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Cell-type specific *cis*-regulatory networks

Insights from Hox transcription factors

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Hox proteins are a prominent class of transcription factors that specify cell and tissue identities in animal embryos. In sharp contrast to tissue-specifically expressed transcription factors, which coordinate regulatory pathways leading to the differentiation of a selected tissue, Hox proteins are active in many different cell types but are nonetheless able to differentially regulate gene expression in a context-dependent manner. This particular feature makes Hox proteins ideal candidates for elucidating the mechanisms employed by transcription factors to achieve tissue-specific functions in multi-cellular organisms. Here we discuss how the recent genome-wide identification and characterization of Hox *cis*-regulatory elements has provided insight concerning the molecular mechanisms underlying the high spatiotemporal specificity of Hox proteins. In particular, it was shown that Hox transcriptional outputs depend on the cell-type specific interplay of the different Hox proteins with co-regulatory factors as well as with epigenetic modifiers. Based on these observations it becomes clear that cell-type specific approaches are required for dissecting the tissue-specific Hox regulatory code. Identification and comparative analysis of Hox *cis*-regulatory elements driving target gene expression in different cell types in combination with analyses on how cofactors, epigenetic modifiers and protein-protein interactions mediate context-dependent Hox function will elucidate the mechanistic basis of tissue-specific gene regulation.

Keywords: *Drosophila*, Hox, homeodomain, tissue-specific, transcription, gene regulation, genome-wide, cofactors

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Hox Proteins: Broadly Expressed Yet Highly Cell-Type Specific Regulators of Gene Expression

Hox genes encode evolutionarily conserved and essential transcription factors (TFs) expressed in precise domains along the anterior-posterior axis of animal embryos where they regulate segment morphogenesis.[1] Besides their role in patterning the early embryo, Hox TFs control organogenesis in later developmental stages[2] and are critical for the maintenance of tissue homeostasis in adult organisms.[3] Hox proteins regulate cellular and tissue identities in a cell-autonomous manner by binding to DNA sequences in Hox response elements (HREs), thereby activating or repressing downstream targets.[4,5] Despite their rather broad expression, Hox TFs execute their regulatory function in a highly context-dependent manner.[6-8] However, the molecular mechanisms underlying the spatiotemporal specificity of Hox TFs have remained a long-standing question.

Hox TFs are characterized by the presence of a homeodomain, a 60 amino acids DNA-binding domain. The Hox homeodomain recognizes DNA consensus sequences containing an -ATTA- core,[9,10] thus Hox TFs show overlapping in vitro binding behaviors.[11,12] Nonetheless, different Hox proteins exhibit very diverse in vivo binding preferences[13] and execute distinct regulatory functions,[5] showing that the loose DNA recognition properties of the homeodomain are not sufficient to confer specificity to the Hox proteins.[12,14,15]

Combinatorial assembly of TF and transcriptional co-regulator complexes on shared *cis*-regulatory elements is a

widespread mechanism driving context-dependent transcriptional responses. Along these lines, it has been proposed that HREs integrate multiple inputs and that the high in vivo specificity of Hox proteins is achieved in cooperation with cofactors and collaborators.[13] Well-established examples of Hox cofactors include members of the PBC and Meis protein families, i.e., Extradenticle (Exd) and Homothorax (Hth) in *Drosophila*.[16] These cofactors improve Hox TF selectivity and permit the differential regulation of target genes through cooperative complex formation.[13,17] As demonstrated by a recent study, complex formation between *Drosophila* Hox TFs and their cofactor Exd has profound effects on the DNA recognition properties of Hox proteins and in particular it reveals novel DNA binding specificities.[18] Strikingly, the Exd-Hox complex derived specificity is unique for each one of the closely related Hox TFs tested in the study,[18] highlighting the crucial role of protein-protein interactions in fine-tuning the in vivo selectivity of different Hox TFs. On the other hand, several TFs coordinate the regulatory activity of Hox proteins without the requirement of complex formation by acting as collaborators.[19] Examples include the collaboration between Ubx and Smads for repressing *spalt* in the *Drosophila* haltere,[19] the collaboration of Ubx and Abd-A with Sloppy paired for repressing Distal-less in the *Drosophila* abdomen,[20] and the regulation of *reaper* expression in the anterior part of the maxillary segment in *Drosophila* embryos by the combinatorial activity of the Hox TF Deformed (Dfd) and 8 other TFs.[21]

Combinatorial Input on Hox-Regulated Enhancers Defines the Cell-Type Specificity of Hox Proteins

As suggested by studies on selected developmental enhancers,[19-21] the integration of a combinatorial transcriptional input on HREs is most likely a frequently employed mechanism for regulating Hox TF activity in vivo. Nonetheless, the information obtained by dissecting a small number of HREs is not sufficient for globally unravelling the regulatory code underlying the spatio-temporal precision of Hox TFs. An in depth analysis of Hox *cis*-regulatory elements on the genome-wide level is undoubtedly essential in order to understand the mechanistic basis of how Hox TFs regulate target gene expression. In recent years, the wide-spread use of genome-wide chromatin-profiling methods such as chromatin immunoprecipitation (ChIP) followed by microarray analysis (ChIP-on-Chip) or coupled to massively parallel sequencing (ChIP-Seq) has resulted in mapping of the in vivo binding profiles of several TFs, among them a number of Hox proteins.[22-26] These studies allowed for the first time the genome-wide identification and characterization of Hox-bound *cis*-regulatory elements.

Analysis of the newly identified HREs revealed several features that appear to be crucial for the cell-type specific regulatory functions of Hox proteins in vivo. In our recent study,[22] genomic regions bound by the Hox TF Dfd in stage 9–12 *Drosophila* embryos were identified by ChIP-Seq. A subsequent analysis of the architectural features of a number of Dfd regulated enhancers led to the conclusion that motif composition and short distance spacing of TF binding sites is critical for transcriptional regulation by Dfd in vivo. Importantly, the analysis of gene classes associated with enhancers displaying different motif compositions and motif pair associations showed that architectural features of Dfd CRMs are sufficient to predict target gene function and expression patterns with high accuracy.[22] Furthermore, a comparison of regulatory regions bound by Dfd[22] and Ubx,[27] two Hox proteins specifying the morphology of different segments in the *Drosophila* embryo, revealed that in contrast to their loose DNA binding specificity in vitro Dfd and Ubx bind to non-overlapping genomic regions in vivo. Analysis of Dfd and Ubx HREs indicated that regulatory specificity/selectivity is encoded by distinct combinations of co-occurring TF binding motifs, highlighting once again the essential role of co-regulatory TFs. Along the same lines, computational analysis of genomic regions bound by Ubx in *Drosophila* haltere and T3 leg imaginal discs revealed the presence of distinct TF motifs in each data set.[25] Interestingly these TFs are locally expressed in either of the two analyzed tissues and therefore they are expected to function as Ubx co-regulators in the respective tissues.[25] Nevertheless, this hypothesis remains to be experimentally addressed. In yet another genome-wide study, the *in silico* analysis of Ubx bound genomic regions in *Drosophila* wing imaginal discs[26] showed that even though no specific Ubx consensus motif was enriched in Ubx HREs, binding motifs for other TFs were over-represented in these sequences, suggesting that these TFs assist Ubx in identifying target HREs and/or in regulating target gene expression.[26] Collectively these studies point out that the restricted spatiotemporal availability of Hox co-regulators dictates the highly precise regulatory function of Hox TFs.

Identification and Analysis of HREs from Isolated Cell-Types is Essential for Unraveling the Hox Regulatory Code

As increasing evidence indicates that the cellular context is an essential determinant of the regulatory output of Hox TFs, it is becoming clear that cell-type specific approaches are required in order to identify all relevant aspects of the interplay of Hox TFs with *cis*-regulatory elements and co-regulatory factors. Genome-wide data obtained from whole embryos or whole tissues (i.e., imaginal discs) represent an averaged signal originating from the mixture of heterogeneous cell types. In such experimental setups, TF-DNA interactions taking place in less frequently encountered cell-types will most likely not be detected, as they will be diluted by signals arising from abundant cell types. Recently, different methods have been presented for isolating pure populations of nuclei from selected cell-types using organisms ranging from plants to animals.[28-30] These methods include affinity purification of nuclei tagged in selected cell types[28,30] and fluorescent activated cell sorting of nuclei that have been fluorescently labeled for a nuclear protein expressed in the cell type of interest.[29] Both approaches were shown to be suitable for genome-wide experiments aiming at identifying TF-bound genomic regions, obtaining gene expression profiles and analyzing epigenetic modifications. The combination of these nuclear sorting approaches with techniques successfully

employed to amplify ChIP signals from limited amounts of starting material[31] will permit cell-type specific genome-wide analyses of Hox-DNA interactions even using less-abundant cell-types. Using isolated nuclei and genome-wide methods to identify *cis*-regulatory enhancer modules bound by Hox TFs is a promising approach for identifying the mechanisms that confer specificity to Hox proteins in the different cellular contexts.

As indicated by our comparative analysis,[22] the genomic regions bound by different Hox TFs show little if any overlap, in sharp contrast to their highly similar in vitro binding properties. Nevertheless, the two data sets used for this analysis were generated using different methods, namely ChIP-Seq for Dfd-bound regions and ChIP-Chip for Ubx-bound regions and embryos of not entirely identical developmental stages, 4–9 h old and 3–8 h old embryos respectively.[22,27] A comparison of *cis*-regulatory elements bound by different Hox proteins in the same cell-type and identified under comparable conditions will provide more detailed insight concerning how the involvement of different co-regulatory TFs properties affects Hox cell-type and segment specific activity.

Interplay of Hox TFs with Epigenetic Modifiers

Epigenetic characteristics including chromatin structure and histone modifications on enhancer modules strongly correlate with spatiotemporal enhancer activity, as shown by genome-wide experiments using cell lines of variable origins and isolated *Drosophila* embryonic mesoderm cells.[29,32,33] Assuming that epigenetic changes resulting in an open chromatin conformation generally potentiate accessibility of DNA to TFs, the observed tissue-specific epigenetic marks are interpreted as a means to control the selective occupancy of TFs in different cellular contexts. As shown in our study, epigenetic regulators bind to Hox enhancers on a genome wide level, suggesting that their interaction with Hox TFs is crucial for the regulation of Hox target gene expression.[22] For instance, the Hox protein Dfd shares a substantial number of target genomic regions with the transcriptional coactivator dCBP.[22] dCBP bears intrinsic acetyltransferase activity and induces histone acetylation and subsequent open chromatin conformation in the vicinity of its binding,[34] thus active histone marks i.e., H3K27ac were expected to be enriched at Dfd/dCBP bound genomic regions. When we analyzed the genome-wide epigenetic data generated by the modENCODE consortium using *Drosophila* embryos,[27] we were not able to detect enrichment for acetylated histone variants. Nevertheless, as the combinatorial binding of Dfd and dCBP takes place only in a small embryonic segment where Dfd is expressed, it is most probably impossible to extract information concerning the epigenetic marks at these loci from data generated using whole embryos. A cell-type specific analysis of histone modifications is required for clarifying whether dCBP-mediated histone acetylation is encountered at Dfd/dCBP bound loci. Generally, the identification of epigenetic marks at genomic regions bound by Hox TFs in different cell-types will determine to what extent chromatin accessibility determines the binding of Hox TFs to their target HREs in the different cellular contexts.

The most obvious explanation for the joint binding of epigenetic regulators and Hox proteins at overlapping genomic regions[22] is that the presence of these histone-modifying enzymes is a prerequisite for inducing open chromatin conformation required for the binding of Hox TFs to DNA. But is this the only plausible explanation? Interestingly, in agreement with the reported activity of histone acetyltransferases to acetylate TFs in addition to histones,[35] we showed that dCBP acetylates Glial cells missing (Gcm), a co-regulatory TF of Dfd and that the interaction of Gcm and CBP is required for gene expression driven by a given Dfd/Gcm regulated HRE.[22] TF acetylation is a commonly used mechanism for modifying/enhancing transcriptional regulatory activity as shown for a number of TFs among which Gcma,[36] Myocardin,[37] EWS-FLI1[38] and GATA-1.[39] Therefore the interplay of Hox TFs and epigenetic modifiers might mediate the local recruitment of such enzymes in order to modulate the transcriptional activity of Hox co-regulators or even of the Hox TFs themselves. Further experiments are definitely required in order to validate this hypothesis and to address whether post-translational modifications of Hox co-regulators mediated by epigenetic regulators are a commonly observed theme and not restricted to the example of Gcm in the *Drosophila* embryo.

In addition to the traditional view that epigenetic patterning of enhancers precedes TF binding, a more complex model was proposed recently for the establishment of lineage-specific epigenetic marks (reviewed in ref. 40). As shown by a number of independent studies, TFs as well as transcriptional co-regulators are often involved in establishing cell-type specific epigenetic signatures by binding at enhancer modules and mediating the recruitment of chromatin modifying enzymes.[40] It would be worthwhile to investigate whether Hox proteins and/or their co-regulatory TFs employ this strategy in order to initiate cell-type specific transcriptional responses.

Protein-Protein Interactions as Determinants of Hox TF Regulatory Specificity

The assembly of transcriptional regulatory complexes on target enhancer elements is a key aspect of transcriptional regulation. After the binding of the first TFs at DNA sequences, further recruitment of regulatory proteins determines the regulatory output, namely activation or repression of transcription and maintenance of the respective transcriptional state. The ability of Hox proteins to bind frequently encountered DNA sequences offers them the possibility to interact with a vast number of genomic regions. Interestingly, even though different Hox proteins are highly similar with respect to their homeodomains, they display pronounced variability concerning other protein domains and amino acid sequences.[13] Thus, amino acid sequences and protein structures outside the homeodomain very likely mediate protein-protein interactions that subsequently refine the binding of Hox TFs to target HREs. Concerning the extensively studied Hox/PBC heterodimers, a hexapeptide motif upstream of the homeodomain has long been described to mediate the interaction of the two proteins (summarized in ref. 13). Interestingly, a recent study

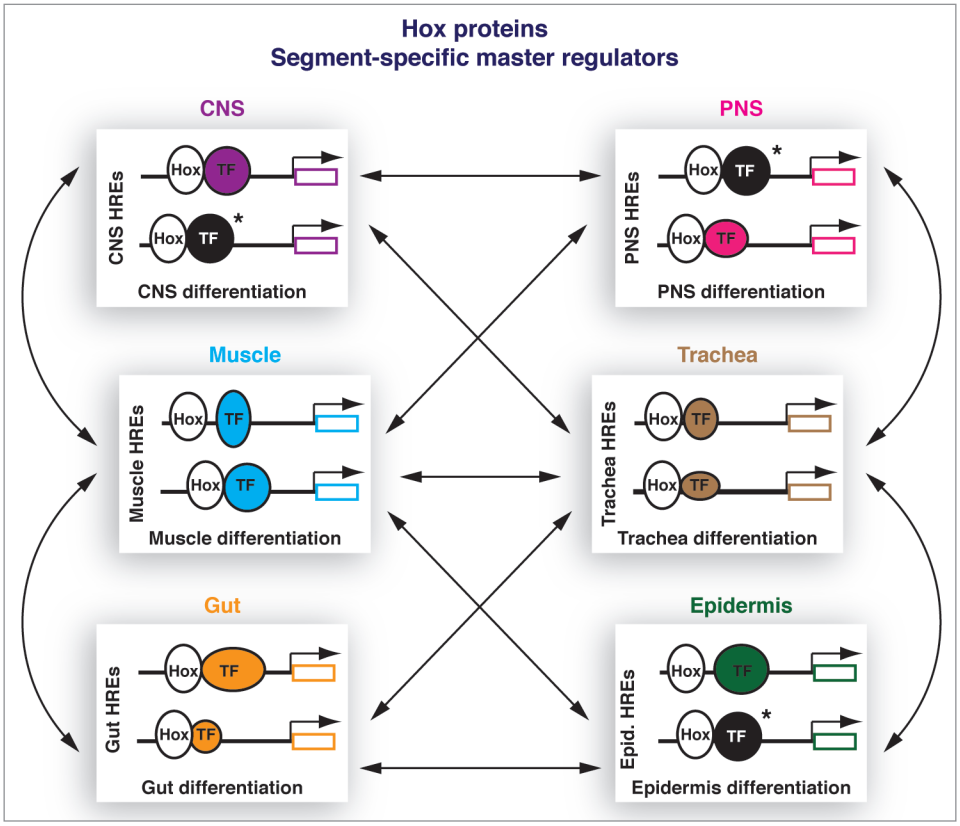

Figure 1. Model: Hox transcription factors function as segment-specific master regulators executing cell-type specific functions. Central nervous system (CNS), peripheral nervous system (PNS), muscle, trachea, gut and epidermis are shown as selected examples of tissues where Hox proteins differentially regulate target gene expression. In the different tissue-types, Hox proteins bind to HREs and promote expression of tissue-specific target genes. Spatially restricted TFs (color-coded depending on the different tissues) fine-tune the regulatory activities of Hox proteins in the different cellular contexts. Co-regulatory TFs active in more than one cell types are shown in black and are marked by an asterisk. Tissue-specific epigenetic modifications implicated in transcriptional regulation are omitted for simplicity reasons. Arrows indicate the interactions between transcriptional regulatory networks active in different tissues, resulting in the coordination of complex processes, necessary for embryonic development and tissue homeostasis.

demonstrated that the picture is probably more complicated, as the hexapeptide seems to be at a certain extent dispensable for the in vivo Hox/PBC interaction, while additional cofactors as well as paralog-specific amino acid sequences are implicated in the protein-protein interaction, resulting in high functional plasticity.[41] Considering the indispensable role of co-regulators in regulating Hox transcriptional activity, identification of Hox protein domains mediating intermolecular interactions with co-regulatory TFs will improve our understanding of how Hox proteins achieve functional specificity. Importantly, as it has been recently suggested by structure-function studies of different Hox proteins, the role of each protein domain should not be considered individually but rather as one of the components of a multifunctional unit that collectively defines Hox-DNA interactions and regulatory activity.[42]

Hox Proteins: Ideal Models for Revealing the Mechanisms of Cell-Type Specific Transcriptional Regulation

Even though cell-type specific transcriptional regulation of gene expression is a fundamental aspect of multi-cellularity, the molecular mechanisms conferring tissue specificity to TFs remain largely unknown. In many cases, the expression of TFs coordinating the differentiation of a given tissue is restricted to the population of precursor cells that will eventually lead to the formation of this tissue. For example, the TF Twist, which drives somatic muscle specification, is only expressed in mesoderm and muscle precursor cells in the *Drosophila* embryo.[43] In sharp contrast to TFs that are tissue-specifically expressed, Hox TFs are active in large embryonic segments containing multiple cell types and giving rise to several different tissues and body structures. This particular feature of Hox TFs makes them ideal candidates for understanding the mechanistic basis of cell- and tissue-specificity. A comparative analysis of the mechanisms employed by a Hox TF for regulating target gene expression in different cell types will allow the identification of general regulatory mechanisms used in more than one cell types and will additionally reveal cell-type specific mechanisms driving target gene regulation solely in a defined cellular context (**Fig. 1**). As discussed above, these molecular mechanisms may include recruitment of cell-type specific transcriptional co-regulators, interaction with distinct epigenetic modifiers and discrepancy in DNA-binding properties emerging from differential protein domain usage and protein-protein interactions. Furthermore, a comparative

analysis of Hox regulatory networks in different cell-types within an embryonic segment will offer valuable insight concerning how gene expression is coordinated in different tissue types in order to ensure the proper implementation of complex biological programs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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