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CORRIGENDUM

for article in the following pages: ANTONY J. DURSTON, HANS J. JANSEN, PAUL IN DER RIEDEN and MICHIEL H.W. HOOIVELD (2011). Hox collinearity - a new perspective. *Int. J. Dev. Biol.* 55: 899-908 (doi: 10.1387/ijdb.113358ad).

The authors would like to apologize for not having duly acknowledged that Fig. 2B is reproduced from Fig. 2 in SOSHNIKOVA, N. and DUBOULE, D. (2009). *Epigenetic temporal control of mouse Hox genes in vivo. Science* 324: 1320-1323 and that Fig. 2C is from Fig. 7 in SPITZ, F., GONZALEZ, F. and DUBOULE, D. (2003). *A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. Cell* 113: 405–417. We regret not having requested permission either from the authors or from the publishers to use these figures. Permission from the latter has now been granted. In addition, in the legend to Fig. 2, we have misrepresented the original legend by stating that "Action on the posterior Hoxd genes is shown as repressing (red) and activating (green) inputs (arrows)." In the original paper, all arrows represent enhancement. This has now been corrected in the present legend to Fig. 2. Finally, we as authors apologize for the oversight in having reproduced most of 3 sentences from a review by YETKA S., TABIN, C. and BARTEL, D. (2008). *Nature Reviews Genetics*. This appears on *Int. J. Dev. Biol.* page 902, right column, starting with: *"Further experiments..."* until ...*"(Gibson and Gehring, 1988)"*. The authors and publisher would like to apologize for any inconvenience, grievance or confusion which may have been occasioned as a consequence.

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Hox collinearity – a new perspective

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ABSTRACT *Hox* collinearity is a spectacular phenomenon that has excited life scientists since its discovery in 1978. Two mechanisms have been proposed to explain the spatially sequential pattern of *Hox* gene expression in animal embryonic development: interactions among *Hox* genes, or the progressive opening of chromatin in the *Hox* clusters, from 3' to 5'. A review of the evidence across different species and developmental stages points to the universal involvement of trans-acting factors and cell–cell interactions. The evidence focuses attention on interactions between *Hox* genes and on the vertebrate somitogenesis clock. These novel conclusions open new perspectives for the field.

KEY WORDS: *hox, collinearity, evolution, Xenopus, Drosophila*

Introduction

Hox complexes are among the most remarkable regions of the genome. A *Hox* complex consists of up to 14 transcription factor genes arranged in tandem. These genes specify patterning along body axes in all bilateria (Gehring *et al.*, 2009; Duboule, 2007; DeRobertis, 2008). 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. 1) 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, 2008; Ronshaugen *et al.*, 2005).

The 3' to 5' sequence of the *Hox* genes in a *Hox* cluster matches the sequence in which they act along body axes; this collinear property links clustering to function, emphasizing that Hox complexes are functional units or meta genes (Mainguy *et al.*, 2007, Duboule 2007). Hox collinearity is crucial in embryogenesis and includes 3 important and interrelated properties: functional collinearity describes the order in which *Hox* genes act along a body axis; spatial collinearity refers to the spatial order in which the *Hox* genes are expressed, and temporal collinearity is the time sequence in which they are expressed (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 very highly conserved in evolution.

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).

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 two mechanistic models. The first and prevailing model 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 second model is that collinearity depends on interactions between 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 Box 1).

In this article, we review the basis of Hox evolution and of the two 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

Abbreviations used in this paper: Hox, homeobox; miRNA, microRNA.

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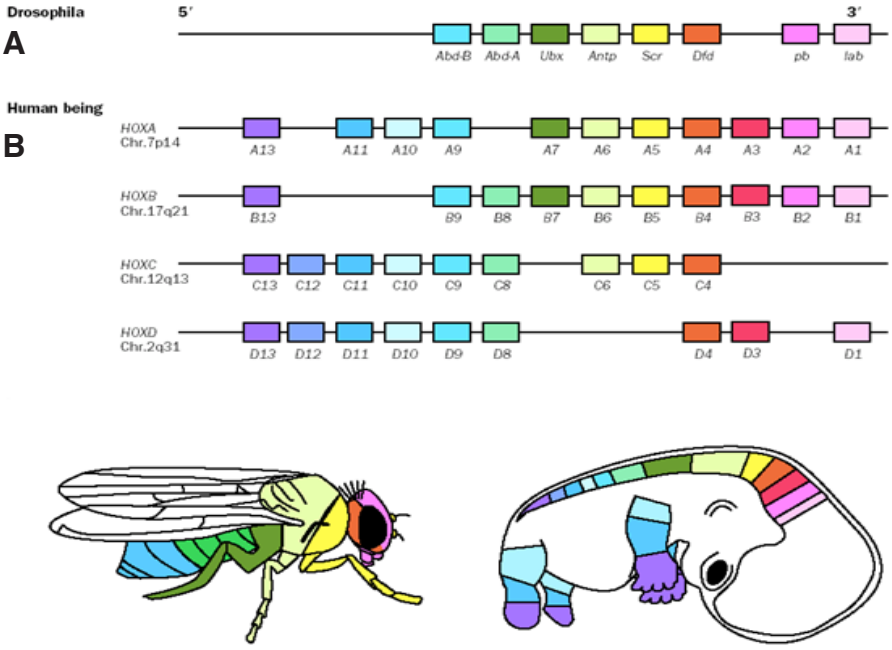

Fig. 1. Hox collinearity. *The four human and one* Drosophila Hox *complexes are homologues. The colour coding in (A,B) shows the correspondence between the genomic order of* Hox *genes in* **(A)** *the* Hox *complexes and* **(B)** *their spatial sequence of expression and action zones along the main body axis in* Drosophila *and human (Goodman et al., 2003).*

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.

The evolution of *Hox* collinearity

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. 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 very similar to each other and are called a paralogue group. *Hox* genes 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.

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. 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* (Seo *et al.*, 2004). These show 'trans collinearity'. It is thus clear that the spatial ordering of *Hox* gene expression does not rely on clustering. Presumably, *Hox* spatial collinearity evolved in an ancestral organism with clustered *Hox* genes 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* collinearity 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) (Box 2). 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 the spatial or temporal sequences of gene expression. Please note that if this is the explanation of collinearity, it obviates any need for an explicit collinearity mechanism (in the sense of an integral mechanism that regulates expression of a whole *Hox* cluster). 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 - as is the case with the gap-segmentation gene hierarchy in *Drosophila.* Nonetheless, we think that collinearity mechanisms

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.

Box 2. An evolutionary explanation of collinearity. It has been proposed that collinearity 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. The upstream mechanism for *Hox* expression will be whatever it evolved to be, in order to regulate the localised expression of the individual *Hox* genes - as with the segmentation gene hierarchy in *Drosophila*. Nonetheless, we think that collinearity mechanisms evolved - see main text.

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 the 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. This idea is discussed further below, in the section 'evolution of posterior prevalence'.

Transcriptional control and chromatin opening

The presently most popular explanation for temporal and spatial collinearity (we do not include functional collinearity because this is a new concept that we introduce in connection with posterior prevalence) suggests that these phenomena are rate-limited by permissiveness for transcription via progressive opening of the chromatin of the *Hox* complexes from their 3' ends towards their 5' ends (Duboule, 1994; Kmita and Duboule, 2003). This view is supported by several observations and experimental studies performed in mouse embryos and in a mouse embryonic stem (*ES*) cell line. During early mouse development, temporally collinear expression of the *Hoxd* complex correlates with the progressive 3'–5' modification of its chromatin from a repressing to an activating state (Soshnikova and Duboule, 2009). Furthermore, elegant experiments showed that transposing a 3' *Hox* gene to a 5' position in the *Hoxd* complex caused later and more posterior expression (Van der Hoeven *et al.*, 1996; Kmita *et al.*, 2000). It has also been shown in ES cells that looping out of genes from their chromosome territory (a correlate of chromatin activation) occurs 3'–5' in coordination with *Hox* gene expression, when retinoic acid is used

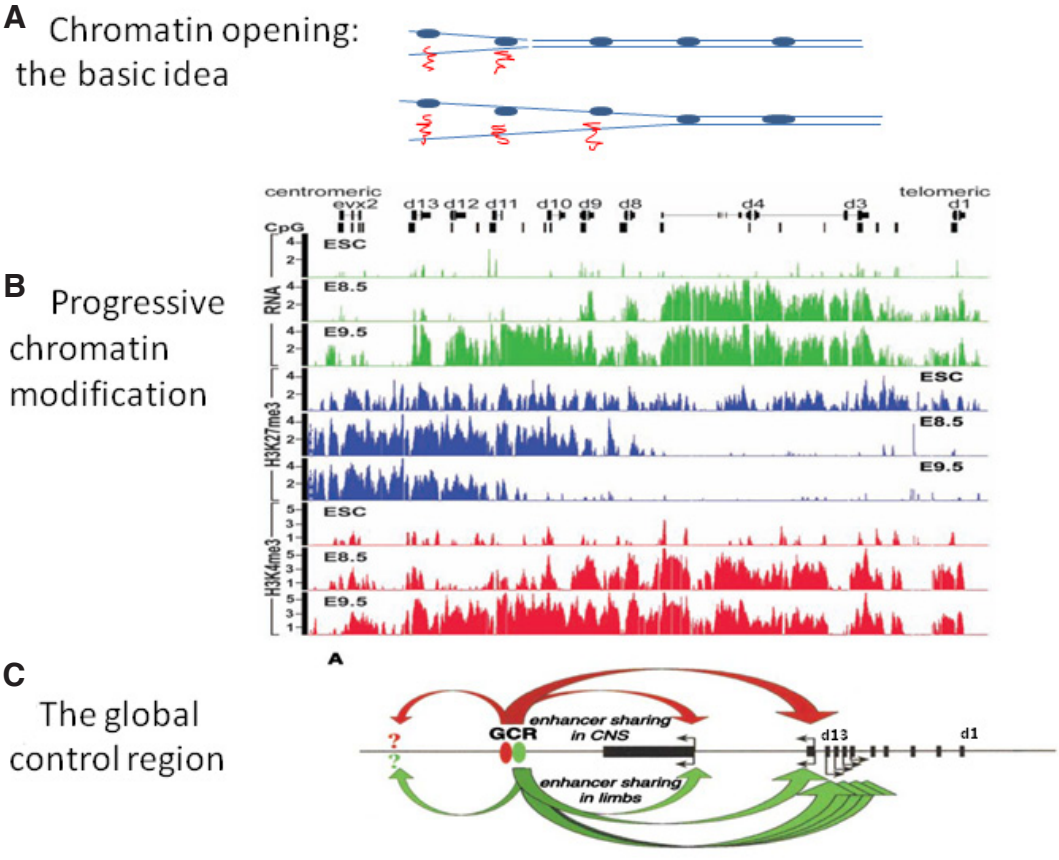

Fig. 2. Chromatin Opening And Transcriptional Regulation. (A) *Chromatin opening: the basic idea. The figure shows two* Hox *complexes. The collinearly ordered* Hox *genes in each complex are shown (on one DNA strand only as blue ovals). As the chromatin opens and the DNA strands separate, the* Hox *genes in the opened part transcribe mRNA (red squiggles).* **(B)** *Progressive chromatin modification in the* Hoxd *complex in mouse development..* ESC*: Embryonic Stem Cells. E8.5 and E9.5: 8.5 and 9.5 days of mouse development. mRNA (green histograms): messenger RNA transcription. H3K27me3 (blue histograms): A chromatin mark for repression. H3K4me3 (red histograms): A chromatin mark for active transcription. As mouse development proceeds from day 8.5 to day 9.5, transcription proceeds from 3' to 5' through the* Hoxd *complex. This is accompanied by a 5' to 3' decrease in the repression mark. and a 5' to 3' increase in the active transcription mark. The embryonic stem cells have a low level of* Hox *expression, high repression mark and low activation mark. Image from Soshnikova and Duboule (2009).* **(C)** *The global control region (GCR). Action on the posterior* Hoxd *genes is shown as red and green arrows. There are also inputs to regions outside the* Hoxd *complex. Image from Spitz et al. (2003). (B,C) reproduced with permission from* Science *and* Cell *respectively.*

to induce temporally collinear *Hoxb* gene expression (Cambeyron and Bickmore, 2004) (Fig. 2).

There is also evidence that transcriptional control of collinearity might occur across entire Hox clusters. A separate study identified a global control region, situated 200 kb 5' of the mouse *Hoxd* complex, that regulates the amplitude of expression of posterior *Hoxd* genes in the mouse limb bud, posterior gut and posterior CNS (Kmita *et al.*, 2002, Spitz *et al.*, 2003, 2005).

These findings are generally considered to be strong evidence that chromatin modification and transcriptional control are involved in establishing the spatial and temporal collinearity of *Hox* genes. However, the available evidence comes only from studies in mouse, and is particularly strong for the *Hoxd* complex. The mouse *Hoxa* and *c* complexes and *Hox* complexes in other bilaterian species have not been investigated. Technical obstacles restrict the possibilities somewhat. Vertebrates other than mouse and human have insufficient genetics for these studies. In invertebrates, *Drosophila* and *Caenorhabditis* could certainly have been investigated but have not. There is no obstacle to investigating the mouse *Hox* a and *c* complexes. The main molecular evidence for involvement of global transcriptional control and chromatin opening in collinearity is as summarised above. As explained below, we think that this mechanism cannot explain all instances of collinearity in the vertebrate embryo. There is also an evolutionary objection against this mechanism (above) in that collinear properties persist in organisms with dispersed *Hox* genes (Duboule, 2007) and it is also found that moving a mouse *Hox* gene out of a *Hox* complex does not destroy its normal axial expression pattern (Krumlauf, 1994).

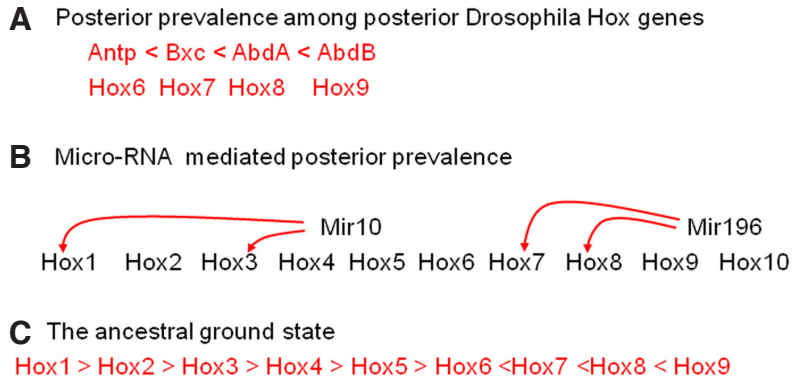

Fig. 3. Posterior Prevalence. (A) *Posterior prevalence among posterior* Drosophila Hox *genes. More posterior genes dominate over more anterior ones.* **(B)** *Posterior prevalence mediated by microRNAs.* Mir 10 *and* Mir196 *each suppress Hox genes as shown. A single* Hox *complex is represented and we show which paralogue numbers are affected. The red lines show interactions in vertebrates.* **(C)** *Gehring's proposal for an ancestral ground state is represented by the function of* Antennapedia *(*Hox 6*) in segment T2. It is dominated both by anterior and posterior* Hox *genes.*

Interactions between *Hox* genes

Posterior prevalence: exception or rule?

A second possible explanation for a part of the phenomenon of collinearity (this addresses the question of how different collinearity properties relate to each other, not their relation to *Hox* complex structure) is that it is mediated by interactions among the *Hox* genes or their products. Support for this model comes from studies on a property of *Hox* genes known as 'posterior prevalence', in which more posterior *Hox* genes are epistatic to more anterior ones (described already above)(Fig. 3). Work in *Drosophila melanogaster* shows that this involves transcriptional, but also post transcriptional and post translational mechanisms.

Working with *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. Further experiments showed that transcriptional cross-regulation is not the only driving force of posterior prevalence. 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). Posterior prevalence is now thought to occur via three mechanisms working in parallel: transcriptional control, posttranscriptional control (via micro RNA's) and posttranslational regulation (involving protein-protein interactions. Posterior prevalence has not been investigated as a mechanism for spatial collinearity because it has generally been assumed that it only occurs once *Hox* genes have already been expressed (rather than it being involved in the establishment of *Hox* gene expression), and is most important for generating unique *Hox* identities in zones in which expression of different *Hox* genes overlaps. Although it is actually clear that posterior prevalence is mediated by transcriptional repression (Hafen *et al.,* 1984; Struhl and White, 1985; Beachy *et al.*, 1988; Miller *et al.*, 2001), this mechanism is paralleled both by post-transcriptional and by posttranslational regulation, specifically by

Box 3. The 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 collinearity 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 miRNA 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 collinearity interaction and that it needs to be dominant, to ensure the 3' to 5' directionality of collinearity.

microRNA-mediated translational control and by protein–protein interactions (see also Box 3) (Plaza *et al*., 2008; Yekta *et al.,* 2008; Woltering and Durston, 2008). Recent exciting findings implicate the *Hox*-associated miRNAs in regulating the translation and stability of *Hox* gene mRNAs. These include the *Hox4*-associated *Mir10* in vertebrates, *Drosophila* and *Caenorhabditis*, the posterior *Mir196* in vertebrates and the posterior *iab4* in *Drosophila* (Yekta *et al.,* 2004, 2008; Woltering and Durston, 2008; Ronshaugen *et al.,* 2005). Therefore, in flies, posterior prevalence mediates functional collinearity via a variety of mechanisms. It is worth noting that any spatial collinearity mechanism is redundant for early *Hox* gene expression in *Drosophila*, where expression of *Hox* genes is turned on by the non collinear segmentation gene hierarchy (Nuesslein-Volhard, 1995). A phenomenon similar to posterior prevalence is also involved in regulating the expression of homeobox-containing genes outside the *Hox* complexes: these genes are expressed in the head anteriorly to the *Hox* gene expression domain and are not contained in the *Hox* complexes (Fig. 3).

Is posterior prevalence the exception or the rule? Posterior prevalence was discovered in *Drosophila.* We know of much evidence (summarised below) that it and other *Hox-Hox* interactions are equally important in vertebrate embryos as in *Drosophila* and invertebrates.

Evolution of posterior prevalence in flies and vertebrates

It has been reported relatively recently by Gehring *et al.,* (2009) that not all *Drosphila Hox* interactions show posterior prevalence. The four 3' *Hox* genes that are expressed anteriorly to *Antennapedia* (*Lab, Pbx, Def, Scr*) are apparently dominant to *Antp. a*nd appear to show anterior prevalence among themselves. Loss of function mutations for these genes leads to posterior transformations and gain of function lead to anterior transformations. Gehring has argued that *Antennapedia* does indeed represent the ancestral *ur-Hox* gene and that both more anterior and more posterior *Hox* genes are derived from this ancestral state by tandem duplication and evolutionary modification as above.This is a beautiful idea that seems very logical and is supported by solid data, but the following points should be considered.

1) Most *Drosophila* axial patterning genes clearly show posterior prevalence. This is definitely true of all of the 5' posterior *Drosophila Hox* genes: *Antennapedia, Ultrabithorax, Abd A, Abd. B,* which are posteriorly prevalent among themselves. It is also true of a number of *Drosophila* non *Hox* homeobox genes: *Ey, Toy, Otx, Ems,* that are early pattering genes in the head. These are all dominated by *Antennapedia* and other *Hox* genes (refs in Gehring *et al*., 2009).

2) There is a reason why *Drosophila Hox* genes might show aberrant collinearity. It is generally accepted that *Hox* collinearity is in process of disintegration and not fully functional in *Drosophila*, which has a *Hox* complex that is split into two. The two halves of the *Drosophila Hox* complex (*Antennapedia* and *Bithorax* complexes) are both greatly expanded, compared to the vertebrate *Hox* complexes and their *Hox* genes are very large. Coordinated regulation of their *Hox* genes will be hindered by this. The anterior *Antennapedia* complex is more degenerate than the posterior *Bithorax* complex. It contains *2 Hox* genes (*Zen, Ftz*) that have been modified in *Drosophila* to mediate different (non *Hox*) functions but whose orthologues are normal functional *Hox* genes in other phyla (Terol *et al.,* 1995; Krause *et al*., 1988). The *Drosophila Hox* genes are also actually turned on individually during early *Drosophila* development by a mechanism (the segmentation gene hierarchy: Nuesslein-Volhard, 1995) that is not related to collinearity. *Drosophila* also has no obvious temporal collinearity (Duboule, 2007).

3) Finally, we should consider whether the *Hox* interactions in *Drosophila* reflect an ancestral *Hox* mechanism that is also conserved in vertebrates. Findings in vertebrates show that *Hoxb4* and the micro RNA *Mir10* act synergistically to repress more anterior *Hox* genes, instead of more posterior *Hox* genes, as with the *Hoxb4* orthologue *Dfd=Deformed* in *Drosophila* (Gehring *et al.,* 2009; Woltering and Durston 2008; Hooiveld *et al.,* 1999). Also, that vertebrate *Hox1* paralogues are required to activate expression of more posterior *Hox* genes back to *Hox* number (paralogue group 6) (McNulty *et al.,* 2005) instead of suppressing these genes, as with *labial* in *Drosophila*. These findings contrast with the situation in *Porcellio* (a Crustacean arthropod), in which the *Dfd* associated *Mir 10* suppresses function of a more posterior Hox gene *(Scr)*, similarly as would be expected in *Drosophila* (Abzhanov and Kaufman, 1999). These findings are not extensive but they open up the possibility that there is a difference between Vertebrates and Arthropods.

We tentatively conclude that the *Hox* interactions in *Drosophila* follow an Arthropod strategy that possibly diverges from the ancestral mechanism in parallel with the disintegration of arthropod *Hox* collinearity and that vertebrates, which have strongly collinear *Hox* complexes, follow a different strategy associated with functional *Hox* collinearity. This may be the ancestral strategy, but the very high degree of collinearity seen in vertebrates is however unique in the animal kingdom and may be associated with a new mechanism. We note that vertebrate *Hox* collinearity, unlike *Drosophila Hox* collinearity features temporal collinearity and we argue below that temporal collinearity requires collinear *Hox* interactions.

A new model: interactions between *Hox* complexes

Temporal collinearity in the vertebrate gastrula mesoderm

To examine the importance of *Hox* interactions in collinearity, we consider the mechanism underlying *Hox* temporal collinearity in a vertebrate embryo. The example we choose is *Hox* expression in the non-organiser mesoderm of the *Xenopus laevis* gastrula, where *Hox* genes are first expressed in the embryo and are expressed with temporal collinearity. This mesoderm manifests a sharply timed temporally collinear sequence of *Hox* gene expression that is translated in time and space to generate a spatially collinear pattern of *Hox* gene expression along the main body axis of the organism (Box 1, Fig. 4).

The 4 *Hox* gene complexes present in most vertebrates arose through 2 rounds of genome duplication during evolution. *Xenopus laevis* and teleost fishes have 8 *Hox* complexes because of 3 genome duplications. A striking feature of the *Xenopus* gastrula's temporally collinear *Hox* expression sequence is that expression of *Hox* genes from different *Hox* complexes is integrated into the same perfectly temporally collinear sequence (Fig. 4). The temporal collinearity of the different *Hox* complexes is therefore synchronised (Wacker *et al*., 2004a; Durston *et al*., 2010). The different *Hox* paralogues (i.e. 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* collinearity simply reflects cis-localised progressive opening of *Hox* complex chromatin for transcription. Trans acting signals

are clearly needed to synchronise the different *Hox* complexes (these are presumably needed for chromatin opening in any case) 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 temporal collinearity is also mediated by trans acting factors.

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* collinearity cannot depend solely on the collinear opening of chromatin. Because the *Hox* complexes are synchronised, *trans*-acting factors 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 initiation 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.

Requirement for extracellular signals and trans-acting factors

Which candidate molecules could mediate the *trans*-acting and intercellular signalling effects described above? Three extracellular signals and one intracellular regulator are known to regulate *Hox* gene expression in the *Xenopus* gastrula mesoderm; however, only one or possibly two of these have the required properties to be involved in triggering the timed and collinear expression of *Hox*-genes during gastrulation. *BMP4, Brachyury* and *Wnt 8* are involved in determining the fate of the part of the mesoderm that expresses *Hox* genes(Wacker *et al.*, 2004b; In der Rieden *et al.*, 2010) (*Brachyury* defines mesoderm and *BMP4* and its target *Wnt8* define the ventral mesoderm). However, all these three regulators are expressed *before* gastrulation, that is, too early; instead, the ideal candidate(s) would be turned on at specific times during gastrulation itself (although *Wnt 8* expression does increase and reach its maximum after the beginning of gastrulation). As such, they could regulate the initiation of *Hox* complex expression or the timed 3' to 5' progression from one *Hox* gene to the next, or both. These signals must be very sharply timed. A fourth regulator, *X Delta2*, is relevant, as discussed below. It is not ruled out that there are other relevant pathways.

Ideal regulators for this function are the *Hox* genes themselves. These are first expressed in the *X. laevis* gastrula mesoderm at the right times, in a temporally collinear sequence. If each *Hox* gene activated its 5' neighbour and its own paralogues, that could cause a temporally collinear sequence. *Hox* genes do, in fact, regulate themselves and each other; they also regulate intercellular signalling, as discussed below.

A potential mechanism for Hox-mediated trans-interactions

What criteria need to be met for *Hox* genes to regulate temporal collinearity? First, 3' *Hox* genes should activate more 5' *Hox* genes. Since activation needs to be sequential according to 3' to 5' position in a *Hox* complex, it is presumably necessary that multiple *Hox* genes, at different 3'-5' positions, do this sequentially. As each *Hox* gene is activated, it should sequentially activate its immediate 5' neighbour and its own paralogues, or the paralogues of its 5' neighbour, or both; second, this activation needs to travel from cell to cell and third, there needs to be a non-*Hox* dependent signal that synchronises the initiation of expression of the *Hox* complexes, presumably by directly regulating the expression of the most 3' *Hox* genes, labial (*Hox1*).

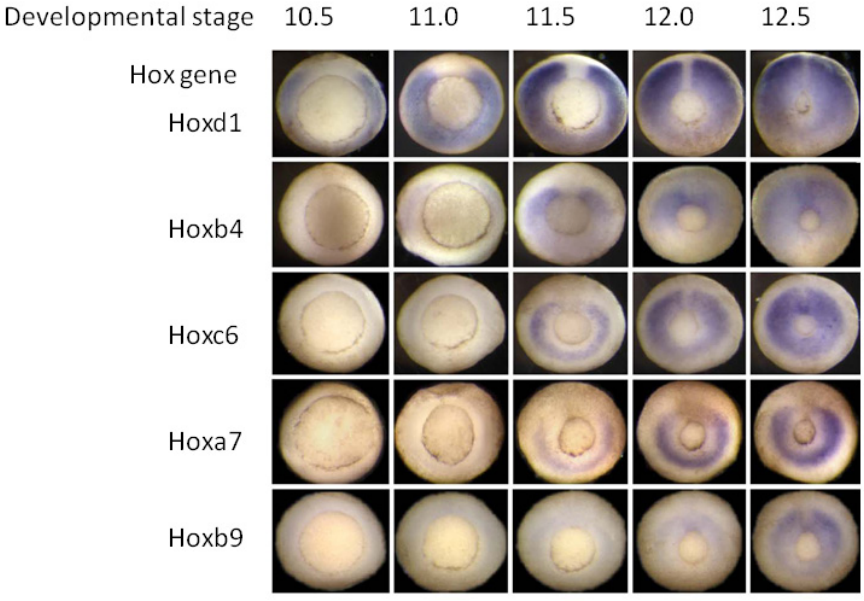

Fig. 4. Temporal collinearity In the *Xenopus* gastrula. *The figure shows* Hox *expression patterns at sequential stages during gastrulation in* Xenopus. *The embryos are seen from underneath, where a ring (the blastopore) shows the position where mesoderm tissue invaginates during gastrulation. This 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, the ring is initially broken. The 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).

Evidence concerning the mechanism

Below we discuss the available evidence supporting the ideal requirements set out in the three points above.

Trans activation

There is evidence from vertebrates that *Hox* genes can activate their 5' neighbours, and thus meet the first criterion listed above. *Hox* genes auto- and cross-activate in early *Drosophila* and vertebrate embryos (e.g. McNulty *et al.,* 2005; Hooiveld *et al*., 1999; Woltering and Durston, 2008; Le Pabic *et al*., 2010; Lobe, 1995; Maconochie *et al.,* 1997; Gould *et al.,* 1997; Bergson and McGinnis,1990; Miller *et al.,* 2001). Ectopic expression of at least two *Hox* genes (*Hoxb4 and Hoxa7*) caused net activation of their own expression and of more 5' *Hox* genes in the *Xenopus* gastrula and in excised gastrula tissues from this organism (Hooiveld *et al*., 1999) We expect, from the sequential nature of temporal collinearity, that these genes would only cross activate 5' neighbours. Indeed, in *Xenopus, Hoxb5* was the only directly activated target of *Hoxb4,* detected so far, apart from *Hoxb4* itself; more 5' *Hox* genes were activated indirectly. Cross activation of other *Hox* genes by a *Hox* gene occurs in another vertebrate embryo (mouse) and in murine embryocarcinoma cells and *Drosophila* (Lobe, 1995; LePabic *et al*., 2010; Gould *et al.,* 1997; Maconochie *et al*., 1997; Miller *et al*., 2001). Expression of 3' *Ho*x genes (Hox1 genes) is also required for more 5' *Hox* gene expression during early *Xenopus* development (McNulty *et al*., 2006) (Fig. 4).

Intercellular signalling

Besides activation of 5' neighbouring *Hox* genes, intercellular signalling is required, to allow *Hox* activation to be transmitted from cell to cell (criterion 2). Much evidence shows indeed that *Hox* genes induce signalling (Bloch- Gallego *et al*., 1993; Chatelin *et al*., 1996; Graba *et al.,* 1995; Bruhl, 2004; Manak *et al.,* 1994; Michaut *et al.,* 2011; Morsi el Kadi *et al*., 2002; Pearson *et al.,* 2005). Known signalling pathways are *Hox* targets in *Drosophila* and vertebrates (eg. Graba *et al.,* 1995; Bruhl, 2004; Manak *et al.,* 1994; Michaut *et al*., 2011; Morsi el Kadi *et al.,* 2002; Pearson *et al*., 2005) and Prochiantz and colleagues have also demonstrated that the *Hox* proteins themselves are unexpectedly translocated from cell to cell, acting as unorthodox intercellular signals (Bloch-Gallego *et al*., 1993; Chatelin *et al*., 1996). Furthermore, in the *Xenopus* gastrula, activation of *Hox* genes by *Hoxb4* is non cell autonomous (Hooiveld *et al*., 1999).

A signal for initiation

There is evidence for a non *Hox* dependent signal that induces expression of *iabial Hox* genes directly in the gastrula. *Wnt 8* induces *labial Hox* genes directly and other *Hox* genes indirectly (In der Rieden *et al.,* 2010). It may not be not the only signal involved in *Hox* complex initiation because it is available from before gastrulation (although its amplitude does increase markedly during gastrulation) and therefore may possibly not initiate the sharply synchronised *Hox* complex expression during gastrulation.

Posterior prevalence

Posterior prevalence occurs in vertebrates (Yekta *et al.,* 2004, 2008; Hooiveld *et al.,* 1999; Woltering and Durston, 2008; Wellik and Capecci, 2003; Carapuco *et al*., 2005; Duboule, 2007) and is an extremely important *Hox* interaction. It is evident in the *Xenopus* gastrula. Expression of more 3' *Hox* genes is downregulated in the *Xenopus* gastrula by early ectopic expression of *Hoxb4* and *Hoxa7*. This is classical posterior prevalence as in *Drosophila* and is entirely logical here. 3' *Hox* genes are expressed earlier than 5' *Hox* genes during temporal collinearity, so their expression is already stabilised by the time 5' *Hox* genes are activated. Therefore they are not expected to be evidently repressed *in vivo*. Expression of 3' and 5' *Hox* genes can overlap as is observed. Repression of 3' by 5' *Hox* genes is presumably required to prevent secondary retrograde activation of 3' genes, which would destroy temporal collinearity. It is especially important to ensure that if a *Hox* gene receives a combination of activating and repressing signals, the repressing signals dominate (see section on Posterior Prevalence and Box 3). Downregulation of more 3' genes by *Hoxb4* has been shown in two early vertebrate embryos: *Xenopus* and zebrafish (Hooiveld *et al*., 1999; Woltering and Durston, 2008). *Hoxb4* acts in synergy with *Mir10*. Posterior prevalence is clearly important in all vertebrates, including the mouse as well as in *Drosophila*. It is probably the most important *Hox* interaction. We think that it is the key collinearity property because it ensures directionality in net *Hox* interactions. Net 3' interactions in gastrula mesoderm *in vivo* should be negative. Net 5' interactions can be positive. The reason posterior prevalence acts at 3 levels may be to ensure that it is always the dominant interaction.

Regulation of Hox collinearity by the somitogenesis clock

What is needed to regulate early collinearity is one or more signals that are turned on at specific times during gastrulation. These could regulate initiation of expression of *Hox* complexes or 3' to 5' progression of expression from one *Hox* gene to the next, or both. They need to be sharply timed. The possibilities are: 1) they come on as a step function; the signal is first off, then sharply on; 2) they are expressed as a pulse; the signal comes on sharply, then disappears. Pulsatile signals are typically oscillatory (i.e. you get periodic pulses). In addition to regulation by interactions among the *Hox* genes themselves, there might be a need for other sharply timed signals. The third intercellular signal known to regulate *Xenopus* gastrula *Hox* expression is actually an oscillatory signal. This is *Xdelta2*, an intercellular signal mediating somitogenesis (i.e. mesoderm segmentation) (Peres *et al*., 2006 and below).

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 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 dynamic process is known to start during gastrulation in chicken and *Xenopus* (Peres *et al.,* 2006; Jouve *et al*., 2002) and 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). *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 al., 2006). This gene could help to drive synchronised temporally

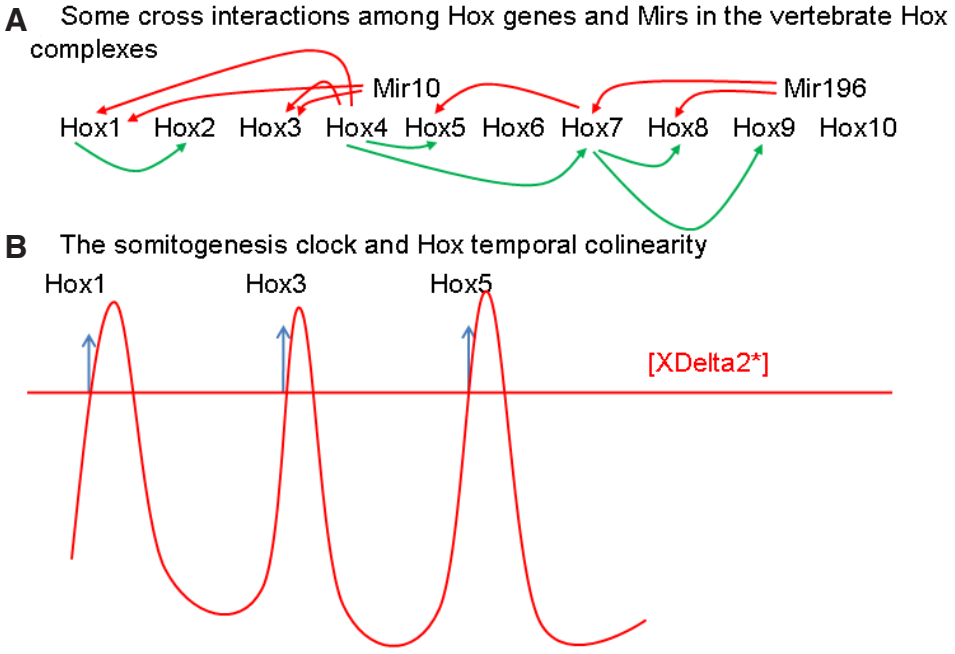

Fig. 5. *Hox-Hox* Interactions And Somitogenesis Timing. (A) *Some cross interactions between* Hox *genes and Mirs in the vertebrate* Hox *complexes during gastrulation. Red: repression. Green: activation.* **(B)** *The somitogenesis clock and* Hox *temporal collinearity. We show an oscillating concentration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

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 labial). If delta 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.*, 2005; Peres *et al.*, 2006). *XDelta 2* may thus mediate *Hox* induced signalling.

Conclusion: a new hypothesis

Vertebrate and *Drosophila Hox* genes undergo *trans*-interactions in early embryos. These putatively mediate the synchronised temporal collinearity of the *Hox* complexes in the vertebrate gastrula stage. These interactions include posterior prevalence, autoactivation and cross activation. Posterior prevalence is a key interaction because it can ensure 3' to 5' directionality in the net *Hox* interactions and can thus generate collinearity. These *Hox* interactions are not necessarily always direct. Besides *trans*-interactions, *Hox* dependent cell interactions are also required. *Hox* proteins activate many signalling pathways and are also signalling molecules themselves. These cell interactions are needed to mediate non-cell autonomous *Hox* interactions. One of the signalling pathways involved in *Xenopus* is the somitogenesis related *Delta-Notch* pathway. *XDelta2* is a timed signalling molecule downstream *of Hox* genes that activates different *Hox* genes during gastrulation. *Hox* chromatin opening may also be involved in early *Hox* collinearity but this mechanism does not require it.

Our ideas about *Hox* interactions and the somitogenesis clock are illustrated in Fig. 5.

Concluding remarks: relationships between different aspects of collinearity

Hox colinearity, which mediates axial patterning in some or all bilateria, is a spectacular phenomenon that has attracted much interest. It is presently generally assumed that its mechanism is progressive opening for transcription of *Hox* complexes. This is presumably important. However, we develop a different mechanistic hypothesis: that collinearity is mediated by *Hox* gene interactions. This idea was already indicated by 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 integral 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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