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Some Questions and Answers About the Role of *Hox* Temporal Collinearity in Vertebrate Axial Patterning.

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Abstract

The vertebrate anterior-posterior (A-P = craniocaudal) axis is evidently made by a timing mechanism. Evidence has accumulated that tentatively identifies the A-P timer as being or involving Hox temporal collinearity. Here, I focus on the two current competing models based on this premise. Common features and points of dissent are examined and a common model is distilled from what remains. This is an attempt to make sense of the literature.

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1/ Introduction. Much evidence points to the conclusion that the vertebrate A-P axis is made by a timing mechanism (Nieuwkoop, 1952, Eyal Giladi, 1954, Collier et al., 2000, Gamse and Sive, 2000, 2001, Vassiliauskas et al., 2001, Selleck and Stern, 2001, Wacker et al., 2004, Stern et al., 2007, Deschamps and Duboule, 2017). The current evidence tentatively identifies the vertebrate axial patterning timer as being or involving *Hox* temporal collinearity (ie, the correspondence of the temporal sequence of *Hox* gene expression during early development with the genomic sequence of *Hox* genes in each cluster). This evidence is presented below. I measure the evidence here against the two current models based on this premise. The following analysis examines, answers and draws conclusions from some of the questions raised. I centre it around comparing and contrasting the two recent models (Durston and Zhu, 2015, Durston, 2015, 2019c, (dur) Deschamps and Duboule, 2017 (dedu)).***Conclusion: An analysis of the important facts around Hox collinearity and timing in axial patterning is required because this is a complex subject where there has been too little discussion . This article strives to make sense of the literature.***

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2/ Does *Hox* Temporal Collinearity Exist? The two recent models depend on *Hox* temporal collinearity mediating a developmental timer (also called a '*Hox* clock'). However, recent publications have also denied the existence of *Hox* temporal collinearity (Kondo et al., 2017. 2019). This denial has been disputed (Durston, 2019 a,b). Does temporal collinearity exist and how does it work (via expression of a full collinear sequence of *Hox* genes or by approximately synchronous expression of temporally sequential blocks of *Hox* genes)? The clearest evidence on this comes from using in situ hybridisation to examine tissue specific spatiotemporal expression of *Hox* genes. These studies, in frog, chicken and mouse embryos, show almost perfectly sequential temporally collinear expression (Izpisua- Belmonte et al., 1989, Gaunt and Strachan, 1996, Wacker et al.,2004, Iimura and Pourquie, 2006, Denans et al., 2015, Gouveia et al., 2015, Moreau et al., 2018). There is only occasional synchrony in expression (eg between *Hoxb8*, *Hoxb9* in Gouveia): not expression in large synchronised blocks. This expression timing generates a nested 'Russian Doll' expression pattern, with the individual *Hox* patterns expanding from a common initiation point.

Conclusion: Hox temporal collinearity does exist and it works via almost fully sequential expression of a collinear sequence of Hox genes (dedu, dur).

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3/ The models propose that *Hox* temporal collinearity (TC) leads to *Hox* spatial collinearity (SC) and axial patterning. Does this occur?

TC leading to SC was first proposed by Duboule and his collaborators (Dolle et al., 1989, Duboule, 1994). Duboule and colleagues made important contributions to the field (cf. Tschopp et al., 2009, Deschamps and Duboule, 2017) but the evidence that TC leads to SC remained elusive. That evidence and insight into the nature of the connection was finally delivered by Wacker et al., (2004), who showed that *Xenopus* temporal and spatial collinearities can be manipulated, are interchangeable (from temporal to spatial collinearity) and are regulated by *BMP*/anti *BMP*. *BMP* rich ventralised gastrula embryos show only temporal (not spatial) collinearity, reflecting the temporal collinearity normally found in the embryo's ventrolateral non organiser (NOM) mesoderm. If they are challenged with anti-*BMP* (*noggin*) solution injected into the blastocoel: (pulse signal) or an anti-*BMP* producing organiser (introducing a continuous signal (step)), they generate parts of the spatially collinear *Hox* axial pattern, the part generated depending on the time of the challenge and its nature. Early challenges generate or initiate at anterior parts (one to a few sequential anterior zones: early *noggin* pulse or axial sequence starting at an anterior level: early implanted organiser). Sequentially later challenges generate more posterior zones or initiate at sequentially more posterior levels in the

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axis***. Conclusion: Hox temporal collinearity leads to Hox spatial collinearity and axial patterning (dedu, dur) The evidence for this comes from BMP-anti BMP regulation of collinearity (dur). The connection between BMP- anti BMP a (a dorsoventral (D-V) patterning antagonism) and A-P patterning reflects the famous connection between vertebrate D-V and A-P patterning (Lane and Sheets, 2002).***

4/ Is *BMP/* anti *BMP* of general importance? The above findings showing *BMP/* anti *BMP* as general Hox regulating factors were made in *Xenopus*. Genesis of a sequence of specific A-P levels in the axial pattern by specifically timed anti *BMP* signals has also been shown in chicken and zebrafish embryos. In chicken, this concerned induction of an A-P sequence of *Hox* genes by *noggin* in the posterior primitive streak (Dias et al., 2014). In zebrafish, this timed sequence (induced by timed heat shock induction of TS-chordin) starts, interestingly, anteriorly in the non *Hox* anterior head part of the axis (Tucker et al., 2008, Hashiguchi and Mullins, 2013). In *Xenopus*, where the zebrafish expt. was repeated and expanded, it continues even further into the most anterior EAD (extreme anterior domain) (Zhu et al., 2019, Jacox et al, 2014)). The fact of an anti-*BMP* dependent A-P time sequence of stabilised induced states implies a *BMP* dependent timer in these

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anterior regions too and indicates that, while the timer includes Hox temporal collinearity, it also exceeds it. In mouse, no *BMP* or anti *BMP* dependence has yet been shown but stabilisation of a series of unstable nascent A-P identities in primitive streak cells by signals from a stable organiser derived cell population has been shown (Wymeersch et al., 2018). This suggests the same mechanism as in the other vertebrates where anti *BMP* signals from the organiser stabilise nascent *Hox* codes in *BMP* rich pluripotent cells, (in NOM mesoderm (Xenopus/anamniote) or in primitive streak (chicken/amniote). ***Conclusion: regulation of Hox collinearities by BMP-anti BMP occurs generally in vertebrates and is central in a core collinearity mechanism. This regulation is central in one of the two models (dur). It is thought, together with the collinear opening of chromatin and Hox-Hox interactions to comprise the basic integral core time-space translation mechanism for collinearity (dur). It is not mentioned in the other model (dedu).***

5/ What is involved in the molecular mechanisms of *Hox* collinearity and axial patterning?

a/ Collinear chromatin opening? There is evidence that a cis-acting mechanism of this nature is involved. It is regulated by TAD's (topologically associating domains), each containing multiple enhancers, there being two: one at each end of each *Hox* cluster studied

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(Deschamps and Duboule, 2017). This cis-acting mechanism appears to involve changes in chromatin architecture, with removal of inhibitory marks on chromatin histones and addition of activating ones (Deschamps and Duboule, 2017). Being cis- acting, this type of process alone cannot account for the synchronisation and coordination of different *Hox* clusters and of different cells that make collinearities observable at the multicellular level of the embryo (Durston 2018).

Conclusion: collinear chromatin opening (dedu) is generally important. It could account for the connection between collinear Hox gene expression and corresponding genomic position. It is presumably part of a core mechanism. This is inherently a cis-acting, single cell mechanism that requires intercellular signalling to synchronise and coordinate it. It is acknowledged in both models. Notably, if chromatin opening is to be visible and detectable in multicellular situations, which it is, this intercellular signalling always needs to be available and active. An 'open by business' chromatin model is indicated.

b/ A Hox-Hox interaction (PI)? Loss of function (LOF) and gain of function (GOF) experiments for *Hox* genes point to involvement of a Hox function in collinearity. Strikingly, antisense Hox RNA treatments of synchronised temporally collinear pluripotent *NT2/D1* human *EC* cells caused cascade LOF phenotypes where LOF for *Hoxb1* or *Hoxb3*

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blocked expression of all later expressed more 5'*Hox* genes in all 4 clusters (Faiella et al., 1994). This indicated that a *Hox-Hox* interaction, posterior induction (PI), where more anterior *Hox* genes induce their posterior neighbours, is involved in temporal collinearity. In *Xenopus* embryos, comparable spatial collinearity phenotypes were obtained, emphasising the connection beween temporal and spatial collinearity. LOF for all 3 *Xenopus Hox1* genes deleted or strongly reduced expression of all more 5' posterior *Hox* genes in all 4 clusters (McNulty et al., 2005). LOF for *Hoxc6* deleted or strongly reduced expression of all more 5' posterior *Hox* genes in all 4 clusters (Zhu et al., 2017b). In addition, *Hox1* LOF enhanced expression of the immediately anterior zonal marker *Gbx2* and *Hoxc6* LOF enhanced expression of the immediately more anterior *Hox* genes *Hoxb4* and *Hoxb5*. The above results emphasise that *Hox* LOF acts in trans. The LOF results were obtained, like the *NT2/D1* LOF results, using antisense technology (in this case morpholinos) and repeats using other approaches (eg CRISPR) would be desirable but the high specificity of the phenotypes obtained leaves no doubt as to the specificity of this approach. In addition to these LOF results, GOF experiments (ectopic expression by microinjection of mRNA) with *Hoxd1, Hoxb4, Hoxa7, Hoxb9* initiated posterior partial axes in ventralised (*Hox* free) and wild type *Xenopus* embryos with the axis starting at the ectopically expressed *Hox* gene in

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each case (Zhu et al, 2017a, Hooiveld et al., 1999). Again, these are very specific phenotypes that indicate a specific result. The facts that these LOF and GOF phenotypes involve effects on all 13 paralogue groups and all 4 *Hox* clusters and that these effects were induced by 8 different manipulations of 7 different *Hox* genes leave no doubt that *Hox* interactions have a general role in collinearity. This role is obviously trans acting between *Hox* clusters, and the fact that *Hox* gain of function can induce a full *Hox* axis with defined coordinated zones indicates that the PI interaction (involved here) acts non cell autonomously. It is also obvious that for PI to be able to work, it needs to be restricted to acting directly only on near posterior neighbouring *Hox* genes. This was tested for one case: *Hoxb4*, acting on *Hoxb5, Hoxb7, Hoxb9*. In this case, *Hoxb5* was indeed the only direct target. *Hoxb7, Hoxb9* were indirect targets (Hooiveld et al., 1999). *Hox* response elements that could mediate a PI like interaction and A regulating response elements (below) have been identified in different *Hox* genes. It is possible that the restriction of PI to close posterior *Hox* neighbours reflects collinear chromatin opening,

The role of PI is proposed only in one model (dur). It is not mentioned in the other (dedu). PI and the other Hox-Hox interactions are proposed to be part of the basic core mechanism for collinearity (dur).

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c/ More Hox-Hox interactions?

Besides PI, other *Hox-Hox* interactions are involved in collinearity. Following the onset of PI in *Xenopus* (which is already active with expression of the first *Hox* gene early in gastrulation), a second interaction starts later. Posterior *Hox* genes begin to repress expression of more anterior ones (Zhu et al., 2017a). This interaction: posterior dominance (PD) is probably required for stabilizing *Hox* zones and thus for the switch from temporal to spatial collinearity. It starts around stages 12-15 (end gastrula to mid neurula) in *Xenopus*. This interaction is imposed by *Hox* genes and also by *Hox* associated *miRNa's*: *Mir10* and *Mir196* (Yekta et al., 2008, Woltering and Durston, 2008). In all cases of *Hox* posterior dominance examined by us and in the known cases of *miRNA* imposed posterior dominance, this interaction involves regulation at the *Hox* mRNA level as well as regulation of *Hox* function. In this respect, this interaction differs from (being broader than) the similar *Hox-Hox* interaction: posterior prevalence, proposed previously by D. Duboule, which, like *Drosophila* 'phenotypic suppression', was proposed to be restricted to action at the posttranslational functional level (Duboule, 1991, Duboule and Morata, 1994). Beside PI and PD, there is a third interaction: autoregulation (A) whereby. for example, mesodermal *Hox* identities are copied over to overlying neurectoderm (Bardine et al.,

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2013). This interaction is clearly non cell autonomous in this particular situation.

These interactions feature in the dur model. Dedu mention and therefore presumably accept only the old studies on 'posterior prevalence' and PD like interactions imposed by Hox13.

d/ Hox directed cell movement?

Experiments in the chicken embryo showed that ectopically expressing a *Hox* gene in a primitive streak cell determines time of ingression and therefore migration of this cell during gastrulation. Ectopic expression of an anteriorly expressed *Hox* gene causes early ingression taking the cell to an anterior position at the end of gastrulation. A more posteriorly expressed *Hox* gene causes later ingression, leading to the correct, more posterior position, later in gastrulation (Iimura and Pourquie, 2006, Denans et al., 2009). This no doubt contributes to the patterning process. This process is putatively important in chicken, where cells ingress individually during gastrulation. It may be less important in anamniotes like frog, where mesoderm cells involute as a sheet during gastrulation.

Conclusion: This movement control likely contributes to axial patterning. It alone is not sufficient to account for the transition from temporal to spatial collinearity (This feature is regrettably, not discussed in either model).

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General conclusion: There is clear evidence for the roles of collinear chromatin opening (dedu, accepted by both models), for the roles of the PI. PD and A Hox-Hox interactions (dur, not mentioned by dedu) and of Hox controlled cell migration during gastrulation (regrettably, discussed by neither), in collinearity. BMP- anti BMP, Collinear chromatin opening and Collinear Hox-Hox interactions together appear to be main components of a basic integral core collinearity mechanism that applies for all Hox genes and interacts with external signalling pathways that each act only on a part of the 3'to 5'Hox sequence (dur, and see below).

6/ How is *Hox* collinearity coordinated/ synchronised at the multicellular level?

Cis- acting or cell localised processes like collinear chromatin opening and possibly like *Hox- Hox* interactions need to be connected, synchronised and coordinated via intercellular signalling to be effective and to be detectable at the multicellular level (Durston, 2018). How is this achieved?

External morphogen signalling pathways? Both the dedu and dur models propose that an A-P series of external signalling pathways synchronise temporal collinearity at different times, corresponding to

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different A-P levels. Dedu mention 3 morphogens: Wnt, (3/3A in mouse), Cdx, Gdf11, working at an A-P series of levels (Deschamps and Duboule, 2017). Dur proposes roles for these and for other morphogens too (Durston 2015, 2019c). The idea is that these three pathways synchronise temporal collinearity at specific times/ A-P levels. Interestingly, the *Wnt* and *Cdx* pathways are known to have response elements acting at approximately the right levels in the axial sequence of *Hox* genes to do this (Deschamps and Duboule, 2017). *Wnt* responsive elements act early in the 3'part of the *Hox* sequence. Some regulate *Hoxa1* directly (Nejits et al., 2016). *Cdx* elements act later in the middle of the axis following *Wnt* induction of *Cdx* (Nejitset al., 2017). *Wnt8* (the Xenopus functional equivalent of murine *Wnt3*) was found to induce only *Hox1* paralogues (*Hoxa1, Hoxb1, Hoxd1*) directly. It induced the other Hox genes examined: *Hoxb4, Hoxd4, Hoxc6, Hoxa7, Hoxc8,* indirectly (In der Rieden et al., 2010). Expression of the earliest, most anteriorly expressed *Xenopus Hox* gene induced by *Cdx: Hoxc6*, was also found to be required for expression of all more 5' posterior *Xenopus Hox* genes (Pownall et al., 1996, Zhu et al., 2017b). A member of *Hoxc6's* immediately anterior neighbouring *Hox* paralogue group: *Hoxa5* was found to *be* induced by *Cdx* loss if function (ie to be repressed by *Cdx)* (Nejits et al., 2017). This recalls the induction of *Hox5* genes by *Hoxc6* loss of function (see above). Perhaps *Hox1* genes and *Hoxc6* are

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the only essential direct *Wnt* and *Cdx* targets respectively for temporal collinearity and perhaps only the first *Hox* gene expressed in each axial domain is the only essential direct morphogen target, the others being capable of being induced indirectly via the PI *Hox-Hox* interaction. A similar conclusion is indicated for action of a third morphogen class: retinoids (Durston, 2019c). Dur noticed that the axial positions where members of the 3 'to 5' axial sequence of morphogen signalling pathways initiate their action correspond exactly to the decision points between sequential anatomical domains on the A-P axis. *Wnt* acts at the boundary between anterior and posterior head; corresponding to posterior/later initiation of the rhombencephalon and of occipital somites; *Cdx* acts at the boundary between neck and thorax, corresponding to termination of rhombencephalon and cervical somites and initiation of spinal cord and thoracic somites (Durston, 2019c). He suggested that these signalling pathways are external to the integral core collinearity mechanism and that their function is to regulate domain switches by being superimposed on it, in each case upregulating the *Hox* gene or paralogue group immediately after a decision point in an extra level of control (Durston, 2015, 2019c). In contrast, dedu assume that these external morphogen signalling pathways are the only means of intercellular communication.

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BMP and non cell autonomous Hox'Hox interactions? In addition to the above A-P morphogens, *BMP*- anti-*BMP* appears to play a general role in mediating the basic integral core collinearity mechanism (see above). In addition, chromatin opening and *Hox* interactions, including PI and A, which, like *BMP*- anti *BMP*, act through the whole *Hox* sequence, appear to be part of this core mechanism. These interactions appear, interestingly, to be non-cell-autonomous. Their intercellular action may enable non cell autonomy of temporal and spatial Hox collinearities in the core mechanism. Perhaps, collinear PI also causes or relies on collinear chromatin opening. Non cell autonomy may be mediated by *Hox* genes activating and being activated by traditional signalling pathways (like *BMP*). It may alternatively be mediated by *Hox* proteins being transported directly from cell to cell (Dupont et al., 2007) (a mechanism not yet accepted by all developmental biologists). It is also possible that `*Hox-Hox* interactions are passed from cell to cell due to cell lineage inheritance (below). Note that none of these features are found in dedu, which assumes that external morphogen signalling pathways are the only relevant means of intercellular communication.

Conclusion: Coordination and synchronisation at the multicellular level Is key to collinearity. It is what makes it detectable. That this is mediated purely by an early-anterior to late-posterior sequence of morphogens, external to the collinearity mechanism (dedu) is

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perhaps unattractive. On the other hand, that these morphogens, which are undeniably involved, feed into and influence an integral basic core functional collinearity mechanism, and that they define axial domains (dur) seems much more likely.

7/ What is the embryology of axial patterning?

There are two main tissues in the vertebrate embryo that carry the A-P axial pattern: First: axial mesoderm: that starts out as involuting/ingressing NOM/primitive streak in the gastrula and goes on to become paraxial/presomitic mesoderm post gastrulation. Second: axial neurectoderm: the precursor of the central nervous system. There are two ideas about how these patterns arise.

Activation-transformation? The classical idea comes from Amphibian embryology. It says that A-P axial levels are first specified in axial mesoderm (we would suggest by time-space translation following an interaction between NOM or primitive streak and the embryo's organiser). These mesodermal A-P levels are then copied over to neurectoderm (which lies adjacent to axial mesoderm in the embryo). This mechanism (activation-transformation) was discovered in Amphibia (ananmniote) but was confirmed and elaborated in Chick (amniote) (Mangold, 1933, Nieuwkoop et al., 1952, Stern, 2005, Bardine et al.,

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2013). This idea is well established and based on much experimental evidence, with explants, recombinates, lineage analysis etc. The evidence is particularly well known in Amphibia but has also been demonstrated in chick. It surely also applies in mouse (Metzis et al., 2018).

Cell Lineage? Second, there are recent exciting findings showing that the embryonic precursors that develop the axial pattern are precursors for mesodermal as well as neural tissue. These pluripotent precursors (NMP's) are postulated to acquire A-P positional information already at their pluripotent stage, then to divide and grow and, at a certain point in time to generate purely mesodermal and purely neural progeny. The ideas for this alternative were developed in mouse, by single cell lineage tracing and other approaches (Tzouacanou et al., 2009, Wymeersch et al., 2016, Metzis et al., 2018). This idea is backed by substantial evidence. It is very attractive because it potentially provides a convenient way to pass on positional information from cell to cell, in parallel to mesoderm and neurectoderm without intercellular signalling being involved, Simply by cell division. This would enable cell autonomous patterning processes like chromatin opening and any cell autonomous *Hox-Hox* interactions to be passed from cell to cell. This general embryology situation is thus complex, with main questions unsolved. It appears that different mechanisms: activation-

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transformation and pluripotent cell lineage are involved in patterning axial mesoderm and neurectoderm in vertebrate embryos. These different modes may possibly operate at different stages of the patterning process and may have different importance in different vertebrates. Please note that intercellular signalling is still nonetheless essential to synchronise and coordinate collinearities and make them observable.

Conclusion: Our two models each (irresponsibly) use only one of the two ideas that have been proposed to underly vertebrate axial differentiation and patterning. Namely, intercellular signalling (dur) and pluripotent cell lineage (dedu). The embryology is unattractively complex at this time.

8/ The precision of axial patterning: how could this be explained?

What are the aspects of precision? Could the mechanisms above explain axial patterning with the necessary precision? Some features of these mechanisms are worrying with regard to precision. For example, if the timing of a cell autonomous function, like chromatin opening, is synchronised at only every 4th to 6th *Hox* gene by external, extracellular A-P signals like Wnt, or FGF, are such extracellular signals external to and independent of, the integrated core collinearity mechanism, with no feedback from it? These aspects require investigation and make the

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potential role of non cell autonomous *Hox-Hox* interactions (which could potentially provide very close control) interesting. Another aspect that provides food for thought is the question of how signals are delivered. Is this a question of a morphogen concentration exceeding a threshold (a typical analogue signal). Could a signal like this time temporal collinearity precisely enough in a sequence of *Hox* genes?

Is high precision timing involved? On the other hand, there is a different, high precision timing device active in the same tissues as *Hox* temporal collinearity that may possibly drive it. This is: the somitogenesis clock; a relatively high frequency oscillator that has a rather constant stable period, presumably due to having limit cycle characteristics. This could measure time with precision; like a quality Swiss watch, by counting the number of elapsed oscillator cycles (ticks of the watch). This timer runs in exactly the same tissues and over exactly the same time course as *Hox* temporal collinearity (Palmeirim et al., 1997, Jouve et al., 2002, Peres et al., 2006, Riedel-Kruse et al., 2007). It is also coupled to collinearity in the way expected if it drives it: different oscillator cycle numbers, generate differently numbered somite boundaries, corresponding to different *Hox* anterior expression boundaries. Loss of function for the somitogenesis clock disrupts *Hox* axial patterning (Peres et al., 2006 Zakany et al., 2001). A recent theoretical model (Kudlicki, 2019) has devised a digital molecular

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mechanism whereby somitogenesis clock cycle No. could be counted, allowing elapsed time to be translated to A-P position.

Conclusion. Precision is possibly a problem. An integrated timing mechanism would help. Involvement of the highly precise somitogenesis clock as a driver and timing by counting oscillation cycles would introduce a much higher level of precision.

8/ What do we know?

Fig. 1 summarises the present knowledge.

9/ Discussion: Best Guess Hypothesis and Future Prospects. How might the vertebrate axial patterning mechanism look?

Best Guess Hypothesis

1/ Hox temporal colinearity (TC) exists. The best evidence comes from in situ hybridisation analysis, which enables detecting onset of *Hox* expression at the appropriate stage in the appropriate tissue. Temporal collinearity appears to be near perfect.

2/ Temporal Collinearity (TC) leads to Spatial Collinearity (SC). Evidence from *BMP*/ anti *BMP* regulation of *Hox* collinearity and patterning in *Xenopus.* Only in the dur model but the evidence is strong.

3/ BMP/ Anti BMP regulation of axial patterning and collinearity is general in vertebrates. Demonstrated in *Xenopus*, chicken, zebrafish. Almost definite in mouse. Strong evidence.

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4/ Collinearities (TC and SC) are mediated by a basic integral core mechanism, involving; BMP/ anti BMP; collinear Hox chromatin opening; collinear Hox-Hox interactions (PI, PD) and A; Hox regulated cell ingression. This core mechanism is proposed only in the dur model, except for Hox regulated cell ingression (Pourquie and colleagues (Iimura and Pourrquie, 2006, Denans et al., 2915)) and collinear chromatin opening (dedu). The evidence for it is strong.

5/ Because some components of the core collinearity mechanism are cis acting/cell localised, intercellular communication is needed to synchronise/coordinate them in the multicellular embryo.

Two types of communication are proposed.

a/ A-P morphogen signalling pathways, external to the basic integral core collinearity mechanism. Eg: *Wnt, Cdx, Gdf11*. These regulate collinearity over particular stretches of the axis. There is evidence that these stretches are axial morphological domains and that the morphogen pathways each serve to co-upregulate expression of the first (most anterior) *Hox* gene in the domain after a particular 'decision point' and that this regulates the other *Hox* genes via PI. Please note that, for two of the three best characterised 'decision points', (retinoids/*Hox1* and *Cdx/ Hox6*), the axial determinant immediately anterior to the decision point is also downregulated by the

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morphogen, as if this drives the *Hox-Hox* interaction PD. I note that there are some pathway response elements that directly regulate *Hox* genes other than the first *Hox* gene after each decision point. These are evidently not essential for morphogen pathway regulation of Hox collinearity.

b/ Communication is part of the core mechanism. The most important here is non cell autonomous *Hox-Hox* interactions (PI, PD, A). These mediate collinearities and participate in mediating domain switches. Apart from these, *BMP/ anti BMP* plays a permissive role, in determining which aspect of the collinearity mechanism is enabled.

6/ The nature of the embryology.

Classical studies revealed that cell interactions are central. Particularly the activation-transformation interactions that mediate transfer of patterning information from axial mesoderm to axial neural tissue. These conclusions are backed by massive experimental evidence.

Recent studies in mouse revealed that common neural-mesodermal precursors develop A-P identities before these cell types diverge. This exciting conclusion is backed by solid experimental evidence.

It raises the exciting possibility that A-P identities can be passed from cell to cell without intercellular signalling. The role of common precursors

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(NMP's) needs to be defined more precisely,

7/ Precision. The requirements for precision are unclear. If required, very precise timing could be imposed via the somitogenesis clock. The role of the somitogenesis clock is unclear.

Future Prospects

1/ Perspectives for medicine.

Can These Insights Be Used in Connection with Stem Cells? The mechanism above is an important part of the body plan program that generates the diversity of cell types and organs that make a vertebrate. The investigation by Faiella et al. (1994) already demonstrated a long time ago that part of this mechanism can operate in a pluripotent cell line. The cells involved in the embryo clearly include pluripotent stem cells too. With the diversity of ES cells now available, it will be important to determine whether this *Hox* mechanism can be used to generate and further new stem cell applications. It should also have perspectives for in vitro organoid culture. I hope someone will explore this. I would do it myself if I weren't too old.

2/ Future investigation of the nature of the mechanism. ***The bones of the axial patterning/ collinearity mechanism are now perhaps***

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becoming clear. There are however key questions that still need to be settled definitively.

1/ Does Hox Temporal Collinearity Actually Exist? Two recent publications questioned whether Hox temporal collinearity actually exists (Kondo et al., 2017, 2019). I have presented the arguments that it does and that it is of central importance (Durston, 2019a, b and see above). This question needs to be settled urgently and definitively if anyone thinks it is still open.

*3/ What Is the Nature of the Timer? Hox t*emporal collinearity drives the timing and spatial sequence of axial patterning. But is temporal collinearity itself the driver or is it in turn driven by something else? Is it itself precise enough to drive a developmental program? This is an important question. The degree of precision required needs to be determined. There is a second very precise time-space translation mechanism active in the early embryo, in the same tissues and with the same timing as *Hox* temporal collinearity. This mechanism (the somitogenesis clock) is presumably precise because it is based on (many ticks of) a relatively high frequency oscillator (the limit cycle characteristics of which should ensure stability) and it is known to be able to drive *Hox* temporal collinearity (Peres et al., 2006). Temporal

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collinearity however also feeds back to drive it (McNulty et al., 2005). These two TST mechanisms are thus clearly connected. What drives what and when and where?

4/ What Is the Nature of Hox-Hox Interactions? The mechanism for generating *Hox* temporal collinearity and translating it to a spatially collinear pattern is complex. Multiple collinear *Hox-Hox* interactions appear to be involved. Temporal collinearity appears to require posterior Induction (PI). PI was deduced from cascade phenotypes in *Xenopus* and in NT2/D1 cells which were all obtained using either ectopic expression (gain of function) or antisense technology (morpholinos or regular antisense oligonucleotides; loss of function). These phenotypes appeared very specific and not artefactual because each generated expression of a very specific sequence of *Hox* genes. However, it would be instructive to see what kinds of *Hox* expression phenotypes other standard gene manipulation approaches (like ectopic expression in mouse, homologous recombination in mouse, CRISPR) give. This is so far llargely unknown. In addition, it is absolutely necessary to identify and catalogue enhancers and any other regulatory motifs that mediate these interactions.

5/ What Are the Roles of Morphogens? There are various morphogens that are thought to be involved in setting up the A–P axis. Their roles in relation to the timing mechanism considered here have been discussed above and elsewhere (eg. Durston, 2019c) . However, this aspect deserves much further attention. There is lots more to be done.

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Fig. 1 legend. Left: (Xenopus) embryos (grey ovals) at sequential stages in gastrulation. The NOM mesoderm (horizontal coloured stripe) runs from ventral to near dorsal. I show some of the successive stages of Hox expression in NOM. It is first blue (Hoxd1 is the most posterior gene expressed at this stage). Then yellow (Hoxb4 is the most posterior gene expressed at this stage). Then red (Hoxc6 most posterior expressed). These are three stages in the first part of the NOM temporally collinear Hox sequence. The yellow background to the figures shows that TC happens in availability of a high BMP concentration, which is available in most of (the left ventrolateral part (V)) the embryos (as shown). Under these conditions, collinear opening of chromatin and the Hox-Hox interaction PI also occur as do Wnt and Cdx inputs into the Hox1 genes and Hoxc6 respectively. These activities all have a yellow background, indicating that they require high BMP conditions. A thin segment at the the right (dorsal:D) side of the embryo has a blue background (shown only for the identical embryos at the right hand side of the figure). This represents anti BMP, which is available in the dorsal side of the embryo (D) only. Under these conditions, successive blocks of cells are frozen at each successive Hox code and these blocks stack up to make an axis. This process involves making mesodermal and neural layers of spatially

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collinear tissue (not shown). It involves two late Hox-Hox interactions, Posterior Dominance, whereby posterior Hox genes inhibit function of and repress more anterior Hox genes and Autoregulation, whereby mesodermal Hox expression is copied over non cell autonomously to neural tissue.