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Hox genes, clusters and collinearity

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ABSTRACT This year marks the 40th anniversary of the discovery by Ed Lewis of the property of collinearity in the bithorax gene complex in *Drosophila*. This landmark work illustrated the need to understand regulatory mechanisms that coordinate expression of homeotic gene clusters. Through the efforts of many groups, investigation of the Hox gene family has generated many fundamental findings on the roles and regulation of this conserved gene family in development, disease and evolution. This has led to a number of important conceptual advances in gene regulation and evolutionary biology. This article presents some of the history and advances made through studies on Hox gene clusters.

KEY WORDS: *Hox gene, evolution, colinearity, patterning, gene regulation*

Perspective

The description of homeotic mutations by Bateson in 1894 has captivated biologists and embryologists for well over a century (Bateson, 1894, Lewis, 1994). How do alterations in these loci lead to the transformation of body parts and tissues? Pursuing an understanding of the molecular basis of these mutations held promise for developing mechanistic insights into the control of morphogenesis in evolution and development. Ninety years later, the convergence of emerging molecular biology techniques and genetics led to the exciting discovery of homeobox motifs in loci of the *Drosophila Antennapedia* (*ANT-C*) and *bithorax* (*BX-C*) homeotic complexes (McGinnis *et al.*, 1984, Scott and Weiner, 1984). The identification and characterization of the conserved Hox homeodomain transcription factor family stimulated a large number of new molecular studies of development in diverse animal systems. This provided new tools and approaches that paved the way for an explosion in our understanding of gene regulatory networks that govern animal development and establishment of the basic body plan.

This year marks the 40th anniversary of the landmark study by Ed Lewis, which described the intriguing property of collinearity, associated with the roles of the *bithorax gene complex* in controlling segmentation in *Drosophila* (Lewis, 1978). Through elegant genetic analyses, this paper provided an impressive description and first glimpse into the components, rules and intricate *cis* and *trans* interactions that underlie the coordinate deployment of *BX-C* in patterning segment diversity. An interesting aspect of Lewis's study is that very early on, before the genes themselves were cloned, it focused attention on the critical importance of understanding gene regulatory mechanisms that control expression of the homeotic complex. In the decades since these initial discoveries of *Hox* genes, *Hox* complexes and collinearity it is remarkable that this gene family has not only yielded important insights on its roles in development, disease and evolution, but it has had a much broader impact and value in revealing and promoting a number of conceptual advances in variety of subject areas.

The cloning of vertebrate *Hox* genes from humans, mice and amphibians revealed a surprising degree of conservation in genes, cluster organization and collinearity between vertebrate and invertebrate systems (Boncinelli *et al.*, 1989, Boncinelli *et al.*, 1988, Dekker *et al.*, 1992, DeRobertis *et al.*, 1985, Duboule and Dollé, 1989, Gaunt *et al.*, 1988, Graham *et al.*, 1989, Regulski *et al.*, 1987). This formed the basis for postulating that vertebrate and invertebrate *Hox* clusters arose by duplication and divergence from a common ancestor and they were associated with an ancient role coupled to axial patterning. However, in *Drosophila, ANT-C* and *BX-C* are separated from each other. Genetic analyses and cloning of homeotic loci in the red flour beetle (tribolium) revealed a single *HOM-C* cluster (Stuart *et al.*, 1991), as opposed to the split between *ANT-C* and *BX-C* in *Drosophila*. This suggested that a tight clustering of *Hox* genes may be more reflective of the ancestral state. The cloning of single *Hox* clusters, with many features shared by vertebrate clusters, in cephalochordates (amphioxus) (Garcia-Fernandez and Holland, 1994) and hemichordates (Freeman *et al.*,

Abbreviations used in this paper: AP, anteroposterior.

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2012) added further support for the concept of a common origin of *Hox* complexes during the evolution of chordates.

In light of the ancient origin of *Hox* clusters, there has been an explosion of evolutionary studies into hypotheses regarding critical evolutionary events or pathways, such as the fin to limb transition or positioning the limb in vertebrates (Nakamura *et al.*, 2016, Shubin *et al.*, 2009)(Shubin *et al.*, 1997). Furthermore, the number and organization of *Hox* clusters have been used as a basis for comparative evaluations of animal phylogeny and whole genome duplications. It is generally accepted that evidence from genome sequencing appears to support two rounds of whole genome duplication in evolution of vertebrates (2R) and in ray-finned fishes an additional fish-specific genome duplication event (3R) occurred (Meyer and Malaga-Trillo, 1999, Meyer and Van de Peer, 2005). This lead as many as eight copies of the ancestral deuterostome genome, creating a rich source of genes that could sub-divide functional roles or adopt new activities. However, there is also evidence in the sea lamprey, based on whole genome sequencing, meiotic mapping and analysis of *Hox* clusters that supports the idea of one round of whole genome duplication followed by independent segmental duplications in vertebrate evolution (Smith and Keinath, 2015, Smith *et al.*, 2018). If similar segmental duplications occurred frequently in chordate evolution, it will be challenging to precisely define parology relationships between *Hox* clusters and many other gene families (Siegel *et al.*, 2007, Smith *et al.*, 2018). Advances in genome sequencing, assembly and lower costs will hopefully bring more examples to bear to better understand the relative degrees to which genome-wide duplications followed by gene loss versus extensive and/or phased segmental duplications played key roles in the emergence of vertebrate traits.

Forty years on, collinearity remains as intriguing today as it did when Ed Lewis first described this unique feature of coordinate regulation. Spatial collinearity refers to the tight correlation between gene order in *Hox* chromosomal clusters and their nested domains of expression along the anteroposterior (AP) axis of animal embryos (Duboule and Dollé, 1989, Graham *et al.*, 1989, Lewis, 1978). This regulatory feature established a combinatorial code for specifying regional diversity of segments and axial structures. Analyses in vertebrate embryos and cell lines rapidly expanded the need to incorporate other features into collinearity, such as timing and response to signaling pathways. Members of the *Hox* clusters were found to display a temporal collinearity, whereby their order along the chromosome also correlated with the relative timing of expression in development (Izpisua-Belmonte *et al.*, 1991). Retinoic acid (RA) is a potent morphogen that impacts patterning of diverse tissues, many of which overlap with sites of *Hox* expression. Treatment of human and mouse cell lines with RA was shown to sequentially activate *Hox* genes in a temporal and dose dependent manner that aligned with their clustered organization (Papalopulu *et al.*, 1991, Simeone *et al.*, 1990, Simeone *et al.*, 1991). Comparing these different types of collinearity, it emerged that genes at one end of a *Hox* cluster were generally expressed in a more anterior regions, activated earlier and were more responsive to RA and the expression of each successive gene in the cluster was progressively more posterior, later and less responsive to RA. Conversely, posteriorly expressed genes were more responsive to FGF signaling compared with anteriorly expressed genes (Bel-Vialar *et al.*, 2000, Isaacs *et al.*, 1998, Pownall *et al.*, 1998).

It was not clear whether these different aspects of collinearity represented distinct properties associated with separate regulatory mechanisms or they were a consequence of a shared process that coupled them to axial patterning. More recent analyses on mechanisms controlling the growth and patterning in vertebrates suggests that these different properties of collinearity are intimately linked to how nested domains of *Hox* expression become established and integrated during axial elongation (Deschamps and Duboule, 2017). Opposing signaling gradients in anterior (RA) and posterior (FGF and Wnt) regions are important for setting up cues that regulate the balance between growth, differentiation and patterning (Bel-Vialar *et al.*, 2002, Deschamps and van Nes, 2005, Diez del Corral *et al.*, 2003, Diez del Corral and Storey, 2004, van de Ven *et al.*, 2011, Young *et al.*, 2009). These axially separated and antagonistic and morphogenetic signals are directly interpreted by *cis*-regulatory regions embedded within and around the *Hox* clusters (Ahn *et al.*, 2014, Neijts *et al.*, 2016, Neijts and Deschamps, 2017, Parker *et al.*, 2016, Parker and Krumlauf, 2017) and indirectly regulated by extensive feedback circuits between components of Wnt, FGF and RA signaling cascades and *Cdx* and *Hox* genes (Deschamps and Duboule, 2017). Members of the *Hox13* parology group appear to represent a break that disrupts this system and leads to termination of axial growth (Denans *et al.*, 2015, Young *et al.*, 2009). Elegant analysis of the timing of *Hox* activation during gastrulation revealed that there was temporal collinearity for members of the *Hoxb* cluster as mesoderm cells progressively ingress along the AP axis (Iimura and Pourquie, 2006). Hence, what at first appeared to be disparate features of *Hox* collinearity have been united by a deeper understanding of the molecular and cellular mechanisms associated with precise coordination of timing, signaling cascades and spatial patterning in vertebrate embryogenesis.

This system may reflect an ancient aspect of the gene regulatory network for how nested domains of *Hox* expression became coupled to axial patterning in deuterostomes. Studies in the hemichordate, *Saccoglossus kowalevskii*, revealed that there is a striking similarity in the deployment of components of signaling pathways and transcription factors, including *Hox* genes, that mirrors their alignment along the AP axis of the prototypical chordate body plan (Lowe *et al.*, 2015, Lowe *et al.*, 2003, Pani *et al.*, 2012). This implies that these signaling centers and transcription factor networks may be established and maintained through a conserved regulatory logic and feedback circuits, that include the ability to generate collinearity and nested domains of *Hox* expression, and they are not dedicated to specifying distinct morphological structures. *Hox*-dependent patterning may therefore become independently coupled to tissues and structures in different animal systems through co-option of this ancient framework (Parker *et al.*, 2014, Parker *et al.*, 2016, Parker and Krumlauf, 2017). This is consistent with the view that functional links between vertebrate and invertebrate *Hox* genes and segmentation evolved independently (Tautz, 2004).

Recent analysis of *Hox* organization and function in a Cnidarian, brings a new perspective to this idea. *Nematostella vectensis* contain a cluster of three *Hox* genes, which display spatial and temporal collinearity in the radial axis of the larval endoderm (He *et al.*, 2018). Gene editing ablation experiments demonstrate that these *Hox* genes have roles in segmentation and segment identity of the endoderm and display a form of posterior prevalence. This work argues that there may be a very ancient link between segmental processes and *Hox* organization, function and collinearity and led to speculation that it is possible the epithelial compartmentation

in endodermal pouches of Cnidaria may be related to processes associated with axial elongation and mesodermal somites in vertebrates (Arendt, 2018).

With respect to function, duplication and divergence, collinearity and nested domains of *Hox* expression presented a series of challenging paradigms to investigate. The idea that combinations of Hox proteins provide a code for specifying regional identities has been validated by genetic gain and loss of function experiments in a wide range of organisms. Yet this raises the question of whether each protein in the combinatorial code has a unique role/contribution or are they interchangeable? The ability to generate targeted mutations and knock in gene variants into the mouse germline provided a powerful opportunity to explore these kinds of questions. This led to a flood of sophisticated genetic analyses of genes in the *Hox* clusters and provided numerous examples of redundancy in a variety of tissue contexts among *Hox* genes within a paralog group and between different paralog groups (Condie and Capecchi, 1994, Greer *et al.*, 2000, Kmita *et al.*, 2005, Mallo *et al.*, 2010, Manley and Capecchi, 1998, Manley and Capecchi, 1997, Sheth *et al.*, 2016, Tvrdik and Capecchi, 2006, Wellik and Capecchi, 2003, Wellik *et al.*, 2002, Wu *et al.*, 2008). Swaps and replacements between homeodomains also raised questions about the degree of specificity of Hox proteins and in some cases showed remarkable plasticity in the ability for Hox proteins to functionally compensate for each other (Greer *et al.*, 2000, Tvrdik and Capecchi, 2006, Zakany *et al.*, 1996, Zhao and Potter, 2001, Zhao and Potter, 2002). This implies that in many contexts it may not be the gene/protein itself that it is important but how it is regulated and the total dose of Hox proteins. The principles uncovered from these findings have been generally applicable to many genes and have helped to uncover cases for redundancy, sub-functionalization and acquisition of novel activities of duplicated genes.

An interesting consequence of collinearity is that in many cases functional roles for *Hox* genes have focused not on individual genes but the global function of the clusters. For example, in heart, limb, hematopoietic and allantois development entire clusters or combinations of clusters need to be deleted or have their expression altered to reveal functional requirements in patterning (Kmita *et al.*, 2005, Lebert-Ghali *et al.*, 2010, Lebert-Ghali *et al.*, 2016, Qian *et al.*, 2018, Scotti and Kmita, 2012, Soshnikova *et al.*, 2013, Vieux-Rochas *et al.*, 2013). This implies that a critical factor in *Hox* functionality is not the individual genes themselves but how they are globally regulated in a coordinate manner.

The interest in global *Hox* gene regulation takes us back to the early focus Ed Lewis placed on the importance of understanding coordinate regulation of *BX-C* and the extensive efforts made over many decades to dissect the molecular mechanisms that underlie collinearity (Deschamps and Duboule, 2017, Kmita and Duboule, 2003, Tschopp and Duboule, 2011, Tschopp *et al.*, 2009). The elegant chromosome engineering experiments by Denis Duboule and his colleagues illustrated the importance of enhancer-sharing, global enhancers, regulatory landscapes and topological domains in controlling the expression of Hox clusters (Lonfat *et al.*, 2014, Tschopp and Duboule, 2011). Again the principles that emerged from this analysis and that of the β-globin gene cluster have been incredibly informative and set the stage for current mainstream efforts in trying to understand how enhancers, promoters, and chromosome topology govern work in a dynamic manner to regulate cell and developmental processes (Furlong and Levine, 2018).

The links and contributions made to understanding other aspects of gene regulation that have emerged from investigations of *Hox* gene regulation are too numerous to mention and review here. However, it is clear that if there is a means of regulating or fine-tuning gene and protein expression (microRNAs, lncRNAs, translational control, specialized ribosomes, promoter competition, bidirectional promoters, mRNA stability, etc.) the *Hox* clusters are likely to incorporate this feature in some context to modulate. During embryogenesis the precisely orchestrated control of developmental processes depends upon getting the appropriate combinations of *Hox* genes expressed at the right levels, times and spatial distributions. Perhaps this is a part of what makes *Hox* genes special and gives them epistemic value?

In closing, I would like to end on a personal note. Ed Lewis would be excited to see the progress made over the forty years since the publication of his landmark paper. It is worth noting that he also had a hand in directly shaping the studies of many others in this area. In 1988, ten years after his study on BX-C, Denis Duboule and I spoke publicly about our discovery of collinearity in the vertebrate *Hox* complexes. Ed heard about this and shared his excitement about the findings. A few months later at a homeobox workshop organized by Walter Gehring and sponsored by EMBO, Ed shared a wealth of ideas from his perspective and many provided many ideas to consider. Fig. 1 is a picture of Ed and I in deep discussion about collinearity and *Hox* expression at this homeobox workshop thirty years ago. It meant a great deal to me to have such a leading

Fig. 1. Ed Lewis and Robb Krumlauf *discussing* Hox *collinearity over a poster at a 1988 homeobox workshop.*

scholar express interest in my work at an early stage in my career. This stimulated a long series of fruitful interactions that continued until he passed. I wish he were here today to help us unravel some of the amazing complexity associated with collinearity.

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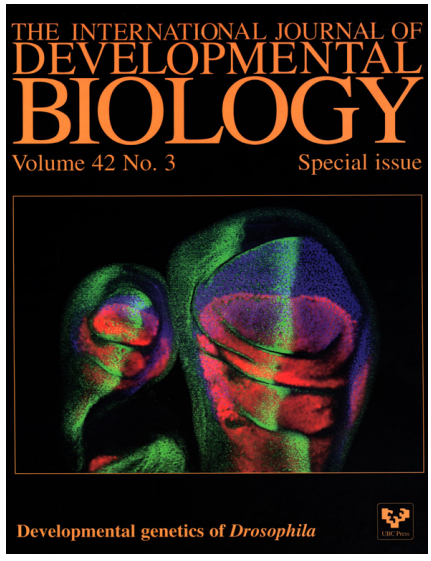

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