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The (unusual) heuristic value of *Hox* gene clusters; a matter of time?

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ABSTRACT

Ever since their first report in 1984, Antennapedia-type homeobox (*Hox)* genes have been involved in such a series of interesting observations, in particular due to their conserved clustered organization between vertebrates and arthropods, that one may legitimately wonder about the origin of this heuristic value. In this essay, I first consider different examples where *Hox* gene clusters have been instrumental in providing conceptual advances, taken from various fields of research and mostly involving vertebrate embryos. These examples touch upon our understanding of genomic evolution, the revisiting of 19th century views on the relationships between development and evolution and the building of a new framework to understand long-range and pleiotropic gene regulation during development. I then discuss whether the high value of the *Hox* gene family, when considered as an epistemic object, is related to its clustered structure (and the absence thereof in some animal species) and, if so, what is it in such particular genetic oddities that made them so generous in providing the scientific community with interesting information.

In 1978, Nature published a remarkable article entitled '*A gene complex controlling segmentation in Drosophila*', authored by Ed B. Lewis (Lewis (1978). The huge impact of this contribution on our way of thinking about development and evolution was recognized by the Nobel prize in 1995. In the meantime, this publication rapidly became a nightmare for any student asked to present it to the departmental journal club, due to the complexity of the underlying genetics and mutant stocks. The work was concerned with a mutational analysis of the *Drosophila Bithorax* gene complex (BX-C), which was reported to have '*a minimum of eight genes*', which would be activated differentially along the anterior to posterior embryonic body axis following an *in-cis* gradient of affinities for a repressor activity. Subsequently, it turned out that only three bona fide homeotic genes operate in this DNA interval (Morata and Lawrence, 2022; Sanchez-Herrero et al., 1985) but complemented by additional long-acting *cis*-regulatory sequences, whose perturbation would impact the expression of these genes in various embryonic and larval segments (Karch et al., 1985), reviewed in (Maeda and Karch, 2009; Peifer et al., 1987). In this respect, Ed Lewis's theoretical view of the system was closer to the vertebrate situation, where about eight genes indeed correspond to the 'BX-C' part of a full *Hox* gene cluster (see below) (Duboule, 1992; Izpisua-Belmonte et al., 1991).

Three years after this publication, I had the chance as a PhD student to attend the 1981 ISDB meeting in Basel, Switzerland, where Ed Lewis presented this work. In the same session, David Hogness reported the efforts of his laboratory to clone the *Drosophila* BX-C through a chromosome walk starting from an inversion breakpoint mapping into BX-C (Bender et al., 1983a; Bender et al., 1983b). This again was a complicated piece of work, mixing genetic and molecular terms but, as many people who were in the audience I suppose, I left the session with the feeling that something important was happening; on the one hand, a genetic approach was reported, that had revealed the existence of a series of genes involved in the organization of body structures. On the other hand, the possibility to characterize these developmental determinants at the molecular level was close to materialize. This was opening the door to a molecular genetic analysis of development, in particular soon after the landmark results of the embryonic lethal screen carried out in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980), which had started to decipher the genetic control of early embryogenesis in flies.

1. A brief, incomplete and biased history of *Hox* clusters

The field moved ahead in 1984 after DNA clones obtained from the *Antennapedia* locus (ANT-C) (Garber et al., 1983), the second cluster of homeotic genes in flies, were cross-hybridized at low stringency and

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revealed the repeated presence of a particular DNA 'H' sequence, the homeobox (McGinnis et al., 1984b). In addition, a cross-hybridizing signal was detected when using a DNA clone from BX-C obtained through the chromosome walk (Bender et al., 1983a). The impact of this discovery from the Gehring's laboratory, which was also reported by Scott and Weiner using a similar approach (Scott and Weiner, 1984) following the work of the Kaufman laboratory on the ANT-C (Scott et al., 1983; Wakimoto and Kaufman, 1981), was immediate and has been discussed in many instances (e.g. (Akam, 1984). Yet two points are of special interest in the context of this short essay, both related to the presence of a homeobox in all homeotic genes belonging either to ANT-C or to BX-C. First, it confirmed the hypothesis proposed by Lewis that BX-C originated through a series of gene duplications that would have stayed clustered together as a way to achieve a coordinated function during the development of the fly (Lewis, 1998). Secondly, it strengthened the proposal (McGinnis et al., 1984b) that ANT-C and BX-C originated from one large ancestral gene cluster, an hypothesis that was unequivocally demonstrated through the subsequent characterization of the full vertebrate *Hox* clusters (see (Akam, 1989), after homeobox sequences had been detected in mice (McGinnis et al., 1984a) and the first vertebrate homeobox-containing gene cloned in *Xenopus* (Carrasco et al., 1984).

In vertebrates, clusters of *Hox* genes of different extents were initially isolated by using either lambda (Acampora et al., 1987; Colberg-Poley, 1985; Hart et al., 1987) or cosmid (Duboule et al., 1986) clones and more genes were rapidly added to the list and linked to particular groups. As various clusters began to be characterized, high sequence similarities between genes mapping on distinct loci led to the idea that clusters had duplicated and that some homeobox genes had paralogous counterparts, either in mice (Hart et al., 1987) or in human (Boncinelli et al., 1988). The final number of four clusters in mammals had nevertheless to await some refinement and was fixed by the early-mid 90's (Duboule, 1994; Scott, 1993), with the complete structure of the *HoxB* cluster finalized a few years later (Zeltser et al., 1996). While the hypothesis of a structural conservation between the mammalian and *Drosophila Hox* clusters, and hence their common ancestral origin, was put forward by Boncinelli and

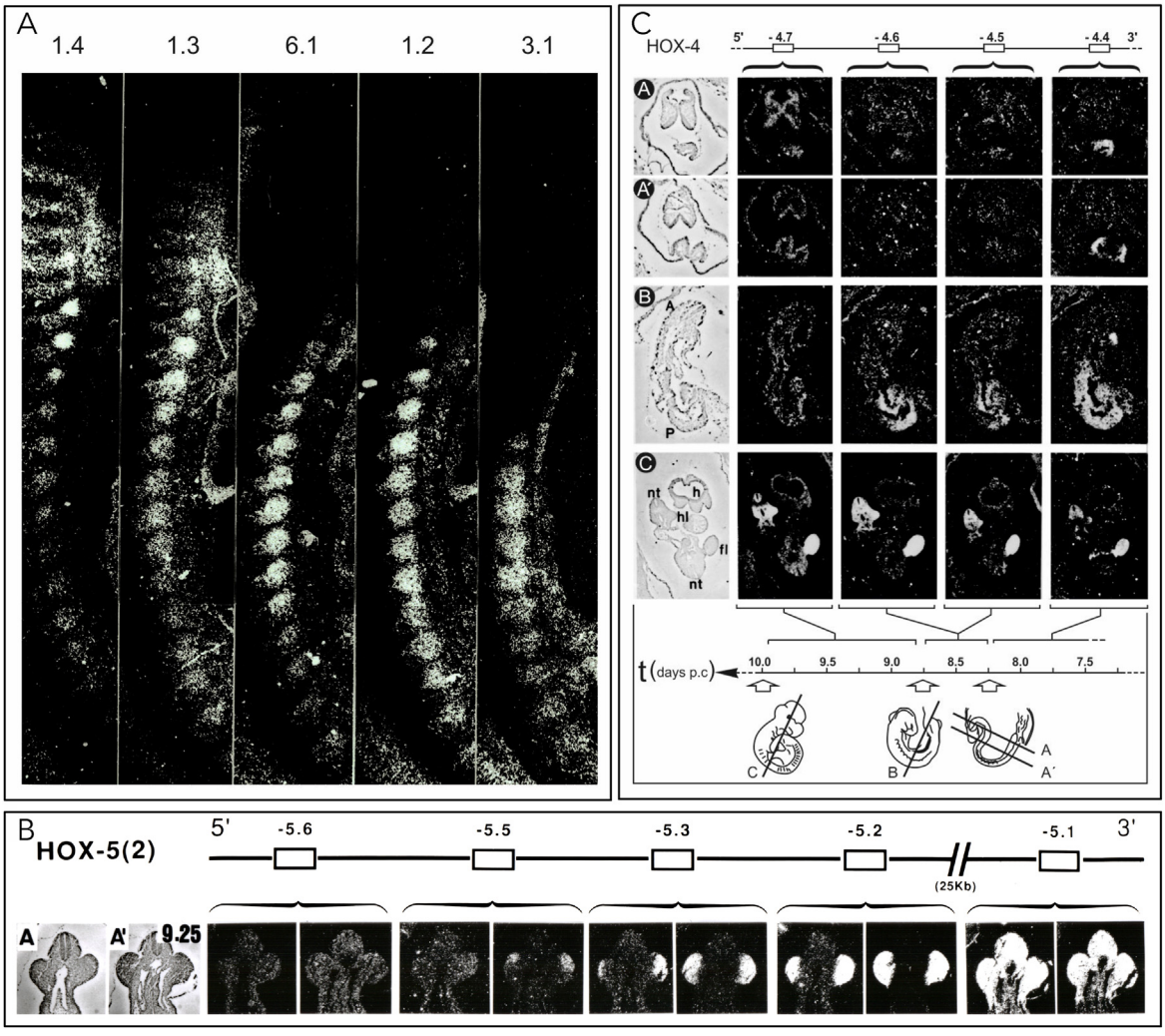

Fig. 1. Spatial and temporal colinearities in vertebrates. A. Original picture describing spatial colinearity along the developing sclerotomes of a 12.5 old mouse fetus (serial sections). Genes are referred to with an old nomenclature and are, from left to right: *Hoxa4*, *Hoxa5*, *Hoxc6*, *Hoxa6* and *Hoxc8*. Expression starts are progressively more caudal levels, except for those two genes belonging to the same group of paralogy. Figure reproduced from Gaunt et al., 1988, Development 104 (supp), 169–179. **B**. Temporal colinearity as initially observed in developing limb buds of a 9.25 days old embryo, serial sections. Genes are referred to with an old nomenclature (different from that in panel **A**) and are from left to right: *Hoxd12*, *Hoxd11*, *Hoxd10*, *Hoxd9 and Hoxd4*. *Hoxd12* transcripts have not yet appeared at this stage. Panel reprinted from Fig. 1 of Dolle et al., 1989), Nature, 342, 767–772. **C**. Temporal colinearity initially observed in serial sections of embryos at three different ages (bottom), stained for four different genes by *in situ* hybridization. Genes are referred to with an old nomenclature (different from that in both panels **A** and **B**) and are from left to right: *Hoxd12*, *Hoxd11*, *Hoxd10* and *Hoxd9*. Transcripts for these genes appear successively along with older embryonic stages. Figure reprinted from Izpisua-Belmonte et al., The EMBO J., 10, 2279–2289.

colleagues in 1988, the implementation of the same functional logic between mammals and insects was not yet formalized: '*Expression analysis in vertebrates is still at a very preliminary stage and does not allow a conclusive statement on a functional other than structural parallelism between Drosophila and vertebrate homeobox complex loci.*' (Boncinelli et al., 1988). Such expression analyses of homeobox genes in mammals had nevertheless started to reveal localized expression patterns, suggestive of a function during development (Awgulewitsch et al., 1986; Gaunt et al., 1986), as early as in gastrulating embryos (Gaunt, 1987).

2. Colinearity goes vertebrate

It is only in 1988 that Gaunt, Sharpe and Duboule were eventually in a position to analyze the expression of series of homeobox-containing genes in-*cis*, using serial histological sections and could thus demonstrate the correspondence between their expression along the AP axis, on the one hand, and their position within their respective clusters, on the other hand (Gaunt et al., 1988) (Fig. 1A) (for a personal account, see (Gaunt, 2019). This illustrated the conservation in mice of the colinear property initially reported by Lewis genetically and further documented in *Drosophila* using molecular probes for *in situ* hybridization (Harding et al., 1985). The cloning and expression analysis of the murine genes lying at the extremities of the orthologous *Drosophila* clusters, i.e., the *labial* (Baron et al., 1987; Mlodzik et al., 1988) and several *Abd-B* (Duboule and Dolle, 1989; Graham et al., 1989; Izpisua-Belmonte et al., 1991; Regulski, 1985) orthologues allowed for a more complete picture to emerge; not only homeobox genes were conserved between mouse and *Drosophila*, not only their colinear expression in space had been maintained throughout evolution, but the entire functional organization of this gene family had been somewhat conserved, with four clusters in mammals and two half-clusters in flies (Duboule and Dolle, 1989; Graham et al., 1989) (Akam, 1989). These observations suggested that the hypothesized ancestral cluster (Boncinelli et al., 1988; McGinnis et al., 1984a) already implemented this peculiar colinear expression strategy. A representation of such a large and unique *Hox* gene cluster was provided some years later by the cloning of the amphioxus counterpart, which revealed one single large group of *Hox* genes clearly orthologous to both the *Drosophila* and mammalian complements (Garcia-Fernàndez and Holland, 1994). Of note, this cluster was subsequently reported even to display some of the general principles of long range gene regulation that had been observed either with the mouse or with the fish counterparts (Acemel et al., 2016; Andrey et al., 2013; Woltering et al., 2014).

Expression analyses in mice also revealed that a comparable colinear regulatory process had been co-opted in the context of the developing limb buds, i.e., during the extension of secondary body axes (Dolle et al., 1989; Lewis and Martin, 1989), which also require patterning cues along with their distal extension (Tabin and Wolpert, 2007). During this set of experiments, another type of colinear relationship was uncovered involving a time sequence in *Hox* gene activation that follows the position of genes in the clusters (temporal colinearity (Dolle et al., 1989) (Fig. 1B). This additional property initially associated with vertebrate *Hox* clusters and not observed in *Drosophila* (see below), was subsequently reported to be implemented during the extension of the major vertebrate body axis (Izpisua-Belmonte et al., 1991) (Fig. 1C). A likely related colinear response was scored when cultured embryonal carcinoma (EC) cells were challenged with various doses of retinoic acid (Simeone et al., 1990), thus illustrating the intrinsic capacity of these gene clusters to respond to external signals in a coordinated manner based on the gene's topology.

3. A booster for Evo-Devo

While they pioneered these exciting developments, the thrill of this period was not only caused by homeobox genes and many more gene families were isolated, not only coding for transcription factors but also for various members of transduction pathways and others proteins of critical developmental interest. This avalanche of new molecular data from different species and the nascent perception that developmental molecular processes were of a universal nature led to the opening of a new chapter in the long-lasting interactions between ontogeny and phylogeny, the revival of an occasionally tumultuous relationship (see e.g. (Duboule, 2010; Gould, 1977; Jaeger et al., 2015), which was referred to as Evo-Devo (see for example (Arthur, 2002; De Robertis, 2008). Undoubtedly however, the work initiated by Ed Lewis catalyzed this revolution and one may wonder whether, in addition to his outstanding contributions, the historical heritage and the scientific context (see (Lewis, 1998), the model system in itself (the *Hox* cluster) may not possess an unusual epistemic value, an ontological property that made it prone to deliver these scientific contributions, much in the same way the *Drosophila* animal model did at the level of developmental processes (e.g., (Jennings, 2011; Martinez-Arias, 2008; Morata and Lawrence, 2022).

While the spectacular nature of homeotic mutations partly explains their interest and historical importance, it is indeed the existence of several related *bx* mutations in-*cis* and the hope to find duplicated genes (see (Duncan and Montgomery, 2002), which triggered Lewis's interest to study this particular locus: '*It soon became evident that the diverse array of existing mutations of the bithorax type held considerable promise of being a cluster of genes rather than a multiple allelic series. It was for this reason that they were chosen for study …* ' (Lewis, 1998). Also, the *cis*-linkage helped to make sense of the hybridization signals obtained when trying to clone homeotic genes and to assign them to either the ANT-C or BX-C clusters (McGinnis et al., 1984b). Ever since these starting points, the *Hox* gene cluster has contributed to a series of interesting observations and concepts, many of which could be subsequently generalized. A few selected examples are discussed below, taken from work carried out on vertebrate *Hox* clusters by many laboratories, which address developmental, evolutionary, structural as well as regulatory issues. Excluded from this short list due to space constraints are some key general principles acquired by studying the function and regulation of *Hox* genes, regardless of how interesting they may be. They include the conservation of HOX 'homeotic' (e.g. (Kessel and Gruss, 1991) (Condie and Capecchi, 1993) and/or 'atavistic' (Dolle et al., 1993) functions in mammals, the concepts of functional redundancy or complementarity between paralogs, mostly through the work of the Capecchi laboratory (e.g. (Condie and Capecchi, 1994; Davis et al., 1995) (Gaunt et al., 1989) and of posterior prevalence or phenotypic suppression (Bachiller et al., 1994; Duboule and Morata, 1994; Gonzalez-Reyes et al., 1990), the transduction of homeoprotein (Prochiantz and Di Nardo, 2022), the functional exchangeability either between mammalian paralogs (Greer et al., 2000; Tvrdik and Capecchi, 2006) or between insect and mammalian HOX orthologous proteins (Zhao et al., 1993) and the dissection of the evolutionary and functional importance of HOX protein binding sites (Crocker et al., 2015; Desplan et al., 1988; Kribelbauer et al., 2019), to name a few. Also, the fruitful use of *Hox* clusters to revisit the evolutionary fin to limb transition (e.g. (Davis et al., 2007; Freitas et al., 2007; Kherdjemil et al., 2016; Sordino et al., 1995; Woltering and Duboule, 2010) cannot be discussed here due to the large amount of related datasets and concepts, ever since the first report of *Hox* expression in developing limb buds (Oliver et al., 1988).

4. *Hox* clusters as time machines?

Spatial colinearity, i.e., the correspondence between the relative positions of *Hox* genes within their cluster(s) and the rostral to caudal distribution of their transcript domains (Fig. 1A) is a hallmark of *Hox* gene regulation in most animals displaying a bilateral symmetry (see (Gaunt, 2019) and a central mechanism during animal development. While this process may appear at first as a regulatory curiosity, it may in fact merely reflect a parsimonious way to use series of related genes to help specify a meristic system. At a mechanistic level, if one considers a chromosomal alignment of genes, which may all respond to similar types of upstream regulations, a colinear-like mechanism may be more

straightforward to evolve than any other, i.e., a mechanism that will start activating one gene located somewhere followed by the spreading of transcription in a directional manner, due either to the structure of chromatin, to fundamental mechanisms associated with the basal transcription machinery or/and to a coordinated response to a graded signaling input (e.g. (Afzal and Krumlauf, 2022). Recent work from many laboratories has given some hints as to how this may happen, for example by the successive release of a repressive structure that is usually found at developmental genomic loci before they become switched on, for instance in ES cells (Bernstein et al., 2005). This general principle is not novel and was already proposed by Emil Zuckerkandl by analogy with position effect variegation, another *cis*-acting phenomenon: '*Such a molecular spreading effect appeared potentially applicable to gene complexes whose member genes are transcriptionally activated or inactivated in the order of their occurrence on the chromosome …* ' (Zuckerkandl, 1990), mentioning as examples the globin gene clusters (see the last item below) and BX-C.

However, the textbook view of the evolutionary conservation of *Hox* clusters, which was initially associated with spatial colinearity considerably changed over the years and we now have a more complete account of the various genomic organizations of this gene family, from a more or less tight clustering to a complete gene dispersion, with various split versions as 'intermediate' conditions (refs in (Duboule, 2007). Interestingly, the kind of genomic organization observed at *Hox* loci in a particular species can hardly be associated with its phylogenetic position and both lophotrochozoans and ecdysozoans contain various types of *Hox* (non-) clusters (e.g. (Ferrier and Holland, 2002). The only robust association that can be observed involves the type of development at work in any given animal taxon. Indeed, all animals developing according to an anterior to posterior morphological progression in time do have a single, non-split *Hox* gene cluster, whereas animals developing according to a time-independent mechanism to produce their main body axis were licensed to split their clusters or even to disperse their *Hox* genes throughout their genomes (Duboule, 2007).

5. The *Hox* conjecture

As a consequence, the reason to keep *Hox* genes fully clustered may not be solely related to spatial colinearity. Instead, strict clustering may be constrained by the deployment over time of this colinear pattern. Accordingly, a *Hox* conjecture was proposed some years ago stating that all animals developing according to an AP time sequence *must* have a complete *Hox* cluster, whereas animals using different developmental modes will have broken their *Hox* gene complement, at least in part (Duboule, 1994; Duboule, 1992) (Ferrier and Holland, 2002). Thus far, after sequencing the genomes of all major animal groups, this conjecture has not yet been proved wrong. However, while the time sequence in the activation of *Hox* genes has been well documented in essentially all vertebrate groups [see however (Durston, 2019; Kondo et al., 2019) for anurans], its causal importance in organizing the colinear sequence of expression domains remains to be functionally demonstrated through perturbation experiments. In this respect, the hypothesis that *Hox* clusters are little machines used to translate time into space (D. Duboule, 1994; Duboule, 2007; Durston et al., 2012; Durston, 2019) (discussed also in (Gaunt, 2015) must still be considered with some caution until the definitive datasets are available. The temporal mechanism at work will have to be understood sufficiently well to allow targeted modifications of its pace to be induced under physiological conditions. This mechanism was initially referred to as the '*Hox* clock' (Duboule, 1994), yet it may rather be considered as a '*Hox* timer' due to its non-recursive structure.

6. *Hox* gene clusters and the evolution of genomes

Hox gene clusters have been used as paradigms in a variety of scientific contexts, including the evolution of genomes and the status of the bilateria ancestor, the interface between development and evolution, as well as the study of developmental gene regulation. As a first illustration, the one to four ratio in the number of *Hox* clusters from ancestral forms to most extant vertebrates was a key element in the re-interpretation of Ohno's hypothesis that vertebrates had evolved through an increase of genome ploidy and neo-functionalization processes following gene duplications (Ohno, 1970). Ohno had hypothesized duplications occurring much before the emergence of vertebrates, yet the existence of the single cluster in amphioxus (Garcia-Fernàndez and Holland, 1994), alongside the global analysis of paralogous relationships (Lundin, 1993), led to both a re-evaluation of genome duplications timing and to the demonstration that duplications were not restricted to *Hox* clusters alone (Holland et al., 1994). Also, the additional genome duplication experienced by teleost fishes was first hinted at by the structure of the *Hox* clusters complement (Amores et al., 1998). At the same time, the clarification of these paralogous relationships, largely triggered by comparing *Hox* clusters, allowed to start throwing a new light on the deep genomic evolution of gene families (see (Garcia-Fernandez, 2005; Holland et al., 1994).

The presence of *Hox* genes in animals, which were phylogenetically even more distant from vertebrates than arthropods (e.g. (Gauchat et al., 2000) and their orthologous relationships based on the prototypic *Hox* cluster was also a critical factor in the re-assessment of animal phylogeny at large, even though it mostly confirmed a then emerging picture based on ribosomal DNA (Aguinaldo et al., 1997). Indeed, the fact that ancestors of each of the two major protostome lineages contained from eight to ten *Hox* genes suggested that the critical period of *Hox* gene duplication and diversification had occurred before the radiation of the three large bilaterian clades (Balavoine et al., 2002; de Rosa et al., 1999), thus supporting the reorganization of protostome metazoans into lophotrochozoans and ecdysozoans (Aguinaldo et al., 1997) (see (Martindale and Kourakis, 1999).

7. The zootype, the urbilateria, the phylotypic stage and Haeckel's pharyngulas

Besides the use of *Hox* clusters to help understand animal phylogeny, this group of genes was also the central element in formalizing the concept of the zootype, i.e., a tentative definition of what an animal is, based on a subset of essential gene expression patterns at a given stage defined as the phylotypic stage (Slack et al., 1993). In their essay, the authors proposed that a '*…system of gene expression patterns comprising the Hox cluster type genes and some others do encode relative position in all animals …. We now suggest that this character should be adopted as (…) a defining character, or synapomorphy of the kingdom Animalia*' (Slack et al., 1993) [it should be noted here that in their view, the defining character was the expression at this stage of those *Hox* genes *present in clusters* in flies and vertebrates, rather than the *existence* of a cluster, which later on turned out to be absent from many animal groups]. Likewise, when De Robertis and Sasai proposed a definition of what the 'urbilateria' ancestor animal at the origin of both vertebrates and arthropods may have looked like, they listed as the first criteria out of five: ' … *an anteroposterior polarity determined by the Hox gene complexes*' (De Robertis and Sasai, 1996), even though their main argument was concerned with a molecular interpretation of the inversion in dorso-ventral polarity, as initially proposed by Geoffroy Saint-Hilaire in 1822 (see (Arendt and Nübler-Jung, 1994). Subsequent genomic analyses revealed that the *Hox* cluster in urbilaterians probably had a larger structure (a 'super-*Hox* cluster') including additional 'ancestral' homeobox genes (Butts et al., 2008).

In 1874, the anatomist Ernst Haeckel, as part of his controversial theory of recapitulation, reported drawings illustrating how vertebrate embryos at the pharyngula stage (Ballard, 1981) resemble each other more than during subsequent developmental stages (Haeckel, 1877). This observation was an extension of the fundamental biological law proposed by Karl Ernst Von Baer in 1828 stating that during animal development, the more general characters of a given group of animals appear earlier in the embryos than more specialized characters (see

discussion and refs in (Abzhanov, 2013). While some doubts were understandably raised (e.g. (Richardson et al., 1997) concerning the acuity of Haeckel's drawings [which could nonetheless be used '*...as phylogenetic hypotheses, teaching aids and evidence for evolution* (Richardson and Keuck, 2002)..] or on the concept itself of a higher 'conservation' of vertebrate embryos at this particular stage (Bininda-Emonds et al., 2003) (Müller and Dallas, 1869) cited in (Richardson, 2022), this observation is generally considered as valid nowadays, despite a level of similarities admittedly below that presented in the original drawings (those copied into the magnifying glass of Fig. 2).

8. The developmental hourglass model

This particular stage where embryos look alike corresponds to what Seidel in 1960 had referred to as the *Körpergrundgestalt*, i.e., a developmental stage in all animal groups, where the body plan is laid down and thus where the future characteristic of an animal group can be seen or somehow anticipated, a stage which was subsequently called the 'phylotypic stage' by Sander in 1983 (for references and discussion, see (Abzhanov, 2013; Slack et al., 1993). As pointed out in these latter contributions, embryos before reaching this particular stage can be very diverse in their appearance, due to their particular environmental conditions. For instance vertebrate embryos can be laid down in water, grown into an egg or implanted into a uterus (Sheng et al., 2021). These early stages were not considered in Von Baer's time as proper embryos (but as 'the germ') and hence there were not integrated into his set of laws, an aspect that currently requires some 'refurbishing' according to (Abzhanov, 2013).

A proper consideration of these 'germs' for what they are, i.e., genuine embryos (regardless of the maternal contribution) led to the vertebrate hourglass model of development whereby all kinds of embryos, irrespective of their shapes and environments transit through this particular period of resemblance, a transition during which they somehow express their belonging to this taxon before starting to materialize species-specific traits (Duboule, 1994) (Fig. 2). The vertebrate developmental hourglass, initially suggested in the writings of His (1875), was subsequently supported by molecular datasets, in particular by a quantitative comparative transcriptome analysis of several vertebrate embryos, showing that the pharyngula stage was more conserved than others when RNAs were taken as readouts (Domazet-Loso and Tautz, 2010; Irie and Kuratani, 2014, 2011; Prud'homme and Gompel, 2010). While the developmental hourglass structure was extended to other animal groups, in agreement with Seidel's early proposal (Kalinka et al., 2010) (discussed in (Richardson, 2012), the question still remains as to what causes the neck of the hourglass, i.e., what does constrain the embryos to express these shared anatomical and molecular features during this specific period. This fundamental question was already asked by His in 1875: '*...the most pressing question is how, from such different developmental starting points, do the similarities in subsequent stages* […] *arise*' (His, 1875), translated and discussed in (Richardson and Keuck, 2022).

The notion of developmental constrains (e.g. (Galis et al., 2018) is old and has multiple roots (see (Dietrich, 2003; Whyte, 1960) and discussions in (Alberch, 1989; Gould, 2002). In the case of the vertebrate hourglass, it is likely that constrains at work apply to (a) particular mechanism(s) that is (are) implemented when passing through the neck, a process that would be less amenable to variations than those occurring either earlier or later. This process would be implemented during a time interval when the adaptability of embryos in response to environmental factors may be traded off against this mechanistic step, which may have little adaptability due to high internal constraints (Alberch, 1989; Gould, 1977) (Fig. 2). When thinking about such a mechanism, it is important to define precisely the onset and duration of the hourglass narrowing (neck) in vertebrates. This period, described as snapshots in Haeckel's drawings, can indeed last from several hours to several days. Therefore, the neck of the hourglass does not correspond to any particular developmental (phylotypic) stage (Slack et al., 1993) but instead, to the deployment in time of a dynamic process and hence the reference to a 'phylotypic

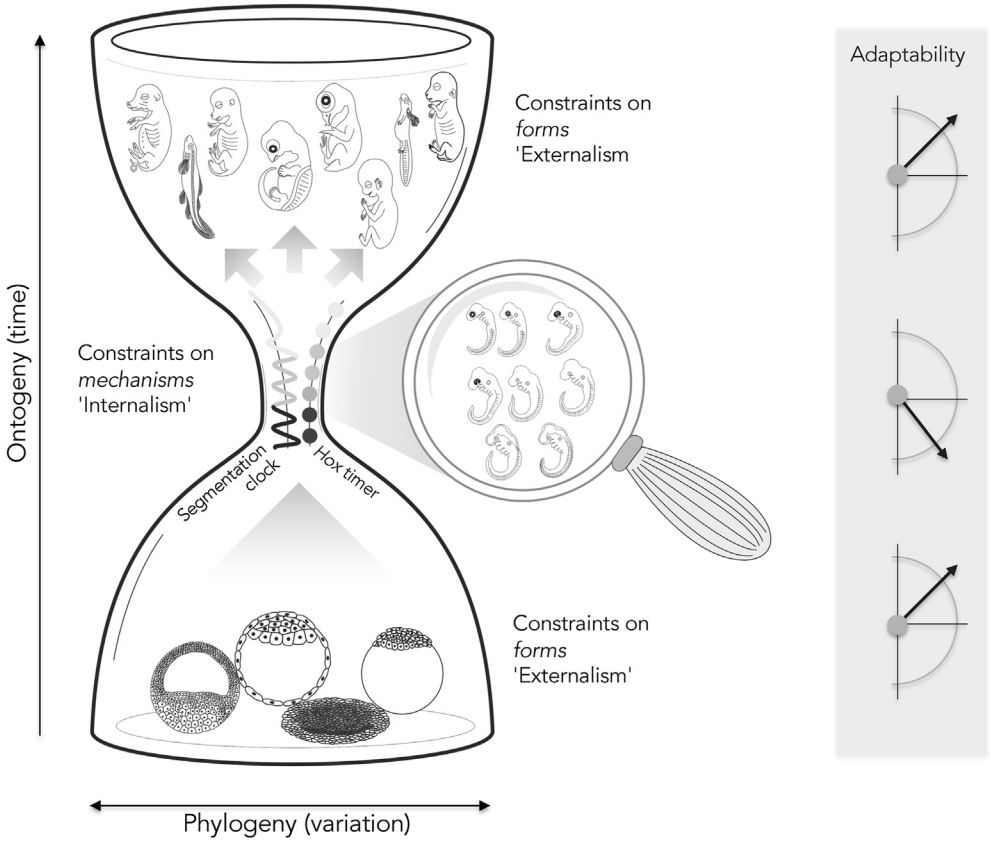

Fig. 2. The vertebrate developmental hourglass model. The horizontal (phylogeny) axis represents the 'amount' of observed variation and the vertical (ontogeny) axis reflect the developmental sequences (not on relative scale). The bottleneck illustrates the developmental period where embryos are most similar to one another, with Haeckel's original drawings copied into the magnifying glass (Haeckel, 1877). The neck of the hourglass corresponds to the coordinated implementation of two time-regulated mechanisms, the segmentation clock (an *in-trans* oscillation) and the *Hox* timer (an *in-cis* progression). This overall process is highly resistant to variation due to the precise crosstalk between two complex mechanisms (modules *sensu* (Raff, 1996), kernels *sensu* (Davidson and Erwin, 2006) and thus represents an obligatory passage for any vertebrate embryo. At these stages, the constraints on internal mechanisms are maximal ('internalism' mode according to (Alberch, 1989) and the 'adaptability minimal. Before and after the hourglass neck, constraints will be mostly on the 'form', with high adaptability to environmental factors ('externalism'). The upper part of the bottleneck is slightly widening to illustrate the progressive loss of precision and higher tolerance for 'variability' in the most posterior pars of the extending trunk axis, according to (Goodrich, 1913). Drawing adapted from (Duboule, 1994).

progression' (Duboule, 1994). Morphologically speaking, this progression corresponds to the laying down of the segmented part of the body plan, from late gastrulation to the end of trunk extension.

9. The nature of constraints

The nature of this (these) constraining mechanism(s) has been subject to various interpretations and there is no reason *a priori* to think that a unique and universal process may impose the same constrains to embryos belonging to all animal groups. Therefore, each group may have evolved its specific phylotypic progression, if any (Richardson, 2012). In vertebrates, where the hourglass was initially proposed, the implementation of temporal colinearity in the expression of *Hox* genes was suggested to be at the core of the neck structure, for the potential evolvability of this mechanism is likely very low. The *in-cis* nature of the underlying regulation, i.e., the fact that the timing of gene activation follows the DNA topology at four distinct loci, makes it understandably difficult to evolve towards a system acting more *in-trans* (for example solely based on signaling molecules and transcription factors), which may be naturally more prone to variation (Duboule, 1994). Adopting a more general view, Raff explained the neck of the hourglass by a period of maximal inter-connectivity between developmental 'modules' (Raff, 1996), i.e., a stage where the interactions and linkages between various independent developmental mechanisms are at their highest thus increasing their non-evolvability.

A related explanation was provided using a system developmental biology approach (and vocabulary) by Davidson and Erwin who proposed the existence of a particularly central class of gene regulatory network components, the 'kernels'. Kernels were defined as highly conserved and densely cross-regulating gene circuits (see (Rothenberg, 2016), likely similar to the most central 'modules' according to Raff, which '*...because of their developmental role and their particular internal structure are most impervious to change*' (Davidson and Erwin, 2006). The conservation of body plans observed during the phylotypic progression may thus derive from the retention since pre-Cambrian time of particular kernels. In fact, the latter definition may somewhat include the former two, provided that 1) the timed activation of *Hox* genes, i.e., the full mechanism including upstream factors be considered as a 'kernel' (it isn't clear whether it really fulfills all criteria thereof-) and 2) this module tightly interacts with at least another well-conserved genetic 'module' implemented during this period, which would also be impervious to change.

10. When a timer meets a clock

The nature of this potential second 'kernel' in vertebrates is somehow suggested by Haeckel's drawings of pharyngulas, which include traces of iterative structures, the somites i.e., the visible expression of the segmented organization of the vertebrate body plan (Stern and Keynes, 1988). The mechanism underlying the segmentation clock (see the article by the Pourquié laboratory) may indeed be taken as such a module, if we consider the *Her* autoregulatory loop and the *Notch* pathway (Hubaud and Pourquié, 2014; Richmond and Oates, 2012), the fact that it is switched on and off by external cues (Hubaud et al., 2017) and that it is relatively well conserved throughout vertebrates (Krol et al., 2011). Within each vertebrate species, the specification along the AP axis (take for example the morphology of the spine as a proxy) will depend on the interactions between this clock and the *Hox* timer, the former producing segments in time, whereas the latter assign them identities over time. The former clock has a recurrent structure, which is part of a 'clock and wave front' process (Cooke and Zeeman, 1976) and is controlled by mechanisms *in trans* (Goldbeter and Pourquié, 2008), whereas the latter timer has a linear movement based in part on a mechanism *in cis* (Deschamps and Duboule, 2017; Neijts et al., 2016; Noordermeer et al., 2014). Potential cross-talks between these two time devices, which may interact with one another following distinct temporal modalities and not necessarily in the same initial cellular territories (see (Deschamps and Duboule, 2017; Diaz-Cuadros et al., 2021), were addressed in several occasions (e.g. (Dubrulle et al., 2001; Ten Broek et al., 2012; Zakany et al., 2001). However, while *Wnt* signaling is certainly to be considered (Denans et al., 2015; Neijts et al., 2016; Ye et al., 2021), direct molecular interactions that would help keeping them in phase with one another are still elusive.

11. Konvergenz, Aehnlichkeit, Divergenz

The developmental hourglass illustrates three distinct types of 'interactions' (*sensu* (Raff, 1992) between development and evolution, matching the three successive developmental periods described by Franz Keibel; 'convergence', 'similarity' and 'divergence' (Keibel, 1906). Initially, from fertilization up to gastrulation, vertebrate embryos are well adapted to drastically different environmental conditions and thus display a large diversity of shapes and functionalities. Even the onset of gastrulation, while being driven by seemingly similar molecular determinants, displays large differences within vertebrate classes (Beddington and Smith, 1993; Sheng et al., 2021). Then, regardless of previous differences, the vertebrate embryo converges towards the entry point to its segmentation process, at around the middle of primary gastrulation, the stage at which both the segmentation clock and the *Hox* timer start to enter the game, along with the extension of the main body axis (Fig. 2). This convergence may be due in part to a canalization process from early on, induced by mechano-geometrical constraints associated to the start of gastrulation mechanisms, as proposed in (Steventon et al., 2021). This period is short when compared to the global developmental timing of the embryos, a period during which the body axis is produced and patterned, thus providing the foundations for the third phase when various segments and other anlagen will realize their fates and when fetuses will start expressing their belonging to particular species, rather than to the vertebrate taxon in general.

In this view, the low evolvability of the hourglass neck may be attributed to three main sources of constraints, two on the independent modules at work, and one on their interaction. First the segmentation clock, which as all biological oscillatory systems has some flexibility to modify framework conditions such as its period (Harima et al., 2013; Herrgen et al., 2010; Liao et al., 2016; Matsuda et al., 2020; Schröter and Oates, 2010), but whose core oscillating mechanism and components are admittedly not easy to modify or replace (Goldbeter and Pourquié, 2008; Hirata et al., 2004; Stauber et al., 2012; Takashima et al., 2011). Secondly, the *Hox* timer, which regardless of its underlying mechanism (discussed in (Deschamps and Duboule, 2017) is understandably the most parsimonious way to progressively activate series of related genes within hours (see (Noordermeer et al., 2014). Third, the interlocking between these two modules at their onsets, and their coordination in time throughout their implementation and termination, such as to associate a genetic progression *in-cis* with a 'morphological oscillation' *in-trans* (Fig. 2).

The necessary combination of two poorly evolvable modules makes the core of this general process phylogenetically hyper-stable. An illustration of this may be found when considering the growth of ES cells-derived pseudo-embryos (gastruloids or stembryos, (Turner et al., 2017; Veenvliet et al., 2021). These structures produce a self-organized extension of a 'trunk axis', following symmetry breaking of a *Wnt*-activated ES-cells aggregate, concomitantly with the implementation of both the *Hox* timer (Beccari et al., 2018) and the segmentation clock (van den Brink et al., 2020). This is also observed in the absence of proper somite formations, i.e., in a situation where both modules are implemented despite the absence of the expected morphological outcome (van den Brink et al., 2020). In this case, the relative 'simplification' of the developmental framework conditions in these *in vitro* system may help uncover the most conserved and robust developmental mechanisms (Steventon et al., 2021).

12. A hyper-stable process with high evolutionary potential

In spite of its high conservation, various possibilities exist to modify the outcome of the phylotypic progression, either by changing parameters associated with one or the other of the time devices, or by changing their interactions. Changes in the coordination between these two modules, for example in the relative timings, are expected to lead to variations in the deployment of morphologies along the AP axis due to the distinct morphological impacts of various HOX proteins (e.g. (Wellik, 2009). In fact, either natural or induced effects of such molecular heterochronies have been associated with shifts either in relative axial positions of tagmata in vertebrates (Burke et al., 1995; Gaunt, 1994), or in the position of limb buds (Moreau et al., 2019) (see also (Gaunt, 2019; Kmita and Duboule, 2003). Also, the entry point of both the clock and the timer into the neck of the hourglass must be rather precisely controlled, for the start of processing corresponds to the post-occipital region where morphologies tolerate less variations (e.g., the cervical region) that in more caudal parts of the embryo where interspecies variation is the rule rather than the exception. This can be interpreted as a progressive relaxation of the phasing between both time devices and hence it may reflect the first signs of entering into the upper part of the hourglass. The result can be observed when comparing adult vertebrate skeletons and was noticed by Goodrich: '*Generally, it (..) is more definite and invariable in the anterior than in the posterior region, and in animals composed of few than in those composed of many segments. It is just as if Nature got tired of counting towards the tail end of a developing animal, and as if her arithmetic became uncertain when dealing with large numbers*.' (Goodrich, 1913). This is illustrated by the slight widening of the hourglass when reaching the upper part of the neck (Fig. 2).

As a final and side comment, the parallel implementation of these two processes at the stage(s) considered by Haeckel as the best conserved morphologically speaking, and associated by Slack and colleagues to Sander's phylotypic point (Slack et al., 1993) is somewhat a metaphor of the relationships between development and evolution, which find part of their roots precisely in the German school of anatomists of the 19th century. Indeed, the intersection between these two historical disciplines of life sciences is complicated by the distinct epistemologies associated with the 'iterative' structure of embryonic development *versus* the 'linear' (*sensu* non-iterative) movement of evolution, much like the molecular structures of the two time-devices presumably fixing this conserved developmental transition in vertebrates. The fact that the timer is associated with the *Hox* cluster, i.e., one of the main elements that triggered the re-birth of Evo-Devo in the mid 1980's (see above (De Robertis, 2008; Slack et al., 1993) adds to this curious analogy.

13. *Hox* and Globin gene clusters and the regulatory genome

The last item I would like to discuss concerns the importance of studying gene clusters in the building of an emerging vision of gene regulation in-*cis,* that has progressively developed over the past 25 years. This vision derives in part from the seminal discovery of enhancer sequences (Banerji et al., 1981; Schaffner, 2015) and the fact that such sequences are often positioned at a distance of their target genes, in particular for genes showing complex and multiple developmental regulations (reviewed in (de Laat and Duboule, 2013; Spitz and Furlong, 2012). While some of the initial key observations and more recent mechanistic aspect were collected on particularly paradigmatic single-gene loci, as exemplified by the *Shh* locus (Lettice et al., 2003; Paliou et al., 2019; Ushiki et al., 2021), many principles associated with global gene regulation were in fact collected by studying either the globin or the *Hox* gene clusters.

Vertebrates globin gene clusters (Fritsch et al., 1980) display some structural analogies with *Hox* clusters; they both derive from series of horizontal gene duplications and, in both cases, this genomic history led to the evolution of regulatory mechanisms that needed to integrate in a way or another the presence of multiple target genes differentially expressed over time. In the β-globin and α-globin gene loci, the discovery of a locus control region (LCR (Forrester et al., 1987; Grosveld et al., 1987; Higgs et al., 1990), see (Li et al., 2002) was the first example of a global regulatory region impacting the transcription of several genes in-*cis*. The detailed analysis of this region and of its interactions with its targets further led to substantial advances in our understanding of long-range gene regulation, for example through chromatin looping (Deng et al., 2012; Tolhuis et al., 2002) (Fig. 3), a process that has now been generalized to many gene loci controlled by the action of remote enhancers. In parallel, studies of how neighboring *Hox* genes are transcriptionally regulated led to complementary observations (Afzal and Krumlauf, 2022; Casaca et al., 2018). For example, the fact that intergenic DNA sequences with a high interspecies conservation had enhancer activity (Bieberich et al., 1990; Puschel et al., 1991; Renucci et al., 1992), that many such sequences were necessary for proper gene regulation (Whiting et al., 1991) and that enhancers could either be shared or used competitively between neighbor genes (Sharpe et al., 1998) are nowadays commonly observed phenomenon when studying all kinds of genomic loci. Similar to the globin clusters, the definition of a global control region (GCR) came from studies of long range regulation at the *HoxD* cluster, as well as the definition of a 'regulatory landscape' (Spitz et al., 2003).

Regulatory landscapes flanking *Hox* clusters were shown to contain series of enhancers partly related to one another in their specificities (Andrey et al., 2013; Berlivet et al., 2013; Montavon et al., 2011) over a distance of close to a megabase. The fact that these landscapes matched the extent of Topologically Associating Domains (TADs), a novel layer of chromatin organization reported in 2012 (Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012) by using chromosome conformation capture (Dekker et al., 2013), illustrated that TADs may sometimes be used as large integrated units of global regulation (Andrey et al., 2013; Marinic et al., 2013; Rouco et al., 2021) (Fig. 3). Noteworthy, long-range acting enhancers at *Hox* clusters were initially hypothesized after the analysis of the *Ulnaless* regulatory mutation in mice (Davisson and Cattanach, 1990; Herault et al., 1997; Peichel et al., 1997; Spitz et al., 2003). Likewise, the LCRs of both the β-globin (Kioussis et al., 1983) and α-globin (Nicholls et al., 1987) loci were initially identified due to their deletions in patients suffering from thalassaemia. In both cases, mutations disrupting a multigenic regulatory organization were instrumental in uncovering the nature of regulations that apply to most pleiotropic single gene loci in vertebrates.

14. Different ways to integrate temporal regulations

Globin and *Hox* gene clusters are nevertheless different in almost all respects, including their cellular specificities, the proteins they produce, their phylogenetic history and species distribution. There is however a common trait between the two systems, which –I would argue-is one of the reasons for their past heuristic importance; they do require a regulatory mechanism that integrates a time sequence essential to their functional outcome, even though these mechanisms are fundamentally distinct in all important parameters; the mechanism underlying temporal colinearity generates progressively increased combinatorial *Hox* transcription patterns during early development, within hours (Kmita and Duboule, 2003) (Fig. 3A), whereas the switch mechanism occurring at globin clusters (Liu et al., 2018; Peschle et al., 1985; Wijgerde et al., 1995) results in an exclusive transition in transcription, occurring at precise timepoints during the life of a vertebrate to ensure the best possible efficiency in oxygen transport by blood cells (see (Grosveld et al., 2021; Oudelaar et al., 2021). The latter is achieved by the change in interactions from the fetal to the adult globin gene promoters with the LCR, a strong (super-)enhancer (Hay et al., 2016) that is being positioned onto these promoters by sets of molecular factors (Liu et al., 2018; Vinjamur et al., 2018), likely through the formation of a transcription hub that may have physical properties of a condensate (a hub-condensate (Grosveld et al., 2021) (Fig. 3B). In this case, a switch occurs in the transcription of genes located in-*cis* within the same cell type.

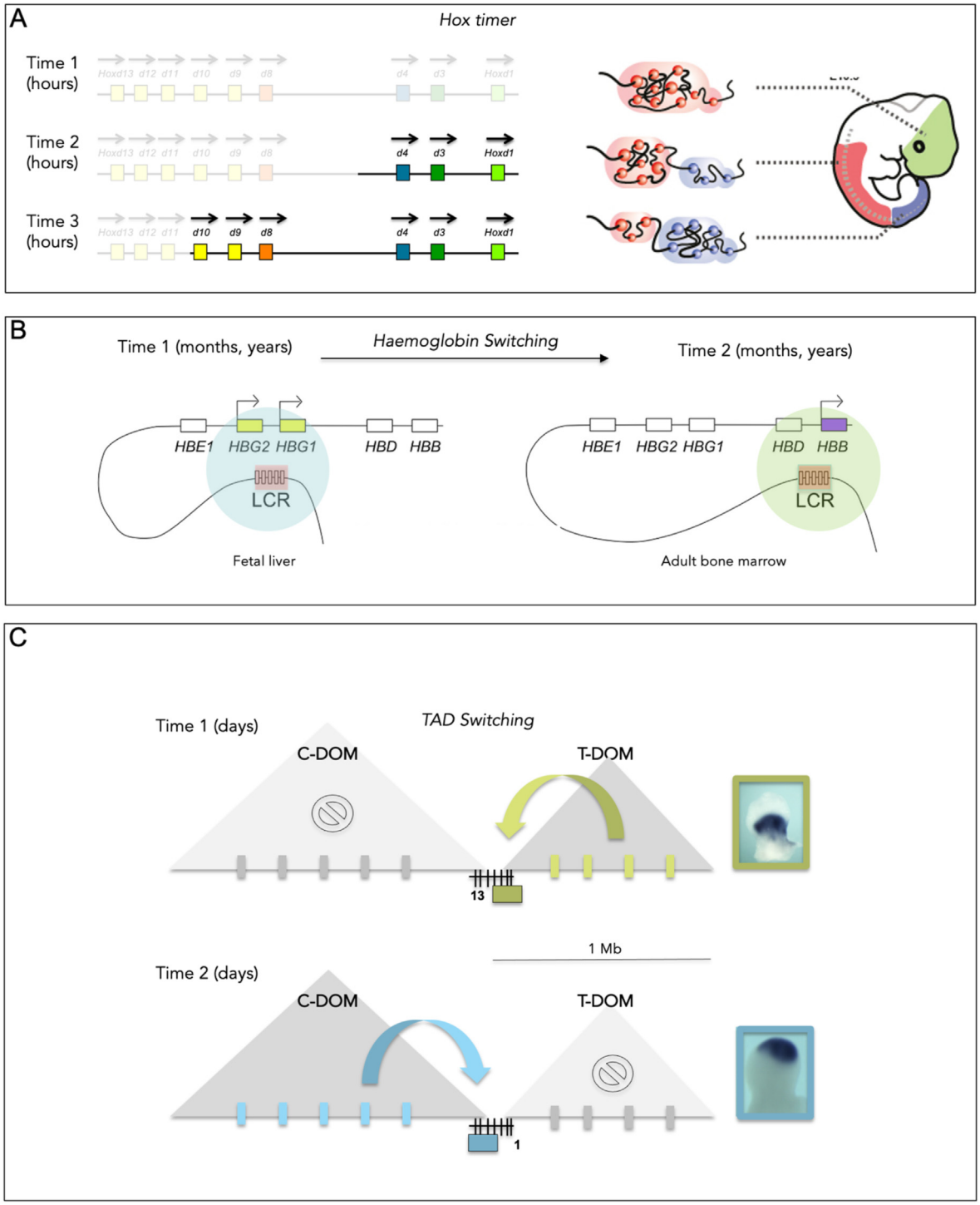

Fig. 3. Various time processes in multigenic regulations. **A**. The *Hox* timer is illustrated in the left with three steps (times 1 to 3) in the colinear activation of the *HoxD* gene cluster during the phylotypic progression. Genes are activated within hours and these various, time-dependent extents in the activation of the gene cluster are memorized at each body level and can be seen as distinct expression territories at later developmental stages (right scheme; modified from (Noordermeer et al., 2011). **B**. Developmental stage-specific gene expression of the β-globin like (haemoglobin switching). Fetal erythroid cells express predominantly the ϒ-globin (yellow boxes), whereas bone marrow derived adult erythroid cells express β-globin (magenta box). In both cases, the Locus Control Region (LCR) acts as a super enhancer, but interacting with different promoters before and after the switch. Due to the presence of specific factors, it forms a transcription hub-condensate (Grosveld et al., 2021), which strongly activates transcription of the target genes (blue and green circles). In this case, the time window is of months to years. The drawing is a modified version of (Vinjamur et al., 2018). **C**. Temporal switch in TAD regulation at *HoxD* during limb bud development. Early on (day 9.5), enhancers (yellow rectangles) located with the telomeric T-DOM TAD and specific for the proximal part of the future limb are active on a series of *Hoxd* genes (large yellow rectangle below the *HoxD* cluster). At this stage, the centromeric C-TAD and its distal enhancers is silent. About 1.5–2 days later, C-TAD enhancers (blue rectangles), which have a distal specificity (picture of the limb buds on the right) are activated and control the transcription of another series of target *Hoxd* genes (large blue rectangle below the *HoxD* cluster). At the same time, the activity of T-DOM enhancers is switched off (Andrey et al., 2013; Beccari et al., 2016). In this case, the time window is in the range of days.

A regulatory switch occurring in the same cells and targeting various subsets of neighboring genes was also described at the *HoxD* cluster during limb bud development, though of a different nature. A first subset of genes is regulated early on by a range of proximal limb enhancers located within the T-DOM TAD flanking the cluster to help organize the proximal parts of the future limbs (Fig. 3C). About 1.5–2 days later, series of distal enhancers located in the TAD lying on the other side of the cluster (C-DOM) are activated, while the T-DOM enhancers are turned off (Fig. 3C) (Andrey et al., 2013; Beccari et al., 2016). This switch occurs between large active and inactive chromatin domains and is thus mechanistically distinct from the case of globins. However, in both instances the switch serves a key developmental function that needs to be regulated in time. The regulatory switch involving *Hox* genes in limb bud cells is also distinct from the temporal colinearity process at work during trunk extension (Fig. 3C), further illustrating the propensity of gene clusters to evolve various regulatory processes with a strong temporal component.

The fetal to adult globin switch was for long causally associated with the LCR itself (Enver et al., 1990), triggering many key experiments to understand how this would work (discussed in (Li et al., 2002). An example was the engineering of inversions at the human β-globin gene cluster in transgenic mice to challenge the timing of their developmental expression (Tanimoto et al., 1999). Likewise, reshuffling *Hox* genes' positions (Kmita et al., 2002; van der Hoeven et al., 1996), as well as the inversion of a *Hox* cluster (Zakany et al., 2004) were produced for the exact same purpose. While none of these experiments (and many others of the kind) gave any clear-cut explanations to the temporal processes at work, they all contributed to reenforce the construction of these epistemic systems, eventually leading to original observations of a different nature, while at the same time progressively building the understanding of the core mechanisms at work.

15. Time for/as a conclusion

How is it that *Hox* gene clusters, ever since their discoveries, have been so generous in providing interesting observations and concepts, making them of unusual heuristic value? The reason why these genes were initially chosen for being studied in depth was partly due to the attractivity of their mutant phenotypes but also to the facts that they were associated to a 'chromosomal event' (gene duplications) of potential evolutionary interest and that the in-*cis* configuration of this genes series was encoding a particular information in itself (Lewis, 1998).

The amplification of the ancestral gene cluster along with genome duplications occurring at the roots of the vertebrate lineage, as well as the subsequent conservation of four copies, facilitated both the definition and functional studies of orthologous and paralogous relationships, as well as of micro-syntenies. For instance, should the clustered genes A, B and C be conserved throughout evolution in the same sequence, one can reasonably assume that the intergenic regions were syntenic too, allowing for the search of significant interspecies non-coding sequence conservation. Likewise, gene clusters generally display some kind of functional coordination and are thus often controlled by multi-genic regulations, opening the door to novel observations in this domain too. Indeed, series of contiguous genes sharing regulations offer a much safer ground to assess these processes due to the potential of cumulative results whereby conclusions obtained with gene A can be controlled by the analysis of gene B.

As a consequence of this functional coordination, vertebrate *Hox* genes were rapidly shown to display redundant or compensatory functions, both as neighbor and as paralogous genes. While the absence of (strong) phenotype after (multiple-) gene inactivation(s) has been -still is-considered as a serious problem by many colleagues, it turned out to be of huge help to decipher complex regulatory mechanisms due to the persistence of a good-looking structure, even after combined genetic modifications (a feature analogous to the relatively easy way to work with terminally differentiated functions such as that encoded by the globin system). The function of *Hox* genes in the development and evolution of the vertebrate limb provides a good example, since the almost full complementation between the *HoxA* and *HoxD* cluster genes during limb bud outgrowth (Davis et al., 1995; Fromental-Ramain et al., 1996) made it possible to study in details the global regulations of each of these two gene clusters separately (Andrey et al., 2013; Beccari et al., 2016; Berlivet et al., 2013; Sheth et al., 2016), while the absence of all *Hox* function led to a severe limb agenesis, making experimental approaches much more complex (Kmita et al., 2005). Considering that this gene family was initially characterized in *Drosophila* because of its realm of remarkable homeotic mutant phenotypes, the fact that some of its functional and regulatory principles could be uncovered because of the quasi absence of phenotype due to a high functional redundancy in vertebrates (e.g. (Soshnikova et al., 2013) adds to the historical oddities of this genetic system.

Also, as noted above and in early work with *Drosophila* chorion genes (Mariani et al., 1988), global transcriptional controls applied to gene clusters often lead to time differences in the responses of the genes, perhaps due to simple mechanistic reasons. Time is the essence of development and the understanding of how various temporal reference frames are built, implemented and how they interact with one another represents major challenges in our understanding of ontogenesis (see (Ebisuya and Briscoe, 2018), also when it comes to the way time is encoded into our genome (Duboule, 2003). Variations in relative temporalities during development have been claimed for long to be a driving force in animal evolution (see (Gould, 1977) and hence any experimental paradigm that includes a time component as a readout on top of quantities or spatial distributions, i.e., a metrics of how these latter two parameters may vary along with the progression of development, may strengthen the confidence in the results and interpretations, regardless whether it works in *trans* (the segmentation clock, the circadian clock) or in *cis* (the *Hox* timer, the globin switch). Our knowledge of developing embryos is largely based on having precisely defined staging series, and molecular mechanisms that are deployed along similar time scales may be more amenable to analytical approaches.

16. Epistemological oddities

While this temporal aspect likely contributed (directly or indirectly) to the heuristic value of *Hox* clusters, it should be reminded that several principles of *Hox* gene cluster function and regulation were uncovered by using *Drosophila* (Lewis, 1978; Sanchez-Herrero et al., 1985), a long germ-band insect that is particularly good in not implementing a progressive time mechanism to activate homeotic genes (discussed in (Diaz-Cuadros et al., 2021; Duboule, 1992). Instead, diptera *Hox* genes use positional information present throughout the early embryo as an upstream system leading to activating signals. From an epistemological viewpoint, this partial breakdown of an ancestral developing system (removing this particular time parameter) may have initially facilitated the interpretation of complex and multiple mutations in BX-C, for instance by 'flattening' the required experimental window of observation, from a 4D to a 3D space. This is particularly well illustrated in textbooks where various series of genes carrying distinct functions (maternal gradients, gap genes, segmentation genes) during *Drosophila* development are usually displayed under the form of epistatic schemes, with vertical arrows, suggesting that 'functional steps' are following one another, without any temporal overlap [which makes it hard to explain to first year bachelor student the double maternal and zygotic components of *Hunchback*!].

Finally, the fact that these principles were initially derived from BX-C rather than from ANT-C illustrates differences in the organization of their regulations in *cis*, BX-C displaying admittedly a more 'integrated' organization than that of ANT-C in this respect (Bender et al., 1983a) and thus being closer to the functional structure of vertebrate clusters (Izpisua--Belmonte et al., 1991; Karch et al., 1985; Maeda and Karch, 2009). This makes sense in an evolutionary context, since the abdominal parts of

many arthropods including short germband insects, is developing following a clear AP temporal sequence and hence the structure of BX-C in diptera might still reflect a recent past when the thorax and abdomen were produced in a time sequence and the BX-C genes activated accordingly. In this view, we currently witness the disorganization of BX-C, an ongoing erosion process that has progressed faster in ANT-C (Duboule, 1992; Negre et al., 2005; Von Allmen et al., 1996). Of note, relating his interactions with Ed Lewis, Zuckerkandl writes: ' … *Ed Lewis remained unconvinced of the above mechanism* [i.e., of a time-dependent spreading process], *primarily because rearrangements that break up the complex do not necessarily alter the sequential activation of the genes.*' (Zuckerkandl, 1990). Even if *Drosophila* indeed no longer implements temporal colinearity, several of the structural and functional reasons that triggered Ed Lewis to work on this wonderful system were somehow remnants of a recent past when diptera ancestors, as many extant insects, were producing their segmented body in a time sequence from anterior to posterior.

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