* and teleost fishes have 8 *Hox* complexes because of 3 genome duplications. Even the individual *Hox* genes are strongly conserved in evolution throughout the animal kingdom (Carrasco et al., 1984; Gehring *et al*. 2009; Duboule 2007, DeRobertis, 2008) and are recognisable by having distinct conserved sequences. The *Hox* genes corresponding to the same position in each of the different vertebrate *Hox* complexes are conserved. They have very similar homeoboxes and hexapeptides and are called a paralogue group. *Hox* genes in invertebrates may be clustered and show collinearity or they may be scattered in the genome to various extents. Different extents of fragmentation, from atomised to fully clustered have been identified. The clustered format is thought to be ancestral.

*Text box 2: A**n evolutionary explanation of collinearity***

It has been proposed that colinearity evolved by repeated tandem duplication of an ancestral ur-Hox gene and sequential evolutionary modifications of the duplicates, leading to generation of an organised gene array from an evolutionary ground state . This idea can conceivably explain how a genomic sequence could relate to a spatial or temporal sequence of gene expression. Please note that, if this is the explanation of collinearity, it is the explanation and obviates the need for an explicit collinearity mechanism involving interactions between or clustering of the Hox genes. The upstream mechanism for Hox expression will be whatever it evolved to be in order to regulate the spatially collinear localised expression of the individual Hox genes- as with the segmentation gene hierarchy in Drosophila. The spatially collinear axial expression pattern of the Drosophila Hox genes is thus secondary and determined by the spatially ordered expression patterns of the gap genes. Nonetheless, we think that explicit collinearity mechanisms evolved- see main text.

Evolution of *Hox* collinearity is particularly important because it can potentially offer an explanation of how collinear properties connect to *Hox* complex structure. The only other

potential explanation for this comes from the chromatin opening model (below). It should be noted that whereas clustered *Hox* genes in organisms having *Hox* clusters show the normal spatially collinear sequence of *Hox* gene expression, so do *Hox* genes in fragmented clusters, from the split cluster seen in *Drosophila* to atomised *Hox* genes in organisms having no clustering- like *Oikopleura* (Duboule 2007, Seo *et.al*. 2004). These show 'trans collinearity' where the spatial sequence of expression of the Hox genes corresponds with their 3' to 5' genomic sequence in the ancestral cluster. It is thus clear that the spatial ordering of *Hox* gene expression does not rely soley on clustering. Presumably, *Hox* spatial collinearity evolved in an ancestral organism with clustered *Hox* genes and and persisted after cluster disintegration during evolution. This already demonstrates that *Hox* collinearity properties can persist in the absence *Hox* clustering and therefore of progressive chromatin opening. It has been proposed that a *Hox* complex, whose function is to pattern an axis, acts as a meta gene or functional unit, where no one *Hox* gene can execute the whole function but the whole complex does (Mainguy *et al*., 2007, Duboule, 2007). It has also been proposed that spatial collinearity has been a selective pressure that drives *Hox* clustering rather than vice versa. (Duboule 2007).

It has been proposed that *Hox* colinearity evolved by repeated tandem duplication of an ancestral ur-*Hox* gene and stepwise sequential evolutionary modifications of the duplicates, leading to generation of an organised gene array from an evolutionary ground state (Lewis 1978 1995; Gehring *et al.*,2009) (**Text Box 2, Fig.3A**). Lewis proposed that the modifications arose by unequal recombination between adjacent *Hox* genes. This idea can conceivably explain how a genomic sequence could generate ordered properties like spatial or temporal sequences of gene expression. Please note that, if this is the explanation of collinearity, it obviates any need for a dedicated collinearity mechanism. The upstream mechanism for *Hox* expression will be whatever it evolved to be in order to regulate the correctly localised expression of the individual *Hox* genes. This is the case with the gap-segmentation gene hierarchy in *Drosophila,* (see below). Nonetheless, we think that dedicated collinearity mechanisms evolved. Lewis showed that 5' posterior drosophila *Hox* genes are epistatic to the *Hox* gene *Antennapedia.* If they are ectopically expressed in the normal *Antennapedia* domain, the most posterior *Hox* gene expressed dominates. If the most posterior *Hox* gene is deleted, the phenotype obtained is that of the most posterior *Hox* gene still expressed. And so on. This interaction was called posterior prevalence (below) and was thought by Lewis to reflect the fact that *Antennapedia* represents an ancestral ground state, while posterior *Hox* genes are derived from the ground state by tandem duplication and stepwise sequential modification (as above). It has been reported relatively recently by Gehring *et al.,* (2009) that the anterior *Drosophila Hox* genes have also evolved from the *Antennapedia* ancestral ground state and that these have developed anterior prevalence.

5. The mechanism of *Hox* collinearity

There are various ideas about this (Fig. 3).

1. In the section above, we have described the idea that collinear *Hox* complexes arose by tandem duplication and sequential modification of an ancestral *ur-Hox* gene. In this case, no special mechanism is required to generate spatial collinearity. The

upstream mechanism for *Hox* expression will be whatever it evolved to be in order to regulate the correctly localised expression of the individual *Hox* genes. This is the case with the gap-segmentation gene hierarchy in *Drosophila* (Nuesslein- Volhard, 1995), where the spatial ordering of the *Hox* genes is secondary. The spatially expressed gap genes are the primary determinats of the spatially ordered *Hox* gene expression pattern (Kehle et al., 1998, Mito et al., 2006) . Later on, other genes, including the *Hox* genes themselves, the cofactor *teashirt*, polycomb group genes and segmentation genes play a role (Gebelein and Mann, 2007, Rusch and Kaufman, 2000 Mito et al., 2006) (Fig 3A, 3B).

2. The idea has developed in the mouse that temporal collinearity is due to progressive opening of *Hox* complex chromatin, from 3′ to 5′ (Fig 3C). (. This idea has become rather popular. There is some evidence for this (Soshnikova and Duboule 2009, Cambeyron and Bickmore, 2004, Van der Hoeven et al, 1996, Kmita et al., 2000) but the idea has limited application. It can not apply in animals with dispersed *Hox* genes that behave colinearly. It is not even the whole story in vertebrates, presumably including the mouse. Synchronised temporal colinearity between the different *Hox* complexes during gastrulation (Wacker et al., 2004, Durston et al., 2010, 2011) indicates the importance of trans acting factors and intercellular signals for temporal collinearity.
3. There is evidence that interactions between *Hox* genes are important. These can obviously not account for the relation between *Hox* complex structure and collinear properties but they are part of the story. Working in *D. melanogaster*, E. B. Lewis showed that loss–of-function mutations in posterior *Hox* genes drive the segmental phenotype towards that of the more anterior thoracic segment T2, which is determined by the *Hox* gene *Antennapedia* (Lewis, 1978, 1995). Struhl used *esc-Drosophila* embryos, which show constitutive activation of gene expression, in combination with *Hox* loss of function mutations to elucidate the functional hierarchy of *Drosophila Hox* genes (Struhl, 1983). All *Drosophila* segments were transformed to the phenotype of the most posterior functional *Hox* gene expressed. Posterior prevalence in *Drosophila* has been thought to underly functional collinearity only, not spatial collinearity. Experimentally derived ubiquitous expression of *Hox* genes under promoters that are known to be transcriptionally irrepressible leads to transformations only in regions anterior to the functional domain of the gene. For example, the thoracic *Antennapedia*, when ubiquitously expressed, suppresses Hox genes of the head, resulting in posterior transformation of head segments towards a thoracic identity while not affecting the abdomen — here, the effect of *Antp* is phenotypically suppressed by *bithorax*-complex genes such as *Ubx* (Gonzalez-Reyes *et al.,* 1990 Gibson and Gehring, 1988). However, posterior prevalence occurs not only postranslationally (Plaza et al., 2008) but also at the levels of transcription (Beachy et al., 1988, Hafen et al., 1984, Appel and Sakonju, 1993, Struhl and White, 1985) and posttranscriptional regulation of mRNA abundance (Yekta et al., 2004, 2008, Woltering and Durston, 2007, Ronshaugen et al.,2005) (Text box 3). It can thus also potentially regulate the mRNA expression of *Hox* genes. Namely, spatial and temporal collinearity. *Hox* interactions also occur during vertebrate gastrulation. These include posterior prevalence (Hooiveld et al, 1999, Woltering and Durston, 2007) but also 3′ to 5′ activation of *Hox* gene expression (McNulty et al, 2006, Hooiveld et al., 1999) See Fig. 3E, Fig. 5.

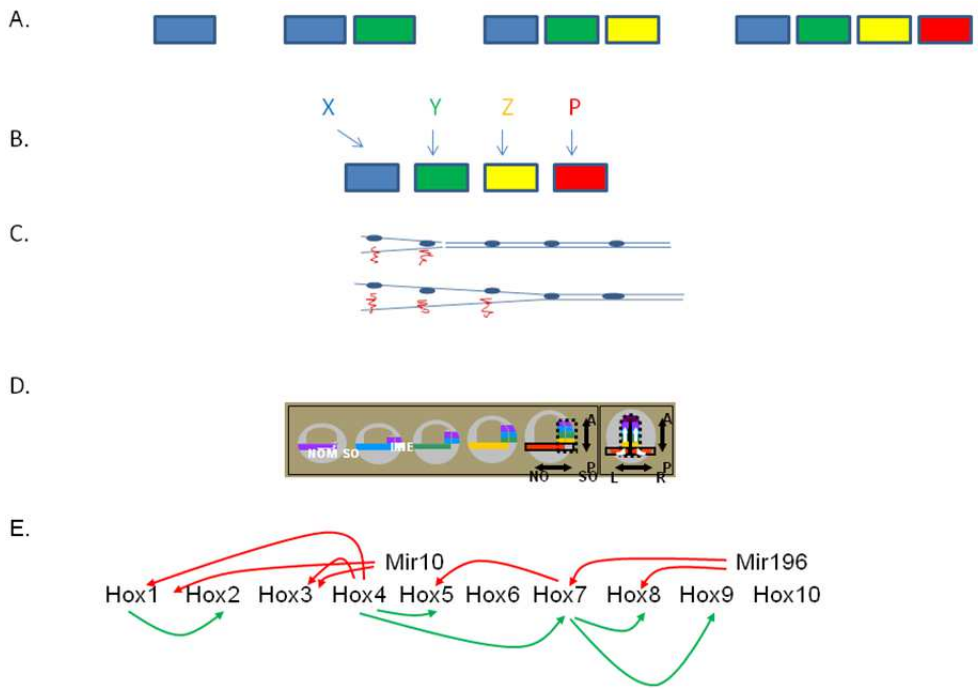

Fig. 3. **Some facts and ideas about Hox colinearity**

A. Tandem duplication and sequential modification. Clustered Hox genes are thought to have evolved by tandem duplication of an ancestral Ur-Hox gene. The duplicates are then thought to have been progressively modified, so they become more and more different from each other. The figure shows tandem duplication and progressive modification towards the right. The ur- Hox gene (left, blue) duplicates and the right hand daughter is modified (green). The green Hox gene duplicates again and its right hand daughter is modified (yellow). The yellow Hox gene duplicates again and its right hand daughter is modified (red). This type of mechanism can give collinear properties.

B. The associated upstream mechanism needed to generate spatial collinearity. If such a Hox cluster is to generate spatial colinearity without an explicit colinearity mechanism, an individual input is needed to turn on each Hox gene to ensure it is expressed at exactly the right axial position. The inputs concerned are going to need an axial pattern themselves. This kind of mechanism is used in Drosophila, where the gap genes provide the inputs. Gap genes specify the primary axial positions where the Hox genes are expressed and segmentation genes, the Hox genes themselves, polycomb group genes and cofactors like teashirt refine this information, restricting Hox expression by specific segment boundaries. In this situation, the Hox genes thus do not provide the primary axial patterning information. They are secondary. It is likely that this kind of mechanism is general in invertebrates, which probably have no temporal colinearity or colinearity mechanism and have had to evolve an ad hoc mechanism to generate spatial collinearity. Something like this may also occur in the vertebrate hindbrain, where the gastrula's colinearity mechanism is presumably the primary patterning mechanism and hindbrain genes confirm or alter the patterning information.

C. Progressive chromatin opening: the basic idea. This is an idea proposed by Duboule and colleagues to account for vertebrate temporal collinearity. The Hox complex chromatin opens from 3' to 5'. This opening progressively permits Hox gene transcription, from 3' to 5'.

D. Time- space translation. Vertebrates show early Hox collinearity. There is a temporally collinear sequence of Hox gene expression in the gastrula. This is used to generate a spatially collinear axial sequence of Hox gene expression. For details, see Fig. 4.
E. Hox interactions.What regulates vertebrate temporal collinearity? Not just chromatin opening, as proposed by Duboule. The different vertebrate Hox clusters are expressed with synchronous temporal collinearity. What may be involved are interactions between different Hox genes. The figure shows some interactions between Hox genes in the vertebrate gastrula.

*Text box 3: T**he Level Of Action***

All effects above on activation or repression of Hox genes during gastrulation result in more or less Hox mRNA.but not all act on transcription. Recent evidence shows that Hox complex mRNA availability is strongly regulated posttranscriptionally, involving such phenomena as polycistronic transcripts, sense/ antisense transcript interactions and alternative splicing. At least one early vertebrate Hox interaction; downregulation of more 3' Hox mRNA's by Hoxb4 is micro RNA mediated (posttranscriptional). We note that the important parameter for colinearity is the sum total of the (activating and repressing) inputs on each Hox gene (there may be many). We think it very significant that posterior prevalence (pp) acts at 3 different levels. If a Hox gene is activated transcriptionally, its mRNA can still be destabilised by pp miR action. If the Hox protein is made, it can still be inactivated by pp protein-protein interactions. We think that pp is the most important Hox-Hox colinearity interaction and that it needs to be dominant, to ensure the 3' to 5' directionality of colinearity

6. *Hox* function in vertebrates

Hox genes have several different roles in development

Vertebrates are unique in being the only type of metazoan animals in which the ancestral *Hox* cluster has been duplicated due to genome duplications. In most tetrapod vertebrates, there are four *Hox* clusters, on different chromosomes, presumably due to 2 genome duplications. Teleost fish have 8 clusters, due to 3 genome duplications.

6.1 *Hox* genes in the developing CNS and hindbrain

There is much evidence that *Hox* genes are important in early anteroposterior patterning of the vertebrate central nervous system. There is an approximately spatially collinear sequence of *Hox* expression in the early neural plate and neural tube. Anterior boundaries for expression of different *Hox* genes distinguish between different parts of the developing CNS- for example, some boundaries distinguish between the different segments in the hindbrain. Much work has been done to characterise the regulatory gene networks that regulate *Hox* expression and *Hox* function in the developing CNS, particularly those that pattern the developing hindbrain: a segmented structure. These networks do not appear to contain any mechanism that mediates collinearity, which is presumably set up earlier in the mesoderm and transferred to the developing CNS. (see below). The hindbrain regulators seem to maintain this early pattern or alter it. They have an ad hoc nature, as do the upstream regulators in *Drosophila*. They do not necessarily maintain spatial collinearity. For example, the primary *Hoxb1* expression domain is at a non collinear position. This work has

been reviewed extensively in recent review articles (Wright, 1993, Krumlauf, 1994, Tumpel et al., 2009, Schneider-Manoury et al., 1998) and will not be discussed further here. The role of *Hox* genes in patterning the developing vertebrate CNS is limited to the hindbrain and spinal cord. The fore- and mid- brain are patterned by other regulators, including the *Otx* and *Emx* gene families (Cecchi et al., 2000) . The patterning of the anterior CNS by these gene families is actually conserved in *Drosophila*, but the anterior CNS region where they act here is very small, compared with the vertebrate forebrain.

6.2 *Hox* genes in axial mesoderm

Besides specifying A-P levels early on, in the developing central nervous system, *Hox* genes specify A-P levels in mesoderm. We are talking here about the axial and paraxial mesoderm. *Hox* genes are expressed in and specify A-P levels in, the presomitic and somitic mesoderm and the lateral plate mesoderm. *Hox* genes are not expressed in and do not specify A-P levels in the early notochord, which is derived from the *Hox*-negative organiser mesoderm in the gastrula. *Hox* patterning of axial mesoderm is covered by excellent recent reviews (Carapuco et al.,2005, Burke et al., 1995). We will not discuss it further here, except for one aspect (below).

The expression of *Hox* genes in the presomitic and somitic mesoderm is interesting because it correlates with the process of somitogenesis, the primary process of segmentation in the early vertebrate embryo, which occurs in this mesoderm. Vertebrate somitogenesis (segmentation of axial mesoderm) works via a mechanism where an oscillating system of gene expression generates a spatial pattern by time–space translation, just as in genesis of the vertebrate axial *Hox* pattern (see below and text box 1). The temporal oscillation in gene expression (somitogenesis clock) generates spatially periodic segments in the axial mesoderm: the somites (Palmeirim *et al*., 1997). This is closely linked to collinear *Hox* expression. *Hox* spatial expression boundaries coincide with somite/segment boundaries and several vertebrate somitogenesis genes are known to regulate *Hox* expression (Peres *et al.,* 2006; Dubrulle *et al*., 2001, Dubrulle and Pourquie, 2004, Zakany *et al*., 2001).

6.3 *Hox* genes in gastrulation

Hox genes are expressed earlier in development than in the developing central nervous system and axial mesoderm. This is interesting because the *Hox* genes set up the primary axial pattern during these early stages. The *Hox* genes are already expressed during gastrulation. For example, in the non-organiser mesoderm (NOM) of the *Xenopus laevis* gastrula, where *Hox* genes are first expressed in the embryo and are expressed with temporal colinearity (Fig.4a). This mesoderm manifests a sharply timed temporally collinear sequence of *Hox* gene expression that is translated in time and space by interactions with the Spemann organiser (SO) to to generate a spatially collinear pattern of *Hox* gene expression along the main body axis of the organism (Wacker et al., 2004a; Durston et al., 2010, 2011) The mechanism for this is shown in Figure 4b. .In short, the temporal sequence of *Hox* gene expression in the mesoderm is sequentially frozen, from anterior to posterior and is transferred to the developing neural plate, which overlies the internalising mesoderm, in the gastrula. (Text Box 1, Fig. 4a,b).

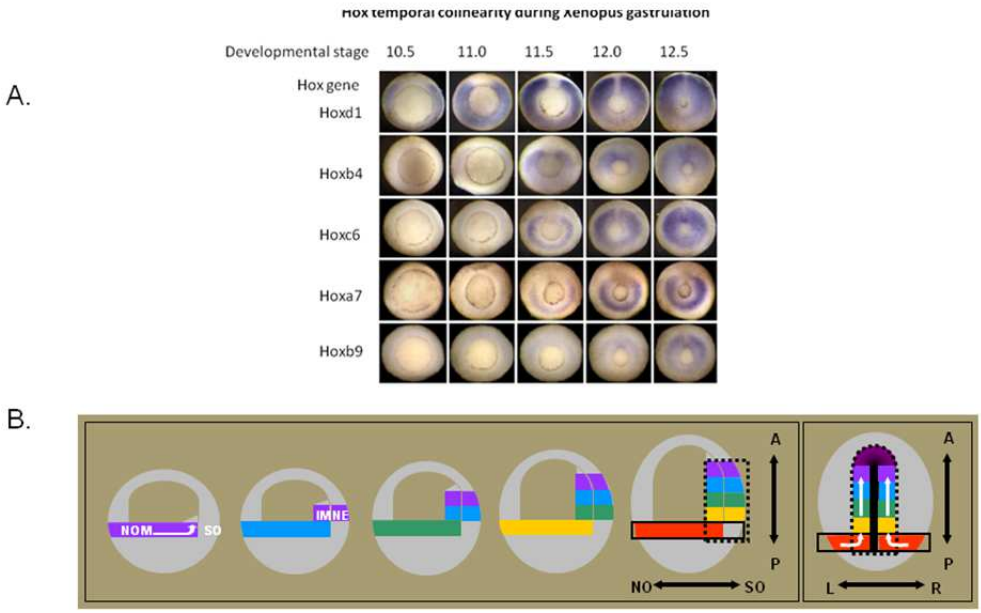

Fig. 4. **Temporal Collinearity AndTime space translation.**

A. Temporal Collinearity In the Xenopus Gastrula

The figure shows Hox expression patterns at sequential stages during gastrulation in Xenopus. From Wacker et al., 2004. The embryos are seen from underneath, where a ring (the blastopore) shows the position where mesoderm tissue invaginates during gastrulation. This ring gets smaller as gastrulation proceeds and the upper tissues in the embryo spread out and cover the lower part of the embryo (epiboly).The expression of several different Hox genes, seen as blue colour by in situ hybridisation, is in each case initially in the gastrula mesoderm in the zone above (outside) the ring. Hox expression is thus seen as a blue ring, and since it is initially only in part of the mesoderm (non organiser nesoderm), the ring is initially broken. This ring of Hox expression gets smaller as the blatopore ring gets smaller and mesoderm invaginates into the embryo.The figure shows expression of a sequence of Hox genes with different paralogue numbers, from 1 to 9. It will be seen that the Hox gene with the lowest paralogue number starts expression first and later numbers start sequentially later. It will also be seen that the Hox genes in this time sequence include members of all of the 4 primary vertebrate paralogue groups (a,b,c,d).

B. Time-space translation

Timed interactions between the Hox expressing non-organiser mesoderm and the Spemann organiser generate positional information during vertebrate gastrulation (Wacker et al., 2004). The drawings show simplified 2-dimensional representations of Xenopus gastrulae. The first 5 drawings show parasagittal (ventral to dorsal) two dimensional representations of gastrula profiles, starting at the beginning of gastrulation and then at sequential stages till the end. The last (6th.) drawing shows the end of gastrulation, from the dorsal side (profile at the level of the dorsal axial mesoderm). Hox expressing tissue (NOM (NO and I) and, late in gastrulation neurectoderm (N)) is represented by different colours, each of which represents a different hox code. Initially, the coloured bar represents

the broken ring of NOM in the wall of the embryo. The later internal coloured blocks at the dorsal side of the embryo represent the involuted NOM mesoderm. The coloured blocks next to them in the wall of the embryo represent the overlying neurectoderm, which also comes to express hox genes. Hox expression is copied from the gastrula mesoderm to the neurectoderm. The SO is shown only in the last drawing, as the heavy median black line. By this stage, it has become the notochord and a head mesodermal portion. The first 5 drawings represent paraxial profiles, where the organiser is not available. The black dotted line in the last drawing depicts the sphere of influence of the SO. N: neurectoderm, NO: non-organiser mesoderm; S,: Spemann organiser; A: Anterior; P: Posterior; L: Left; R: Right. N nonorganiser; S Spemann organiser. The white arrows reflect directions of cell movement flow. To dorsal, anterior and internal(drawings 1 and 6). -There is a collinear time sequence of hox expression in non involuted non-organiser mesoderm (NOM) in the gastrula (depicted by the spectral sequence of colours). -During gastrulation involution movements continuously bring cells from the NOM into the inside of the embryo See stack of blocks of different colours, reflecting a history of the collinear hox mesodermal time sequence, in the internal involuted mesoderm. -Stable (ectodermal) Hox expression is induced by a combination of signals from the SO and the Hox expressing NOM. See corresponding blocks of sequential spectral colours in the gastrula's mesoderm and outer layer, reflecting a vertical transfer of the Hox codes from involuted mesoderm to overlying neurectoderm. A "Hox stripe" as part of the anterior–posterior Hox pattern is thus formed at the dorsal side.

A striking feature of the *Xenopus* gastrula mesoderm's temporally collinear *Hox* expression sequence is that expression of *Hox* genes from different *Hox* complexes occurs in the same perfectly temporally collinear sequence (Fig. 4A). The temporal collinearity of the different *Hox* complexes is therefore synchronised (Wacker et al., 2004a; Durston et al., 2010, 2011) . The different *Hox* paralogues (ie the different copies of each different Hox gene type, produced by the vertebrate genome duplications) in the different complexes are on different chromosomes, ruling out that *Hox* colinearity simply reflects cis-localised progressive opening of *Hox* complex chromatin for transcription. Trans acting signals are clearly needed to synchronise the different *Hox* complexes and, since we are dealing with a cell mass rather than a single cell, intercellular signals are also required. We note that these *trans*-acting factors and intercellular signals must be very sharply timed to enable synchronisation of the different *Hox* complexes and are probably timed to trigger expression of different *Hox* genes at different times. This conclusion was not a complete surprise. It is known that trans acting factors must mediate collinearity in organisms with dispersed Hox genes. This is, however, the first evidence that vertebrate temporal collinearity is also mediated by trans acting factors.

The involvement of trans acting factors and intercellular signals has been investigated and three sectors of the regulatory gene hierarchy have become interesting.

1. There is evidence that the *Hox* genes themselves are involved, via *Hox-Hox* interactions including posterior prevalence and via interactions involving micro RNA's. These interactions drive initiation of *Ho*x complex expression as well as progression of temporally collinear expression through the *Hox* complexes (Hooiveld et al., 1999, Woltering and Durston, 2008, McNulty et al., 2005) (Fig. 5A). There is much evidence that *Ho*x genes in vertebrates and *Drosophila* show activating as well as repressive interactions, including posterior prevalence McNulty *et al.,* 2006; Hooiveld *et al*., 1999;

Woltering and Durston, 2008; Le Pabic *et a*l., 2010; Lobe. 1995, Maconochie *et al.,* 1997; Gould *et al.,* 1997; Bergson and McGinnis,1990 ; Miller *et al.,* 2001, Wellik and Capecchi, 2003) and that they drive conventional intercellular signalling pathways(eg. Graba *et al.* 1995, Bruhl 2004, Manak *et a*l1994, Michaut *et al*., 2011, Morsi el Kadi *et al.,* 2002, Pearson *et al*., 2005) as well as acting as signalling molecules themselves (Bloch-Gallego *et al*., 1993, Chatelin *et al*., 1996).

2. There is evidence that the signalling factor *Wnt 8* acts as a signal to initiate synchronous expression of the different Hox complexes. (In der Rieden et al., 2010)
3. There is evidence that the somitogenesis clock is involved (Fig. 5B). Vertebrate somitogenesis (segmentation of axial mesoderm) works via a mechanism where an oscillating system of gene expression generates a spatial pattern by time–space translation, just as in genesis of the vertebrate axial *Hox* pattern (see above and text box 1). This dynamic process is known to start during gastrulation in chicken and *Xenopus* (Peres *et al,* 2006; Jouve *et al*., 2002) and it drives activation of Hox gene expression. *Xdelta2* is a *Xenopus* oscillating somitogenesis gene (Jen *et al*., 1997, 1999). It is already expressed during gastrulation and then generates presomitic stripes so its expression is already oscillatory. It regulates expression of *Ho*x genes during gastrulation (Peres *et a*l., 2006). This gene could help to drive synchronised temporally collinear expression of the *Hox* complexes. It could do so either by regulating only initiation of expression of *Hox* complexes (via *labial Hox* genes) or by driving initiation and 3′ to 5′ progression, (repeatedly inducing expression of different *Hox* genes). We note that *XDelta2* drives expression of at least 3 different *Hox* paralog groups including *labia*l). If *delt*a drives progression as well as initiation, a repeated periodic pulsatile signal is required. The idea that the somitogenesis clock drives *Hox* temporal collinearity is very attractive because both of these timers are known to operate already in the gastrula and because of the evidence linking *Hox* patterning and segmentation (above). Such a signalling pathway might act separately from the *Hox* genes or be downstream of them. *XDelta 2* is indeed downstream of Hox genes as well as upstream. There is a positive feedback loop (McNulty *et al*., 2006, Peres *et al*., 2006). *XDelta 2* may thus mediate *Hox* induced signalling. These findings indicate that the axial segmentation mechanism may help to drive Hox expression in vertebrates, just as in *Drosophila.*

The *X. laevis* example was chosen because the data are most complete for this system; however, the conclusions are strongly supported by many findings in other vertebrates (zebrafish, chicken and mouse) (Gaunt and Strachan, 1996, Alexandre *et al.* 1996, Deschamps *et al*., 1999). This example illustrates that *Hox* colinearity cannot depend solely on the collinear opening of chromatin. Because the *Hox* complexes are synchronised, *trans*-actingfactors and intercellular signals must be involved — *trans*-acting factors would be necessary for coordinating the sequential 3′ to 5′ activation of *Hox* genes in and between *Hox* clusters, and intercellular signals would enable the coordinated activation of *Hox* gene expression between cells in a tissue. An alternative explanation is that only the most 3′ *Hox* genes (*Hox1*) transactivate, and the remaining timing is provided by synchronised opening of the *Hox c*omplexes. The different structures of the 4 primary vertebrate *Hox* complexes (with different *Hox* paralogues missing from each) would, however, make it difficult for progressive opening of different *Hox* complexes to stay synchronous. Since the gastrula mesoderm is a cell mass, not a single cell, trans-activation needs to be accompanied by intercellular signalling.

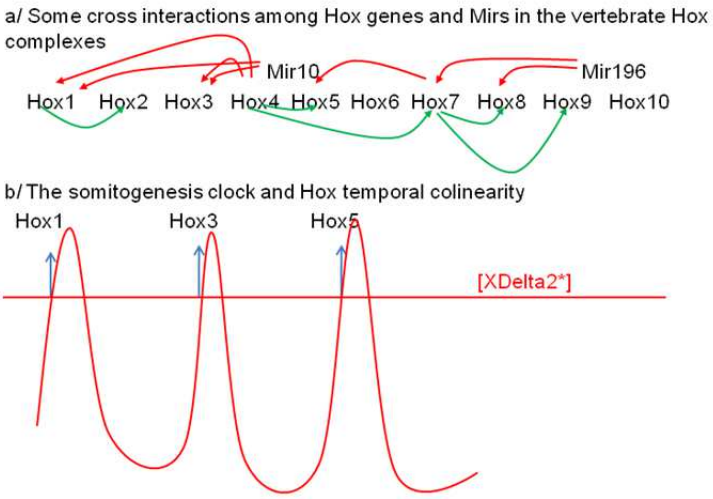

Fig. 5. **Regulators of vertebrate Hox temporal colinearity: Hox-Hox interactions and Somitogenesis oscillations**
A. some cross interactions between Hox genes and Mirs in the vertebrate Hox complexes during vertebrate gastrulation. Red: repression. Green: activation
B. The somitogenesis clock and Hox temporal collinearity. We show an oscillating concentration of XDelta2. Sequential peaks of XDelta2 activate expression of different Hox genes. [XDelta2]; The threshold concentration of XDelta2 at which Hox expression is activated.*

6.4 *Hox* genes in later development and in. limbs, hairs, haematopoiesis, the pancreas, etc

Vertebrate *Hox* genes have many other functions than specifying levels in the main body axis, in the central nervous system and in axial mesoderm. They regulate the axial patterning of limbs (Zakany and Duboule, 2007). They mediate patterning and differentiation in hairs (Awgulevich, 2003), the gut (Kawazoe et al., 2002), the pancreas (Gray et al., 2011), the blood (Magli et al.,. See articles for details. These other functions will not be discussed further here. Many of these *Hox* functions have been elucidated by gain and loss of function expts. In general, loss of function mutation in a single vertebrate *Hox* gene delivers a deceptively mild phenotype. This has bedevilled the analysis of *Hox* function using mouse genetics. It is because each vertebrate *Hox* gene is a member of a paralogue group of at up to 4 or 8 *Hox* genes which have parallel and shared functions. Where measures have been takn to knock out a whole paralogue group, a suitably dramatic phenotype is obtained. See Fig. 1.

6.5 Modified use of *Hox* genes in elongated vertebrates: Snakes and Caecilians

The elongated, snake-like skeleton, as it has convergently evolved in numerous reptilian and amphibian clades, is from a developmental biologist's point of view amongst the most fascinating anatomical peculiarities in the animal kingdom. This kind of body plan is characterized by a greatly increased number of vertebrae, a reduction of skeletal

regionalization along the primary body axis and loss of the limbs. Recent studies conducted in both mouse and snakes now hint at how changes in gene regulatory circuitries of the *Hox* genes and the somitogenesis clock could underlie these striking departures from standard tetrapod morphology. These studies show that particular snake Hox genes have changed their specificities by mutations in the homeobox. This leads to their failing to specify the expected axial boundaries and enables particular body regions, especially the thorax, to become drastically extended (Woltering et al., 2009, Di Poi et al., 2010).

7. Conclusions

Hox genes are upstream regulators in the developmental hierarchy that are of great importance for the bodyplan. They specify and differentiate between different zones along the main body axis. These genes show collinearity- clustering associated with acquisition of ordered properties within the gene cluster- a spectacular phenomenon that has attracted much interest. A *Hox* cluster is actually a metagene. It, but not an individual *Hox* gene, can fulfil a developmental function- patterning the body axis. In Drosophila, and probably in all other invertebrates- the full potential of the *Hox* genes is not realised. The expression of each individual *Hox* gene is regulated by other spatially regulated genes and so *Hox* collinearity is not used to pattern the main body axis. In vertebrates, temporal collinearity has been developed and this is used to pattern the main body axis and develop spatial collinearity, by time-space translation. It is presently generally assumed that the mechanism of temporal collinearity is progressive 3′ to 5′ opening for transcription of *Hox* complexes. This may be important. However, we develop a different mechanistic hypothesis: that collinearity is partly mediated by *Hox* gene interactions. This idea was already indicated by earlier investigations of posterior prevalence. We review new evidence that trans-acting factors and intercellular signals mediate vertebrate *Hox* collinearity; that these include interactions among *Hox* genes, including posterior prevalence, as well as somitogenesis signals. We propose that these *Hox* interactions have a role in generating *Hox* temporal and spatial collinearity as well as functional collinearity. We note also that an evolutionary explanation for collinearity actually probably obviates any requirement for a dedicated collinearity mechanism. Our conclusions open new perspectives for research into the mechanisms underlying collinearity. Testing this model will require a much more extensive investigation and description of early vertebrate *Hox* temporal collinearity.

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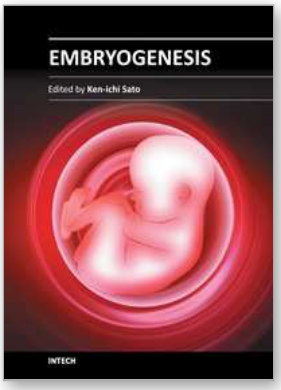

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