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A Tribute to Lewis Wolpert on the 50th. Anniversary of the Publication of his Paper 'Positional Information and the Spatial Pattern of Differentiation'. Evidence for a Timing Mechanism for Setting Up the Vertebrate Anterior-Posterior (A-P) Axis.

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Abstract

This article is a tribute to Lewis Wolpert on the occasion of the recent 50th anniversary of the publication of his article 'Positional Information and the Spatial Pattern of Differentiation'. This tribute relates to another of his ideas: his early 'Progress Zone' timing model for limb development. Recent evidence is reviewed that a mechanism sharing features with this model patterns the main body axis in early vertebrate development. This tribute celebrates the golden era of Developmental Biology.

Keywords: *Hox* genes; limb development; main body axis; timing; time space translation

1/ Introduction: Lewis Wolpert and His Ideas

Lewis Wolpert is a giant in the arena of scientific discovery. Together with others, including Francis Crick, Sydney Brenner, Brian Goodwin, Janni Nuesslein Volhaard , Ed Lewis, the Physicist Morrel Cohen and the Mathematicians Rene Thom and Christopher Zeeman, his ideas and discoveries in a period starting in the 1960's helped establish that

Developmental Biology is absolutely a key area of science. This was a period when ideas, rather than only technological developments were recognised as the true currency of science. These pioneers' original thinking and novel concepts reanimated the sleepy discipline of embryology. One sometimes fears that Developmental Biology's pioneering spirit is now dead. We must ensure that it never dies. Original ideas and audacity lie at the root of any exciting science. They must be nurtured! They guarantee the future.

Wolpert's key proposition, contained in (Wolpert 1969), has been held to be that concentration gradients of morphogens acting on concentration thresholds can establish a developmental map in the embryo. This idea is undoubtedly of great importance and inspired a generation. It motivated many young scientists, including myself and inspired us to become developmental biologists. However, Wolpert was also responsible for other important ideas and discoveries: to name just two; a very important early developmental timing model, his progress zone model for limb development (Summerbell et al., 1973, Wolpert et al. 1979, Wolpert 2002 a,b, Tabin and Wolpert, 2007); and his idea that an asymmetric molecule is the basis of left-right asymmetry (handedness) in the embryo (Brown and Wolpert, 1990). 2019 was an important year for Lewis Wolpert. Not only was it it the 50th anniversary of 'Positional Information'; it was the 40th anniversary of his key publication reporting

experimental evidence supporting his 'progress zone' model (Wolpert et al. 1979), although the original idea for this (Summerbell et al., 1973) was already published 6 years earlier; it was also his own 90th anniversary; and he was recently deservedly awarded the Royal Society's Royal Medal. In this article, as a tribute to Lewis to celebrate these anniversaries, I point out evidence that a developmental timing mechanism, sharing properties laid bare by his 'progress zone' model is the prime candidate for patterning the vertebrate anterior-posterior (A-P) or rostro-caudal axis.

2/ Wolpert's 'Progress Zone' Model.

The Progress Zone (PZ) Model states that a 300um deep zone of mesodermal cells in the distal tip of the developing limb is under the specific influence of a signal from the limb's most distal (and external) apical feature: the apical ectodermal ridge (AER): a known organiser. These mesodermal cells are growing and dividing and while they are in the progress zone, their positional identity changes (it is dynamic and labile). The longer they or their progeny remain in this zone, the more distal their identity becomes. A timing mechanism is thus clearly indicated. Once these cells leave the PZ, their positional identity becomes fixed and can be manifested. See Fig.1. **Fig. 1 here.**

This model was supported by the results of early X-irradiation of the developing limb tip (Wolpert et al., 1979). It was found that this (which inhibits cell division and growth) causes defects in the proximal but not distal parts of the limb. This was interpreted to mean that a decrease in cell division and growth rate will cause cells to remain longer in the PZ so that their identity becomes more distal. There will be very few proximal cells that are able to make proximal structures.

This was a beautiful, simple and elegant model that clearly described a (or is it the only?) way in which a timing mechanism can pattern an axis. It was very influential in that it kicked up a storm of controversy (Dudley et al., 2000, Saunders, 2002, Towers and Tickle, 2009, Wolpert 2002a, b). This controversy inspired much new experimentation and brought on the limb field immensely. It soon became clear that the simplest form of the PZ model alone could not account for all of the data concerning proximodistal limb patterning. For example, it did not account for intercalary regeneration in grafts of proximal to distal limb bud sections (Maden, 1980). That is unimportant. The PZ model and Lewis Wolpert's pioneering spirit had already served their purpose. Lewis had continued the traditions of the pioneers. It is also clear that the PZ timing mechanism actually is a part of the proximodistal limb patterning

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mechanism. See below. (Tabin and Wolpert, 2007, Rosello et al., 2014, Saiz-Lopez,et al., 2015).

Proximodistal limb patterning is clearly a complex process with multiple components including signalling factors and a timer which appear to be relevant at different phases.   Wolpert's progress zone ideas also seem relevant for patterning the main vertebrate anterior-posterior or rostro-caudal (A–P) body axis. See below. The currently popular limb patterning mechanism has many components and features in common with the current proposed main A-P axis patterning mechanism. These include: some of the morphogens involved; *FGFs* and retinoids, *BMPs* and *Wnts; Hox* genes (*Hox a and d 9-13* in common); timing via trichostatin A sensitive histone acetylation: presumably because this leads to collinear opening of Hox cluster chromatin; collinear *Hox-Hox* interactions; stage/PD position dependence for sensitivity to different morphogens. Wolpert's progress zone model forms part of the current thinking. There is a candidate for the AER's PZ signal: *FGF8.* (Lewandoski et al., 2000). Other *FGF's*: *FGF10, FGF4* have also been proposed to be relevant (Zakany and Duboule, 2007, Niswander et al., 2004). The molecular nature of the PZ timer is currently under investigation. I have a suggestion for that below.   (Cooper et al., 2011, Rosello-Diez et al, 2014, Saiz-Lopez et al., 2015, Sheth et al., 2014,

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Zakany and Duboule, 2007, Deschamps and Duboule, 2017. Tabin and Wolpert, 2007). See below.

3/The Vertebrate A-P Axis is Also Generated in a Timed Manner.

Many findings show that the vertebrate A-P axis is generated in a timed manner (eg. Nieuwkoop et al., 1952, Eyal Giladi, 1954, Collier et al., 2000, Selleck and Stern, 1991, Gaunt and Strachan, 1996, Gaunt, 2000, Gamse and Sive, 2000,2001, Vasiliauskas and Stern, 2001, Wacker et al., 2004, Tucker et al., 2008, Stern et al., 2006, Deschamps and Duboule, 2017). The current evidence points strongly to the idea that the axial timer is or involves *Hox* temporal collinearity (below). It is interesting to ask whether this vertebrate axial timing mechanism has anything in common with Wolpert's PZ model and/or with what has recently been established for the limb PD patterning mechanism.

4/ What is the Nature of the Vertebrate Axial Timing Mechanism?

There is evidence that A-P axial patterning in vertebrates is mediated by a timing mechanism and time space translation (TST) from gastrula stages onwards (Wacker et al., 2004, Durston and Zhu, 2015, Dias et al., 2014, Tucker et al., 2007, Hashiguchi and Mullins, 2013, Deschamps and Duboule, 2017, Wymeersch et al., 2018.). (Fig. 2). **Fig. 2 Here**. To

be brief: An integral core *Hox* collinearity mechanism which applies for all *Hox* genes and employs: collinear chromatin opening, collinear *Hox-Hox* interactions and the antagonism: *BMP*-anti *BMP* mediates: a developmental timer: *Hox* temporal collinearity (TC) and time space translation (translation from *Hox* temporal collinearity to *Hox* spatial collinearity: TST, which is the basis for the axial pattern in the embryo). This main axis TC/TST machine matches Wolperts's PZ model in that operation of the timer depends on a diffusible signal. In the main axis, this is *BMP* and there are also various external inputs (below). In the PZ model, the signal is *FGF8*. The main axis TC/TST mechanism   is also regulated by external inputs: morphogen signalling pathways, which act by being cofactors for expression of particular Hox genes, namely, in each case, the first *Hox* gene after a particular 'decision point' between two morphological domains on the body axis. Examples are: *Wnt,* acting at the anterior head-posterior head decision point; retinoids, acting at the same decision point, *FGF*, acting at the neck-thorax decision point, *Gdf11*, acting at the thorax-lumbar abdomen decision point. Please note that each of these external pathways acts only over a certain limited developmental period and only over a certain limited part of the A-P axis. The (extensive) evidence for this mechanism is given in three recent reviews: Deschamps and Duboule, 2017, Durston, 2019c and Durston, 2019d. Space prohibits repeating it here again; Please note that many

of the features of this mechanism are also relevant for limb P-D

patterning: namely, retinoids, *FGF, Hox* genes, chromatin histone

acetylation, collinear opening of *Hox* cluster chromatin, *Hox-Hox*

interactions, action of different morphogens at different stages.. Both

the limb workers and the A-P axis workers have made hypotheses about

how all this applies in their particular field. We need to evaluate the

possibility that there is a mechanism with common features that applies

for both the limb P-D and main axis A-P mechanisms.

5. Do P-D limb patterning and A-P body axis patterning have a common basis?

Table 1 shows the correspondence between components of limb P-D

axis patterning and A-P body axis patterning. There are many parallels.

This is perhaps not surprising considering that these are very welll

known developmental components**. Table 1 here**.

I note the following points;

!/ Both the axial patterning mechanism and the limb patterning

mechanism operate in the main axis of the structure concerned. In the

body, this is the A-P axis; in the limb, the P-D axis, which are both

patterned by *Hox* genes. However, the limb's P-D axis is patterned

together with the limb's A-P axis, which is a minor axis in the limb.

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2/ The axial patterning mechanism and the limb patterning mechanism are each regulated by a timer and time-space translation.

3/ Both show *Hox* temporal collinearity that precedes and apparently generates *Hox* spatial collinearity, which generates the body's A-P axial pattern and and the main (P-D) axis pattern and structure in the limb. This is called time space translation (TST). TST mediates patterning and growth in the body axis and although the evidence is somewhat less complete, it clearly does the same in the limb. See below. The evidence is thus clear that *Hox* temporal collinearity is the developmental timer both in the main A-P axis of the embryo and in the P-D axis of the limb.

4/ The *Hox* timer (TC) in the body axis is *BMP* dependent.The diffusible signal *BMP* (produced in the NOM mesoderm/primitive streak, where the timer runs) allows/generates a timed sequence of nascent positional values. TST and stopping of the timer is induced by timed anti *BMP*. An A-P sequence of positional values are stabilised at an early to late sequence of times. In the limb, *FGF8* (Lewandoski et al., 2000) (and perhaps other *FGF's*) seems to be the AER's signal that corresponds to *BMP*, that enables the timer and presumably, *FGF* antagonism or subthreshold *FGF* would stabilise positional values (as with anti *BMP*). The limb story is however complex. Unlike in the main body axis, different *Hox* clusters are very differently regulated. Only the ho*xa* and *Hoxd* clusters are relevant during limb development. Deletion of the

Hoxb and *Hoxc* clusters does not affect it (Zakany and Duboule, 2007) (Fig. 3). **Fig. 3 here.** The *Hoxd* cluster is best studied. Investigation of its function shows clearly that the *Hoxd* cluster regulates limb P-D as well as A-P development. I should mention here that a great deal of the excellent work that has been done on the role and regulation of *Hoxd* genes in limb development has been by D. Duboule and his colleagues (eg. Tarchini and Duboule, 2006. Zakany and Duboule, 2007). Regulation of the *Hoxd* genes is complex in that it shows two phases. In phase 1, during earlier development of the limb, *Hoxd9-13* are expressed sequentially in time in nested 'Russian doll' patterns, centred in the distal-posterior part of the limb bud. The more 3'*Hox* genes are expressed sequentially too but their expression shows no localisation. It is clear that these patterns are regulated by A-P as well as P-D signals. The expression patterns are very similar to the expanding Russian doll patterns seen in the developing mouse main axis when TC generates SC. I suggest that the same is happening here too. (Fig 4). **Fig. 4 here.** The posterior-distal location of the TC focus indicates that as well as being regulated by the limb's P-D factor: *FGF8*, this is regulated by the limb specific A-P factor, sonic hedgehog (*Shh*). Both of these factors are presumably required for temporal collinearity in this phase. In a second phase, during later development of the limb's autopod (hand plate), the patterns remain posteriorly distal for *Hoxd* genes but become purely

distal for *Hoxa* genes. There are thus two diffusible factors (*FGF8*/ other
relevant *FGF* and *Shh*) potentially involved in limb *Hox* collinearity.
Possibly, only *FGF8* remains relevant for later expression of *Hoxa*
genes. However, there is also an indirect role for *BMP*-anti *BMP* too.
This regulates the viability and availability of the AER and therefore
presumably regulates the *FGF* signal emitted by the AER that permits
the limb timer to run in the progress zone (Pizette and Niswander, 1999).
This signal is thus repressed by *BMP* and enhanced by anti-*BMP*. These
BMP dependent functions in the limb are clearly different from those in
the body axis.

5/ 5' *Hox a* and *d* genes are clearly centrally involved in limb
development. See above. Their importance has been examined in the
forelimb by combined loss of function for both paralogues, leading to
combined *Hox a* and *d* loss of function. Loss of function for the *Hox 9* or
10 paralogues causes defects at sequential levels in the stylopod
(presumptive upper arm) (Fromental-Ramain et al., 1996a, Wellik and
Capecchi, 2003, Tickle, 2006, Zakany and Duboule, 2007), loss of
function of *Hox11* causes defects in the zeugopod (presumptive
forearm) (Davis et al., 1995, Wellik and Capecchi, 2003, Zakany and
Duboule, 2007), loss of function for *Hox 12* or *13* paralogues modifies or
deletes the autopod (presumptive hand and wrist) (Wellik and Capecchi,
2003, Dolle et al., 1993, Fromental-Ramain et al., 1996b, Zakany and

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Duboule, 2007). The developmental timer (*Hox* temporal collinearity) thus operates sequentially throughout early and mid limb development (the stages when the stylopod, zeugopod and autopod are specified sequentially). *Hox 9-10* are involved sequentially in specifying the stylopod, *Hox 11* in specifying the zeugopod and *Hox 13* in specifying the autopod (Zakany and Duboule, 2007). In addition to being regulated by *FGF's* and *Shh*, the *Hox* genes regulate these factors themselves. *Hox 9* and *10* upregulate *FGF 10* and *Shh. Hox 13* downregulates *FGF10.* This feedback presumably relates to the obvious connection between limb A-P and P-D patterning. These data are all consistent with Hox temporal collinearity mediating Wolpert's PZ timer for proximodistal limb patterning. I note that the timer's action during earlier limb development is somewhat masked by the fact that the timer's necessary distal diffusible signal: *FGF8* is antagonised by a proximal signal: retinoic acid (Cooper et al., 2011, Rosello- Diez et al., 2014, Saiz-Lopez et al., 2015). The period of temporal collinearity operation is thus potentially the same period of operation as tor Wolpert's progress zone timer and the idea that *Hox* temporal collinearity mediates this timer has been proposed previously (Rosello-Diez et al., 2014,). The reasoning here supports this view. An alternative idea has been that the timer is cell cycle based (Summerbell et al., 1973, Saiz-Lopez et al., 2015)

6/ It was at one point proposed previously that limb P-D patterning is mediated by morphogens alone, without any intrinsic (timing) mechanism (Rosello-Diez et al, 2011). It actually seems most likely that these diffusible morphogens interact with an endogenous timing mechanism to pattern the limb's P-D axis (Cooper et al., 2011, Rosello-Diez et al., 2014, Saiz-Lopez et al., 2015 and see this paper). Some of the morphogen signals involved in patterning the main body axis have their best characterised effects early and anterior in the axis. Too early and too anterior to be relevant for limb patterning. However, the same morphogens have multiple effects including (so far less well characterised) effects on the posterior axis. We can for example expect retinoids to have a function in the abdominal part of the axis or at the abdomen/tail boundary, a position where they can truncate the axis or induce limbs in place of a tail (Kessel and Gruss, 1991, Mahapatra et al., 1994). Some of these more posterior functions may well relate to morphogen functions in the limb.

6/ Questions for the Future.

1/ Does *Hox* Temporal Collinearity Actually Exist? Two recent publicati ons question 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). This question needs to be settled urgently and definitively if anyone still doubts this.

2/ Can These Insights Be Used in Connection with Stem Cells? The mechanism above is an important part of the program generating the diversity of cell types and organs that make an animal. 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. 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.

3/ What Is the Nature of the Timer? *Hox* temporal collinearity drives the timing and spatial sequence of A-P axial patterning and probably also of limb PD 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. There is a second time-space translation mechanism active in the early embryo as well as in the developing limb (Pascoal and Palmeirim, 2007), in the same tissues and with the same timing as Hox temporal collinearity. This mechanism is active in NOM mesoderm (anamniotes) or primitive streak

(amniotes) and later in presomitic mesoderm. It is active during

gastrulation (chicken and *Xenopus* (Jouve et al., 2002, Peres et al.,

2006) but is also already known to be active by the zebrafish's earlier

blastula stage (when the head starts its specification) (Riedel-Kruse et

al., 2007). This mechanism (the somitogenesis clock) is presumably

precise because it is based on (many ticks of) a relatively high frequency

stable oscillator (the limit cycle characteristics of which should ensure

stability and which can deliver precision if elapsed cycles are counted)

and it is known to be able to drive *Hox* temporal collinearity (Peres et al.,

2006). Temporal collinearity however also feeds back to drive it (McNulty

et al., 2005). These two TST mechanisms are thus clearly connected.

What drives what?

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 an

interaction where each *Hox* gene induces its posterior neighbours.

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) (

Faiella et al., 1994, Hooiveld et al., 1999, McNulty et al., 2005, Zhu et

al., 2017a,b). These phenotypes appeared very specific and not

artefactual because each generated expression of a very specific

sequence of *Hox* genes either starting with the treated *Hox* gene and

running towards late/posterior (gain of function) or running from

anterior/early up to and stopping with the marker before the treated *Hox*

gene (loss of function). However, it would be very 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 largely unknown.

There have been investigations using ectopic expression and knockouts

as well as genetic rearrangements in mouse that provide information

about one interaction: PP/PD (Zakany and Duboule, 2007, Tarchini and

Duboule, 2006). These are informative but much more needs to be

done.

5/ What Are the Roles of Morphogens? There are various morphogens

that are thought to be involved in setting up the main body's A–P axis

and the limb's P-D axis. Their roles in relation to the timing mechanism

considered here have been discussed above and elsewhere (Durston,

2019c, d). However, this aspect deserves further attention. There is lots

more to be done.

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6/ Do the limb P-D and body axis A-P timing mechanism and TST have a common molecular basis? This complex problem needs to be solved. See above

7/ Hearty congratulations to Lewis on these important anniversaries.

May creative science in general, Developmental Biology in particular and Lewis himself, thrive!

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Figure Legends

Fig. 1 The Progress Zone Model

A cell´s proximo-distal positional value may depend on the time it spends in the Progress Zone. Cells continually leave this zone at the tip of the limb

under the apical ectodermal ridge. Cells that leave early form proximal structures while cells that leave last form the (distal) tips of the digits.

Fig. 2. Timing, Axial patterning, and Time Space Translation

Left and right: (*Xenopus*) embryos (grey ovals) at 3 sequential stages in gastrulation. The NOM mesoderm (horizontal coloured stripe) runs from ventral to near dorsal. I show some of the successive stages of temporally collinear Hox expression. First, blue stripe (*Hoxd1* is the most posterior *Hox* gene expressed). Then yellow stripe (*Hoxb4* is the most posterior *Hox* gene expressed). Then red stripe (*Hoxc6* is the most posterior *Hox* gene expressed). These are three stages in the first part of the NOM temporally collinear (TC) *Hox* sequence. The yellow background shows that TC happens in availability of a high *BMP* concentration, which is available in the ventrolateral part (V) of the embryo (as shown). Under these conditions, Collinear opening of chromatin and the Hox-Hox interaction PI (Faiella et al., 1994, Hooiveld et al., 1999, McNulty et al., 2005, Zhu et al., 2017a, b), which is necessary for TC, also occur as do *Wnt* and *Cdx* inputs into *Hox1* genes and *Hoxc6* respectively (Durston 2019c). A thin segment at the the right side of the embryo has a blue background (shown in detail for the embryos at the right side of the figure). This represents anti *BMP*, which is available in the dorsal side of the embryo (D). Under these conditions, successive blocks of cells are frozen at each successive *Hox* code and these blocks stack up

from early anterior to late posterior to make the A-P axis. This process involves making mesodermal and neural layers of spatially collinear tissue (separation 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 from mesodermal to neural tissue (Zhu et al., 2017a, Bardine et al., 2013).

Fig 3. Irrelevance of the *Hoxb* and *Hoxc* clusters for limb development.
On top, the full complement of *Hox* genes is shown (left), along with the associated wild-type morphology (right). The various schemes below illustrate full cluster deletions. Only the removal or either *Hoxa* or *Hoxd* leads to a detectable phenotype, which is not drastic and mostly affects the digital plate. However, the combined deletion of both *Hoxa* and *Hoxd* leads to an early arrest of limb growth, pointing to a large functional redundancy between these two clusters (S: stylopod, comprising the humerus and defining the upper arm; Z: zeugopod, comprising the radius and ulna and defining the lower arm; A: autopod, comprising the ensemble of carpus and digits).

Fig. 4. *Hoxa* and *Hoxd* expression in the developing limb.
Two phases of expression of *Hoxa* and *Hoxd* genes. During phase 1, genes are activated sequentially in both clusters,, following the same general

collinear strategy, with 'posterior' genes (e.g. group 13) transcribed in

progressively more posterior cells of the limb buds. During the second (late)

phase, *Hoxa* and *Hoxd* patterns are still quite comparable, but with obvious

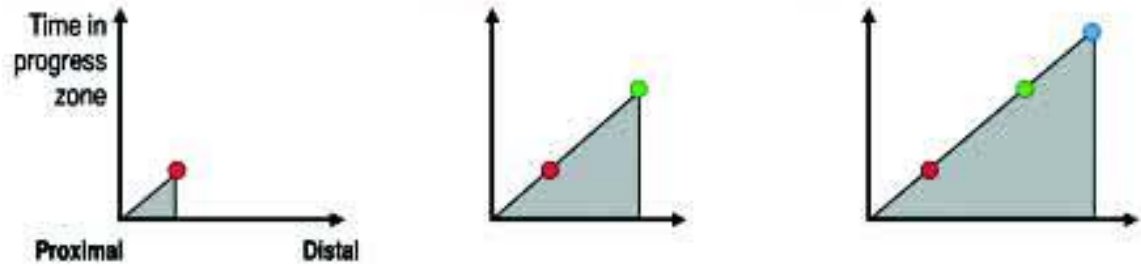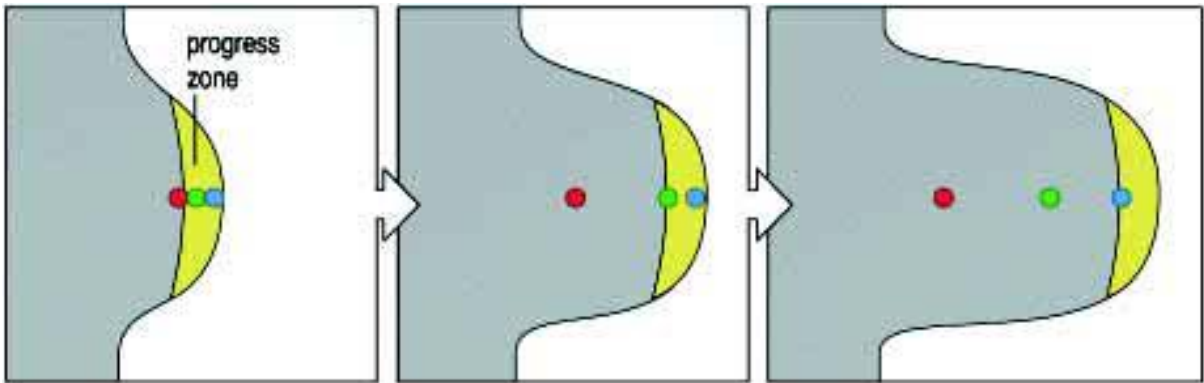differences, suggesting that different regulations are now implemented

Figures

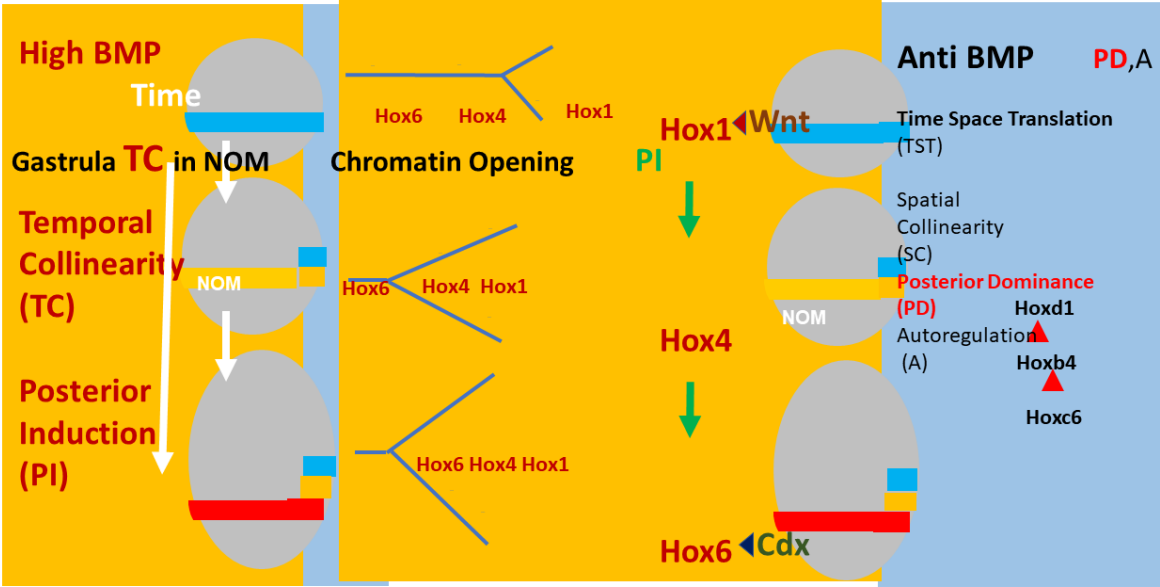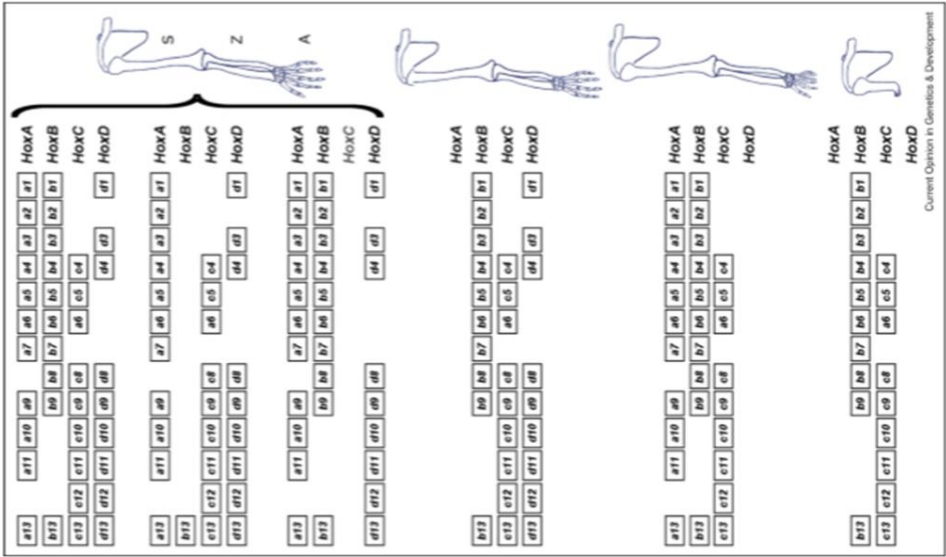## Fig. 1

Fig. 2

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Fig, 3

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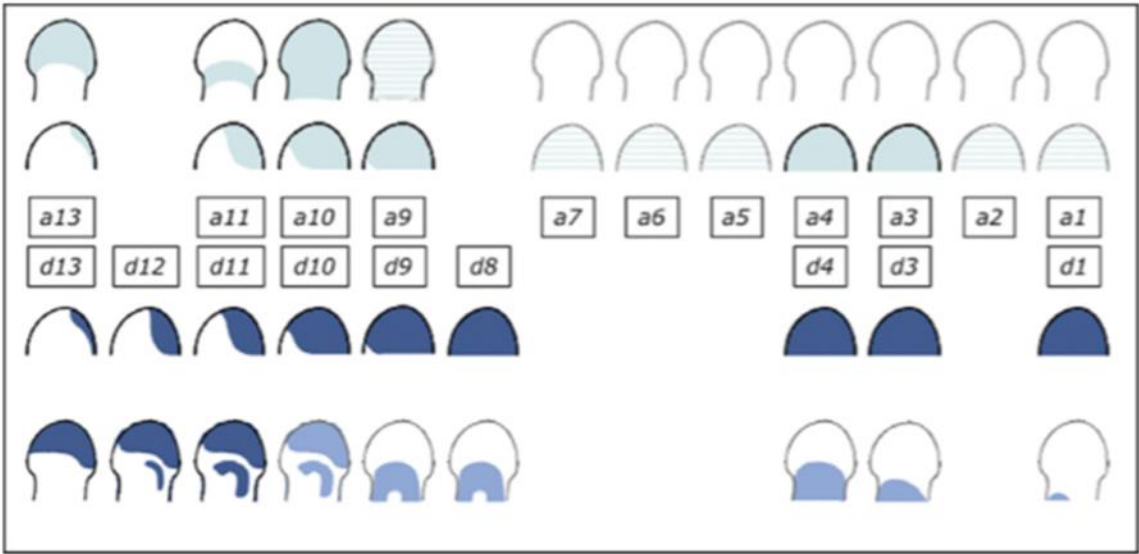
Table 1: Common features, between main body A-P axis and limb P-D axis development

	A-P main axis	PD-AP Limb
Characteristic		
Morphogens		
FGF	+ (Hox6)	+ (AER)
Retinoid	+ (Hox1)	+ (proximal)
Wnt	+	+
BMP	+ Axis connection D-V-A-P	+ Axis connectio D-V-P-D
Chromatin		
Histone acetylation	+	+
Hox genes	+ (1-13) TC,SC.	+ (9-13) TC, SC.
Timer	+	+
Stage dependence	Early anterior	Early proximal