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The Hox protein conundrum: The "specifics" of DNA binding for Hox proteins and their partners

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Abstract

Homeotic genes (*Hox* genes) are homeodomain-transcription factors involved in conferring segmental identity along the anterior-posterior body axis. Molecular characterization of HOX protein function raises some interesting questions regarding the source of the binding specificity of the HOX proteins. How do HOX proteins regulate common and unique target specificity across space and time? This review attempts to summarize and interpret findings in this area, largely focused on results from *in vitro* and *in vivo* studies in *Drosophila* and mouse systems. Recent studies related to HOX protein binding specificity compel us to reconsider some of our current models for transcription factor-DNA interactions. It is crucial to study transcription factor binding by incorporating components of more complex, multi-protein interactions in concert with small changes in binding motifs that can significantly impact DNA binding specificity and subsequent alterations in gene expression. To incorporate the multiple elements that can determine HOX protein binding specificity, we propose a more integrative Cooperative Binding model.

Keywords

Hox; Binding specificity; Co-factors; TALE; Cooperative Binding model

1. *Hox* genes and development

One of the critical ongoing questions in developmental biology surrounds the regulatory mechanisms for determining segment identity, whether particular regions of developing embryos will become heads or tails. Homeotic genes (*Hox* genes) are homeodomain-transcription factors that confer segmental identity along the primary body axis (reviewed by McGinnis and Krumlauf, 1992) and are implicated in the regionalization of the body plan of all bilaterally symmetrical animals. Mutations in homeotic genes described to date that involve overexpression, overactivation or inactivation have often resulted in dramatic

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transformations in segment identity leading to body structures forming incorrectly or in the wrong place, as seen in *Drosophila* and mice (Lewis, 1978, 1982, 1992; Vinagre et al., 2010; Wellik and Capecchi, 2003; Zhao and Potter, 2001, 2002). The *Hox* genes are highly conserved across Metazoan groups and the genes in the *Hox* cluster largely maintain their chromosomal organization, 5' to 3', in parallel with their spatial and temporal expression patterns in the organism (McGinnis and Krumlauf, 1992). The selective pressures that have led to this conservation are reflective of the pivotal role that *Hox* genes play in developmental processes.

Hox genes function as selector genes (genes conferring segmental identity) in vertebrates and invertebrates, but they have a wide variety of additional roles in morphogenesis and patterning. The *Hox* family of genes evolved through two to three rounds of duplication and divergence, which has resulted in multiple gene paralogs with overlapping expression domains and functional redundancy, depending on the Metazoan group. The *Drosophila Hox* complex is split into two regions, 700 Kb apart on the same chromosome (HOM-C). These two complexes are known as the Bithorax (BX-C) and Antennapedia (ANT-C) complexes. The Antennapedia complex consists of labial (lab), *Proboscipedia (Pb), Sex-combs reduced (Scr), and Deformed (Dfd).* The Bithorax complex consists of three genes, *Ultrabithorax (Ubx)*, abdominal A (abdA) and *Abdominal B (AbdB)* (Carroll, 1995; Lewis, 1978; Powers et al., 2000). Mammalian genomes contain 39 *Hox* genes organized into four complexes (*HoxA, HoxB, HoxC* and *HoxD*) per haploid set, located on four different chromosomes that, together, constitute a paralogous group as they have arisen as a result of gene duplication (Boncinelli et al., 1988; Hoegg and Meyer, 2005; Krumlauf, 1994, 2018; Scott, 1992; Duboule, 2007). Many paralogous *Hox* genes display functional redundancy, meaning they can functionally compensate for each other (Tvrdik and Capecchi, 2006). As a result, compound mutations of paralogous group genes can reveal functional compensation and synergism between genes within the same group. Further, paralogs often have overlapping expression patterns (Kiecker and Lumsden, 2005) and their loss-of-function mutations partially resemble each other (Barrow and Capecchi, 1996; Ramirez-Solis et al., 1993). A sequence alignment between the *Drosophila* chromosome region that bears the *Hox* code, and vertebrate *Hox* complexes suggests that the four mammalian *Hox* complexes arose from a single ancestral cluster by gene and chromosome duplications during evolution (Duboule and Dolle, 1989; Graham et al., 1989). There is evidence that variation in the body plan among the arthropods is due to variation in the expression and regulation of *Hox* genes (Hughes and Kaufman, 2002; Ronshaugen et al., 2002). The sequence variations in homeodomain proteins also contribute to changes in target specificity and are subject to evolutionary changes (Ekker et al., 1994; Li et al., 1999; Ronshaugen et al., 2002). Indeed, the impact of *Hox* genes in evolution has been reviewed and discussed at length (Alexander et al., 2009; Carroll, 1995, 2005, 2005; Mallo et al., 2010; Wellik, 2009). What is readily apparent from the conserved organization of these *Hox* genes at the chromosome level, in conjunction with HOX protein impact on segmental patterning, is that the *Hox* gene family codes for critical developmental regulators.

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2. *Hox* gene function determined through the window of mutation analysis

Analysis of various mouse mutants supports the idea that there is unique function for individual *Hox* genes which might be attributed to some inherent, latent properties of HOX proteins. Interpretation of these studies is challenging because of the potential for functional redundancy associated with paralogs in mice. Greer and colleagues swapped complete coding regions between *Hoxa3* and *Hoxd3* (Greer et al., 2000). Their analysis supported the ideas that *Hox* genes are functionally equivalent and that different functions arise from differences in temporal and spatial domains of expression (Greer et al., 2000). This has been interpreted as an "equivalency model" suggesting that it is the quantity of Hox protein that is important in determining functions and not the variations in the proteins, themselves (Duboule, 2000). However, in similar types of experiments, Zhao and Potter reported that a homeobox swap between *Hoxa11* and *Hoxa13*, generating chimeric *Hoxa11* (A1113hd) alleles, results in mice that develop normally and give rise to normal skeletons, kidneys and male reproductive tracts (Zhao and Potter, 2001). Conversely, in limb and the female reproductive tract development, $Hoxa11^{13hd}$ acts as a dominant-negative allele, significantly disrupting normal development (Zhao and Potter, 2001). In the female mice, the uterus is transformed into a cervix/vagina. This latter result likely stems from the observation that the *Hoxa13* expression domain overlaps these structures in the normal female reproductive tract (Zhao and Potter, 2001). Swapping the homeodomain of *Hoxa10* into *Hoxa11* ($Hoxa11^{10hd}$) leads to a hypomorphic phenotype in appendicular skeleton, kidney and reproductive tracts but the mutant animals show no defects in axial skeleton development. Swapping the homeodomain of *Hoxa4* into *Hoxa11* ($Hoxa11^{4hd}$) generates animals similar to a *Hoxa11* null phenotype (Zhao and Potter, 2002) with surprisingly normal axial skeletons.

The results from these domain swap experiments imply tissue-independent and tissue-specific roles for different homeodomains. The results further lend support to the idea that defining segmental identity may have been a common or primitive function of the homeodomain acquired before functional divergence between different paralogous groups, with context playing a more significant role in segment identity after chromosome duplication in the phylogenetic tree. Despite this apparent redundancy, there is also strong evidence to support distinct functions for the different *Hox* paralogs, particularly across developmental time and space (Vinagre et al., 2010; Wellik, 2007; Wellik and Capecchi, 2003). This implies that each HOX protein has some level of unique function, although many HOX proteins might have common targets and function in similar developmental pathways (Minoux et al., 2009; Vieux-Rochas et al., 2013). A recent review by Luo and colleagues highlights the contrast between aspects of the *Hox* code that are clear versus those that remain incomplete or unanswered (Luo et al., 2019).

What remains to be explored is to what extent these unique functional roles relate to differences in domains of expression between the genes or to subtle variations in the HOX proteins, themselves, and their downstream target loci. This distinction leads to a series of fundamental questions: How do different HOX proteins regulate common and unique targets? What is the source of HOX protein specificity? Are there distinct *cis*-sequences that are key to differential HOX specificity? How do tethered binding modules, for example, HOX, HOX:cofactor and/or cofactor motifs separated by a spacer, and their

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underlying binding sites dictate rules for specific binding of different HOX proteins? How do sequence differences at the amino acid level contribute to HOX protein binding and functional specificity? These issues are, in general, highly relevant not only to HOX proteins but to many other families of transcription factors, particularly in terms of characteristic recognition sequences (target sequences) associated with transcription factor groups. Hence, the general rules and knowledge gained from analysis of HOX proteins should be relevant for other types of factors or, at the very least, provide a useful basis for comparison.

3. The Hox protein family: commonalities and differences

Given the level of sequence similarity and functional redundancy, how do different HOX proteins regulate common and unique targets and what is the source of that specificity? There are common structural domains among the *Hox* genes, including the homeodomain, that are critical for function (Alexander et al., 2009; Mann et al., 2009). All homeotic genes share a characteristic motif called the homeodomain, coding for 61 amino acids, that recognizes a specific DNA sequence and is required for activating and repressing downstream target genes (Fig. 1A). The HOX proteins have a characteristic dimensionality and fold into 3 helices, with helix 2 and 3 generating a helix-turn-helix conformation that is a hallmark of transcription factors that bind to the major groove of DNA. While the HOX protein N-terminal region that precedes helix 1 contacts the nucleotides of the minor groove of the target DNA, the third helix (the recognition helix) recognizes a four-base motif, TAAT, which is conserved in nearly all sites recognized by the homeodomain (Gehring et al., 1990; reviewed by Mann and Chan, 1996; Otting et al., 1990). The sequences flanking and inside the homeodomain also aid in target specificity and are subject to evolutionary changes (Ekker et al., 1994).

In general, the 39 mammalian HOX proteins recognize AT-rich binding sites, while the ~60 basic helix loop helix (bHLH) proteins recognize the 5'-CACGTG-3' or E-box domain (Berger et al., 2008; Conlon et al., 2001; Jones, 2004). In *Drosophila* more than 50 homeodomain-containing proteins bind to a six base pair core binding sequence such as 5'-TAATTG-3' and 5'-AATTA-3' (Noyes et al., 2008). All invertebrate and vertebrate HOX proteins show binding affinity to such AT-rich sequences in *in vitro* monomeric binding assays. However, the issue of binding affinity is further complicated by the differences in *in vitro* and *in vivo* properties ofthese proteins. In *Drosophila*, Antennapedia (Antp) recognizes 5'-[C/T][C/A] ATTA-3' and binds DNA with high affinity while Labial (Lab) and Proboscipedia (Pb) bind to 5'-nTGATTGATnnn-3'. Deformed (Dfd) and Sex combs reduced (Scr) preferentially bind to 5'-TGATTAATnn-3' (Slattery et al., 2011). Such sequences are abundant in the genome and one such sequence can be found at an approximately 500 base pair intervals at a genome-wide level. However, when tested in *in vitro* binding studies, Ultrabithorax (UBX) and Antp binding properties are indistinguishable (Berger et al., 2008; Mann et al., 2009; Noyes et al., 2008). *In vivo* Antp is involved in control of leg (Casares and Mann, 1998; Mann and Hogness, 1990; Schneuwly et al., 1987a, 1987b) versus antenna segment specification, while UBX is involved in haltere versus wing specification (Castelli-Gair and Akam, 1995; Konopova and Akam, 2014; Roch and Akam, 2000). These commonalities beg the following questions. Does the underlying binding sequence have a role in binding specificity that results in these distinct functional roles? Do cofactors modify

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binding properties of HOX proteins and lead to specific interactions masked in the *in vitro* binding assays? How relevant are these binding and regulatory "rules" across transcription factor groups? The goal of this review is to address some of these critical questions in the context of mutagenesis and binding studies, highlighting key elements that integrate to fine-tune HOX binding specificity during development and building toward a "cooperative" binding model for HOX proteins.

4. Hox proteins and cofactors

In light of their common origin and evolutionary conservation, HOX proteins have very similar homeodomains and overall structures which bind to simple sequences with relatively similar sequence preferences (Berger et al., 2008). Hence, their individual specificity for target sites *in vivo* is likely to be modulated through the involvement of cofactors or interacting proteins. Evidence to support this comes from studies of two major classes of HOX cofactors that are members of the Three amino acid loop extension (TALE) protein family. These include Pre-B-Cell Leukemia Transcription Factor (PBC) and Myeloid Ecotropic Viral Integration Site (MEIS) that have three additional amino acids between helix 1 and 2, thereby altering the 3-dimensional conformation of the binding site. PBC and MEIS are classes of TALE-homeodomain containing proteins that can have HOX-dependent and HOX-independent functions (Longobardi et al., 2014; Penkov et al., 2013; Stanney et al., 2020). The most commonly characterized PBC proteins in mice are the Pbx family whose evolution predates Hox genes (Bobola and Merabet, 2017; Merabet and Mann, 2016). The most studied members of the MEIS class are the MEIS and PREP proteins (Penkov et al., 2013). Many of the HOX binding sites or HOX response elements identified *in vivo* at gene enhancers and promoters are found in association with adjacent PBX and/or MEIS/PREP binding sites (Manzanares et al., 2001). Mutating the PBX and MEIS binding sites in downstream HOX targets, which can also include Hox genes, can prevent their expression (Ferretti et al., 2000; Gould et al., 1997; Maconochie et al., 1997; Manzanares et al., 2001).

The TALE protein family is also evolutionarily conserved, which supports the idea that they have an ancient role in potentiating the binding specificity of HOX proteins even in jawless vertebrates such as Lamprey and in invertebrates (Hudry et al., 2012; Parker et al., 2019; Slattery et al., 2011). Mutation in *EXD* (*Extradenticle*; homolog of *Pbx*) causes homeotic transformation in *Drosophila* without altering *Hox* expression, while in mice and zebrafish *Pbx* mutants can recapitulate *Hox* loss-of-function mutant phenotype in the hindbrain and other tissues (Moens and Selleri, 2006; Peifer and Wieschaus, 1990; Popperl et al., 2000; Rauskolb et al., 1993, 1995; Selleri et al., 2004; Vitobello et al., 2011; Waskiewicz et al., 2001, 2002). Furthermore, *Pbx* and *Hox* genes have been genetically shown to interact, as partial knockdown of *Hoxb1a* in *Pbx4* heterozygotes shows synergistic effects in control of motor neuron migration and paralogous group1 *Hox* genes show synergistic interactions with zygotic *Pbx4* in specification of rhombomeres (Cooper et al., 2003; Waskiewicz et al., 2002). These results together suggest that the TALE family of proteins can act as cofactors to confer DNA binding specificity to HOX proteins through an evolutionarily conserved mechanism.

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What are the conserved binding domains in HOX proteins? The hexapeptide region adjacent to the homeodomain of HOX proteins has been found to be an important site of interaction for the PBC DNA-binding partners. Indeed, targeted mutations in the hexapeptide domains of HOX proteins prevents them from binding to PBX (Chan et al., 1996; Hudry et al., 2012; Medina-Martinez and Ramirez-Solis, 2003). Alterations of the hexapeptide domain of *Hoxb8* result in dominant homeotic transformations similar to that observed in *Hox7* and *Hox9* null mice (Medina-Martinez and Ramirez-Solis, 2003). What is important for interpreting these results is that the expression of the *Hox7* and *Hox9* genes was unaffected despite the dramatic shift in segment identity associated with the hexapeptide domain changes in *Hoxb8*. This also suggests that in the absence of HOXB8/PBX binding, non-HOX target genes were transcriptionally mis-regulated in a segment-specific manner. There is also evidence to suggest that the PBC class of proteins is shown to be acting as pioneer factors that facilitate the opening of poorly accessible chromatin and recruitment of cofactors, including the HOX proteins, themselves (Grebbin and Schulte, 2017). Together, these studies highlight the importance of cofactors, such as PBX and MEIS, in guiding HOX proteins to their appropriate and tissue-specific targets essential for their *in vivo* functions. However, they also illustrate critical gaps in understanding the underlying mechanisms controlling differential HOX specificity *in vivo*, indicate alternative roles for the HOX cofactors, and lend support toward building a more comprehensive model of HOX protein binding and function.

5. How do the Hox proteins and cofactors come together?

Biggin and McGinnis (1997) proposed two models to explain HOX-cofactor interactions on DNA. They called these the “widespread binding model” (Fig. 1B) and the “co-selective model”. These two models differ in terms of the requirement for TALE proteins to facilitate HOX binding and do not account for any other cofactors facilitating HOX recruitment on genomic targets. Interestingly, in cases where TALE cofactors are not required for HOX binding, such as in the widespread model, they still may play a context-dependent role in regulation of target genes. In the widespread binding model, HOX proteins bind to clustered HOX-response elements without the aid of cofactors. Many of these binding sites might not relay a functional output. However, cofactor binding could alter the ability of HOX proteins to regulate target genes. Once activated, they could serve to activate or repress transcription depending upon their context and the influence of other recruited proteins. Evidence to support this model comes from the fact that EXD/PBX proteins are required along with HOX proteins (*i.e.* Dfd) for activation of target genes (Casares and Mann, 2000; DiMartino et al., 2001; Gonzalez-Crespo et al., 1998; Gonzalez-Crespo and Morata, 1995, 1996). *Hox* control of haltere development in *Drosophila* and distal appendages in arthropods and other vertebrates (limb), does not need *Pbx/Exd* or *Hth (Homothorax)/Meis* for proper development (Biggin and McGinnis, 1997; Peifer and Wieschaus, 1990; Pinsonneault et al., 1997; Vinagre et al., 2010; Wellik, 2007; Wellik and Capecchi, 2003). In *Drosophila*, UBX binding sites in the *sal cis*-regulatory region define the overall strength of repression in an EXD-independent manner (Galant and Carroll, 2002). However, these studies do not rule out the possibility that another cofactor(s), apart from EXD/PBX or HTH/MEIS, may be involved in this process (Galant et al., 2002). The challenge arises, then, as to how to distill

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out common themes from evidence across species and different HOX proteins to amend our view of HOX binding specificity.

In the “co-selective model” proposed by Biggin and McGinnis (1997), HOX proteins do not significantly bind with high affinity to any response element unless cofactors such as EXD/PBX and HTH/MEIS are present. This model employs a bipartite site for HOX-binding and EXD/PBX binding, such that specificity is imparted by the composite influence of multiple binding sites that are spatially separated and yet present within a neighborhood. In this case, the HOX-PBX bipartite site refers to a binding site where half the bases recruit one factor while the remaining nucleotides recruit the other factor. In this case, binding of PBX to the motif may be required for HOX binding to the other half. These sites are also known to contain PBX-HOX-MEIS ternary complexes (Ferretti et al., 1999, 2000, 2005). Strong support for this model has arisen from genome-wide studies to identify binding targets of Hox proteins and their regulatory functions in *Drosophila* and vertebrates. For instance, when *Hoxa1* is expressed in embryonic stem (ES) cells where cofactors are absent, HOXA1 binds with low affinity and fidelity to target domains (De Kumar et al., 2017b). Of note, the HOX-PBX sites are underrepresented in the bound region. Upon retinoic acid (RA) treatment, cofactors such as PBX, MEIS, TGIF and PREP are expressed which leads to HOXA1 high affinity binding on HOX-PBX enriched sequences (De Kumar et al., 2017b). This type of diverse binding capacity is also displayed in zebrafish where HOX:PBX binding sites are highly enriched near MEIS/PREP sites, specifically in the segmentation stage embryo (Ladam et al., 2018). The identification and characterization of HOX-response elements associated with auto-, para-, and cross-regulatory interactions between HOX proteins have revealed that bipartite HOX-PBX sites are commonly used for HOX binding and functional activities (Alexander et al., 2009; Gould et al., 1998; Mann and Chan, 1996; Mann et al., 2009; Manzanares et al., 2001; Slattery et al., 2011; Tümpel et al., 2009).

The HOX-PBX bipartite site is also an integral part of the HOXB1 responsive enhancers of *Hoxb1, Hoxb2, Hoxa2, Hoxa3* and *Hoxb4* in mice and *labial* in *Drosophila melanogaster*. The prevalence of these bipartite elements in so many HOX-response elements in different species and the deep utilization of the PBX and MEIS TALE proteins as cofactors with HOX proteins in bilaterians (Hudry et al., 2011, 2012; Slattery et al., 2011) lends support to the co-selective model (Biggin and McGinnis, 1997). What is interesting is that PBX/EXD-HOX interactions have been shown to be involved in repression as well as activation of target genes (Rauskolb and Wieschaus, 1994), indicating that multiple proteins combine to regulate the nuances of expression. A clear example of this type of cooperative binding is shown in hindbrain specification. Within the vertebrate *HoxB* cluster is a well characterized rhombomere (r) 4 autoregulatory region involved in maintenance of *Hoxb1* in r4 that contains HOX-PBX bipartite sites 5'[T/A]GAT[T/A]GA[T/A]G-3'. Deletion of these conserved blocks results in a pronounced segmentation defect and abolishes r4 expression. It has been shown that this autoregulation is dependent upon the *labial* group of genes (*eg. Labial, Hoxa1* and *Hoxb1*) when tested in both mice and *Drosophila* (Pöpperl et al., 1995; Studer et al., 1998). This interaction is through cooperative binding of HOXB1 with PBX (Marshall et al., 1994; Pöpperl et al., 1995; Pöpperl and Featherstone, 1992) and results

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in expression of *Hoxb1* in r4 of the developing hindbrain in mice, providing clear *in vivo* evidence of complex protein interactions impacting binding site specificity.

Layered upon the protein-protein interactions at the DNA targets is the specificity inherent within the nucleotide target sequences. In *labial*-mediated autoregulation in *Drosophila*, for example, just two base pair alterations in the HOX-PBX site changes its specificity from Lab to Dfd (Chan et al., 1994a, 1997) with dramatic consequences for gene expression. Changing the central base of the bipartite HOX-PBX site from GG to TA resulted in an expression pattern in mice and *Drosophila* that resembled those observed in *Deformed* or *Hoxb4* mutants for both species. Interestingly, similar binding sites with GG as the central base pairs can be found in the *Drosophila Dfd* gene and in the mouse *Dfd* ortholog, *Hoxb-4*. These results indicate that the DNA sequence is central to HOX binding specificity and probably provides a platform for context-specific interaction of HOX proteins with its EXD/PBX and HTH/MEIS cofactors (Joshi et al., 2007; Mann et al., 2009; Ryoo et al., 1999; Sanchez-Hi-gueras et al., 2019; Slattery et al., 2011), thereby defining regional regulatory domains. Manzanares et al. (2001) identified two HOX-PBX bipartite sites and a PREP-MEIS binding site within a conserved block near the *HoxA3* gene. Using *in vitro* binding assays, they showed that the *Hoxa3* PBC-B site can bind HOXB3, HOXD3 and HOXA3 with increasing efficiency. Competition with the HOXA3/PBC-A/B site was effective at blocking other known HOX/PBC sites from HOXB1 and HOXB2 to inhibit binding activity. Furthermore, multimerized HOX/PBC-B sites (five copies) were able to direct reporter expression in r6/7 and r5. The HOX/PBC site at *Hoxa3* differs slightly with other known HOX-PBX bipartite sites and contains TA or TT in the center instead of GG (Manzanares et al., 2001). Comparing results from binding and transgenic assays with sequence information, it appears that subtle changes in binding sites can lead to changes in the nature of binding through the same set of HOX proteins and cofactors with the critical result of altered domains of expression regulated in a gene-specific manner.

Evidence for other cofactor involvement in binding specificity has been demonstrated with UBX and ABDA that have been shown to interact with other cofactors such as Motif1 binding protein (M1BP) through release of transcriptional pausing (Zouaz et al., 2017). Further, it was demonstrated that *Abdominal-B* (*Abd-B*) regulates apoptosis and proliferation of serotonergic neurons important for adult mating behavior in a sex-dependent manner using the sex-specific isoform of Dsx as cofactor (Ghosh et al., 2019). Recently, it has been shown that HOXA1 can regulate expression of genes involved in the balance between pluripotency and differentiation through interacting on NANOG-bound genomic loci (De Kumar et al., 2017a). In addition, TALE proteins such as TGIF also may function as a cofactor of HOXA1 De Kumar et al., 2017b. Interestingly, there is no evidence to support that HOX proteins absolutely require TGIF or PREP proteins for recruitment into genomic targets, leaving this as an open question for exploration. In an elegant study by Sanchez-Higueras et al. (2019), the authors determined that the binding of different *Drosophila* HOX proteins to a critical *cis* regulatory module was dependent not only the ability of HOX proteins to bind alone, but also dependent on cofactors and other “collaborative” proteins. Based on recent studies where Hox genes seems to be cooperating beyond canonical HOX-TALE sites, we propose to elaborate the “Co-selective model” beyond the HOX-TALE paradigm. Instead, we propose a “Cooperative Binding model” (Fig. 1B) where binding

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cooperativity between various cofactors and HOX proteins results in the variation and specificity associated with *Hox* regulation of downstream targets.

6. Clustering and synergy between tethered binding sites

Clustering of low affinity sites and the presence of spatially separated tethered binding sites may provide alternate mechanisms for achieving binding specificity. We define a tethered binding site as having a spatially separated configuration of adjacent sites for HOX and cofactor binding. Structural closeness of tethered sites is dictated by interactions of cofactors bound on these spatially separated sites and contributes an additional layer of regulation. *Antp* P2 *cis*-regulatory element contains 41 Ultrabithorax (UBX) binding sites while the *Dfd* autoregulatory region contains four moderate-to high-affinity binding sites for Dfd protein (Regulski et al., 1991). The two highest affinity sites share a 5'-ATCATTA-3' consensus sequence (Appel and Sakonju, 1993). Recently it has been shown that HOXA1-bound regions contain clustering of HOX, MEIS and HOX-PBX binding sites (De Kumar et al., 2017a; De Kumar et al., 2017b), lending support to the idea that there is a combination of cross- and auto-regulatory mechanisms that control gene expression, depending upon the transcription factor/cofactor landscape. It appears that HOX proteins can use a series of weak binding sites in an additive manner to achieve binding required for regulatory activity. In such cases, multiple sites may increase the overall strength of binding through cooperativity or through increasing the chances of occupancy. This also raises the possibility that HOX proteins can regulate their targets through monomeric binding on a binding site cluster without using cofactors like EXD/PBX or HTH/MEIS. It seems that clustering of HOX binding sites is an important mechanism to achieve high affinity binding from low affinity sites and, hence, specificity in binding (Kuziora and McGinnis, 1988; Regulski et al., 1991; Sanchez-Higueras et al., 2019; Zeng et al., 1994). Binding activity of HOX proteins and cofactors are highly dependent upon chromatin accessibility. Desanlis and coworkers have shown that HOXA11 occupies binding sites for HOX13 paralogous when ectopically expressed in distal limb bud; a domain whose segmental patterning is regulated by *Hox13* genes (Desanlis et al., 2020). Interestingly, these sites are present in a region previously shown to have HOX13-dependent chromatin accessibility (Desanlis et al., 2020). In further support of this idea, HOXA1 can occupy open chromatin regions with HOX binding sites with low affinity and fidelity (De Kumar et al., 2017b). These results indicate that availability of open chromatin is an important aspect in ectopic binding of HOX proteins and is the key aspect for binding specificity of HOX proteins (Porcelli et al., 2019). The role of chromatin accessibility is an area ripe for investigation regarding HOX protein binding capabilities and the role of cofactors in this process.

While investigating the binding specificity of HOX proteins will require consideration of the chromatin landscape, it is also critical to take into account the full context and importance of the array of potential cofactors and their binding sites present in that landscape. In support of a combinatorial approach for regulating gene expression, there have been binding sites found for PBX-MEIS heterodimers adjacent to many HOX-PBX bipartite sites in HOX response elements (Ferretti et al., 2000, 2005). For example, HOX, PBX, and MEIS can bind and form ternary complexes using these adjacent binding sites. Specifically, MEIS can be immunoprecipitated with HOX and PBX in mice. MEIS can also be found as a component

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of the HOX-PBX complex or MEIS can act in the absence of PBX with posterior *Hox* gene products through a cooperative binding mechanism (Chang et al., 1997; Ferretti et al., 2000; Jacobs et al., 1999). Ferretti and colleagues mapped at least three PREP-MEIS sites near the HOX-PBX bipartite site in mice and demonstrated that these are essential for functional activity of the enhancer (Ferretti et al., 2005). Berthelsen and coworkers have shown assembly of a PBX1, PREP1, and HOXB1 trimeric complex on the R3 element of *Hoxb1* (Berthelsen et al., 1998). Interestingly, most of the complexes identified also contain a combination of PBX/PREP-MEIS (PM) and PBX-HOX bipartite (PH) sites suggesting that ternary complexes of HOX-PBX and MEIS may be a common feature on HOX response elements (Ferretti et al., 2000; Gould et al., 1997; Manzanares et al., 2002; Ryoo et al., 1999). Though the *Hoxb1*, *Hoxa2* and *Hoxb2* enhancers generate an r4-restricted expression pattern in transgenic assays, the specific organization and numbers of the PM and PH bipartite sites vary among them and also can vary within the same gene between species (Ferretti et al., 2000, 2005).

Considerable evidence supports the idea that there is synergy between the PM and PH sites in the *Hoxb2* enhancer that ultimately defines its spatio-temporal activity. Ferretti and colleagues argued that differential affinity of PM1; PM2 and R2PM3 sites determine formation of a ternary complex on R3. Increased levels of PBX1 upon RA (Retinoic Acid) induction may change binding affinity of PBX/PREP-MEIS complexes on PM sites through increased availability of PBX for interaction on R3. As a result, these changes may alter configurations from repression to activation. A fine balance in this mechanism is achieved through addition of tethered PREP-MEIS sites (Ferretti et al., 2000, 2005). It has also been argued that PBX and MEIS may interact with this *Hoxb1* r4 enhancer first to open chromatin with the consequence that the availability of *Hoxb1* changes the nature of the interactions and switches this to an active state (Choe et al., 2009). In another example, Tümpel and colleagues identified a conserved region in the *Hoxa2* intron containing three bipartite (PH-1–PH3) sites and a single PBX-PREP (PM1) site (Tümpel et al., 2007). This region served as an r4 enhancer in chick electroporation assays. Hence, these spatially restricted yet separated sites form the basis of recruitment for separate HOX:cofactor, Cofactor:Cofactor complexes which, in turn, form a ternary structure to regulate expression of downstream targets.

Another critical mechanism to consider with regard to complexity of factor binding sites in gene regulation is the potential for direct competition for sites or cofactor binding. Jacob and colleagues presented analyses that the anterior HOX protein (HOXB1 and B2) targets sites for PBX and MEIS may compete with each other to generate a hierarchy of heterodimers (Jacobs et al., 1999). They also suggested an alternative interpretation that the factors may cooperatively interact to establish a higher order and hierarchical DNA binding complexes (Jacobs et al., 1999). The authors verified that HOXB1 binds to the *Hoxb2* enhancer along with MEIS and PBX. MEIS appears to be important for specificity of the ternary complex binding to DNA. They argued that the PBX-MEIS pairing is able to bind DNA without a stringent requirement for the half site, suggesting that the amino terminal domain of TALE proteins may be sufficient for heterodimer formation between TALE proteins. As a result, this would leave their homeodomains free to interact with DNA in various configurations. This latter model would allow assembly of ternary complexes on DNA consisting of a

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site for HOX and PBX with a distinct flanking MEIS site. Despite the differences in models, their work further supports the idea that inclusion of MEIS in HOX-PBX interaction complexes appears to help in increasing specificity (Jacobs et al., 1999). Interestingly, recent results using HOXA1 genome-wide binding indicate another mechanism whereby the TGIF protein may have an independent input into the HOXA1-dependent enhancer without physical interaction (De Kumar et al., 2017c). This latter result leaves open the possibility of as-of-yet unidentified cofactors as mediators in this interaction or another mechanism of interaction, entirely, that could be incorporated into an ever-evolving Cooperative Binding model.

7. Hox proteins–Determinants of their own specificity

The amino acid sequences in the HOX homeodomain add another layer of complexity to the determination of HOX protein binding specificity. Six amino acid motifs, referred to as hexapeptide motifs and located near the homeodomain, play an important role in determining HOX specificity. Many HOX proteins contain a conserved hexapeptide motif containing tyrosine (Y)-proline (P)-tryptophan (W)-methionine (M) in series (YPWM). The importance of this motif varies in different HOX proteins and contexts (Mann et al., 2009). HOXA1 and DFD require the YPWM motif for interaction with EXD/PBX (Green et al., 1998; Joshi et al., 2010). In contrast, UBX and ABDA do not require the YPWM motif, but rather these homeotic proteins interact with a distinct six amino acid motif known as UbdA that is located C-terminal to the homeodomain motif (Galant et al., 2002; Hueber et al., 2013; Merabet et al., 2003, 2007; Saadaoui et al., 2011; Tour et al., 2005). Furthermore, UBX and ABDA have conserved C-terminal residues that are important for their *in vivo* function (Chan et al., 1994b). Phalen and Featherstone have shown that the N-terminal residues are crucial for monomeric and hetrodimeric binding specificity of HOX proteins (Phelan and Featherstone, 1997). They confirmed that this position is contacted by the HOX N-terminal residues and differs between HOX proteins. It is important to mention that the N-terminal difference has moderate to no effect on binding affinity based on *in vitro* studies (LaRonde-LeBlanc and Wolberger, 2003; Phelan and Featherstone, 1997). In the case of HOXA1, Arg5 in the N-terminal arm makes contact with the minor groove and the hexapeptide plays an important role in the interaction with Pbx. In many posterior *Hox* genes across species, the hexapeptide has diverged from the consensus (LaRonde-LeBlanc and Wolberger, 2003). Unlike HOXA1 and HOXD4 proteins, HOXD9 and HOXD10 bind to 5'-TTAT-3' and 5'-TAAT-3' motifs in monomeric binding assays and can bind to 5'-TTAT-3' in heterodimeric binding (Phelan and Featherstone, 1997). The residues responsible for heterodimeric binding can be mapped to Lysine (Lys)-3, Lys-6 and Lys-7. This adds support for the idea that N-terminal residues can alter specificity of monomeric and hetrodimeric binding of HOX proteins from different paralogous groups. One more intriguing aspect of this study comes from the surprising observation that the R3-*labial* binding site is not the site with highest hetrodimeric binding affinity. This raises a question regarding the relative balance between specificity and affinity. Site selection in an *in vivo* context may be more weighted towards specificity rather than to affinity (Phelan and Featherstone, 1997). Neuteboom and Murre selected similar affinity binding sites for HOXC6, HOXB7, HOXB8, and HOX-PBX using the PCR selection method in mice (Neuteboom and Murre, 1997).

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This analysis provided further support for the idea that specificity, rather than affinity, may play a heavier role in complex binding outcome in regulating gene expression.

Additional motifs have been identified that help govern binding specificity. Lelli and colleagues reported that a Tryptophan-based motif is important for interaction with EXD (Lelli et al., 2011). In the case of ABDA, an additional Tryptophan-containing motif with Y followed by aspartic acid (D) (YDWM) differs from the classical HOX hexapeptide motif (Lelli et al., 2011). In addition to these conserved domains, the C-terminal sequences are important for specific interaction with *EXD*-dependent targets. In contrast, UBX does not seem to be dependent on C- or N-terminal regions for context-specific regulation of target genes. Altering the UbdA domain severely affects the binding property of the homeodomain in monomeric or heteromeric (HOX-PBX) binding assays. Lelli and colleagues argued that the presence of the extra Tryptophan-containing motif in posterior HOX proteins may be the basis for dominance of posterior genes over anterior genes (Lelli et al., 2011; Noro et al., 2011). Interestingly, many proteins, other than HOX proteins, such as Engrailed and MyoD, also use Tryptophan to interact with EXD and PBX. In the case of Scr, a single Tryptophan residue in the YPWM motif is sufficient for interaction with the EXD-dependent functional domain (Knoepfler et al., 1999; Peltenburg and Murre, 1996). In a recent paper, Singh and coworkers have demonstrated that even a six-amino-acid-long, highly conserved DNA-binding region is able to confer sufficient specificity and function, thereby dictating the ancestral role of paralogous group 1 genes in mice (Singh et al., 2020).

HOX proteins and cofactor association, together, have the potential to yield a new specificity and binding site recognition, which is not shown by either of them in monomeric binding. In other words, though HOX proteins may show comparable monomeric binding properties on similar AT-rich sequences, their “latent specificity” is unlocked by cofactor association (Slattery et al., 2011). Slattery and coworkers demonstrated that HOX proteins acquire novel binding specificity when they bind together with cofactors. The authors indicated that “latent specificity” occurs when *“differences in the amino acid sequences of transcription factors within the same structural family may only impact DNA recognition when these factors bind with co-factors.”* They argued that this mechanism is distinct from cooperativity, where binding kinetics are important and cofactor association interferes with binding energetics (Slattery et al., 2011).

The source of latent specificity may well be the N-terminal and linker sequences of HOX proteins. Co-binding of EXD with HOX protein secures interaction of the YPWM or hexapeptide region of HOX proteins to DNA. In the case of the Scr homeodomain, two Arginines (Arg, R) at the 3rd and 5th positions are localized to the minor groove by YPWM and EXD interactions (Joshi et al., 2010). An adjacent Glycine (Gly, G) to 3rd position Arg, which is unique to paralogous group 2 *Hox* genes, is required for this interaction. An Arginine (R)-Glutamine (Q)-R (RQR) motif with R in the 3rd position is a unique feature of group 2 proteins and may favor a conformation allowing insertion of both R side chains into the minor groove (Joshi et al., 2007). In the case of class 3b genes (namely *Ubx*, *abdA* and *AbdB*), the homeodomain contains an R at the 2nd position. Crystal structures of HOXA9-PBX complexes reveal that this critical R makes contacts with the minor groove through water-mediated hydrogen bonds (LaRonde-LeBlanc and Wolberger, 2003; Mann

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et al., 2009). Two paralog-specific residues in the HOXA9 Homeodomain define HOXA9-TALE interaction (Dard et al., 2019). This suggests that small changes in one or more amino acids in homeodomains are utilized by cofactors to modulate DNA binding specificity to refine the overall transcription factor landscape at specific target sites. All preferred binding sequences form a narrow groove and Arg5 makes contact with, or is located near to, this region based on available crystal structures (Rohs et al., 2009). Interestingly, minor groove topologies show distinct features based on interactions with HOX proteins. Anterior HOX proteins (group 1 and group 2) prefer narrow minor grooves while posterior proteins achieve specificity through interactions with a wider minor groove. Different HOX proteins bind to distinct DNA sequences but seem to have a similar overall DNA topology and structure (Slattery et al., 2011). Slattery and coworkers argued that the presence of the TpR motif tends to widen the minor groove in the middle of the binding site to accommodate Arg3 and Arg5, while the TpA in group 3 proteins prevents insertion of Arg3.

The target DNA sequence, PBX and MEIS cofactors, and the HOX proteins, themselves, together contribute to modulating binding specificity in concert, thereby impacting gene expression outcome. Small variations in choice of the amino acid in the hexapeptide or the homeodomain, in turn, could lead to recognition of slightly different DNA sequences or cofactor associations. In this context, DNA sequence *per se* seems to be less important than topology generated by a specific sequence, or combination of factors and sequence, to generate a transcription factor landscape at a single gene target or at coordinately regulated gene targets. HOX proteins might bind to different DNA sequences if they are capable of generating a similar overall topology. Many binding and structural studies seem consistent with this possibility. It is also true that the TALE cofactors play a very important role in determining functional output of HOX binding. These cofactors determine stability of the binding complex(es) in addition to recruitment of co-activators and co-repressors that can affect the final outcome of binding (Fig. 2).

In conclusion, monomeric binding sites, negative and positive regulatory interactions, impact of cofactor interaction, chromatin accessibility, subtle amino acid differences, and the spatio-temporal expression profile of *Hox* genes, collectively, define DNA binding specificity. The end result of the complexity of regulation is a supremely fine-tuned system that establishes regional identity with incredible temporal and spatial resolution that shares elements of both the Widespread and Co-Selective models. We suggest that this is more aptly named a “Cooperative Binding model” that incorporates more than just differential co-selection of bound factors at target sites. While the nuances of binding specificity and regulation for the HOX proteins are an area of active investigation, it is important to consider whether or not these types of context-dependent regulation models might be applicable beyond the HOX paradigm. If so, then it will be critical to reconsider some of our current models for transcription factor-DNA interactions in terms of incorporating components of more complex, multi-protein interactions in concert with minute changes in binding motifs that can have considerable impact on DNA binding specificity and alterations in gene expression. Regardless, it is apparent that the binding specificity and rules of function associated with the *Hox* genes are nowhere near as clear-cut as the segment identities they help to define.

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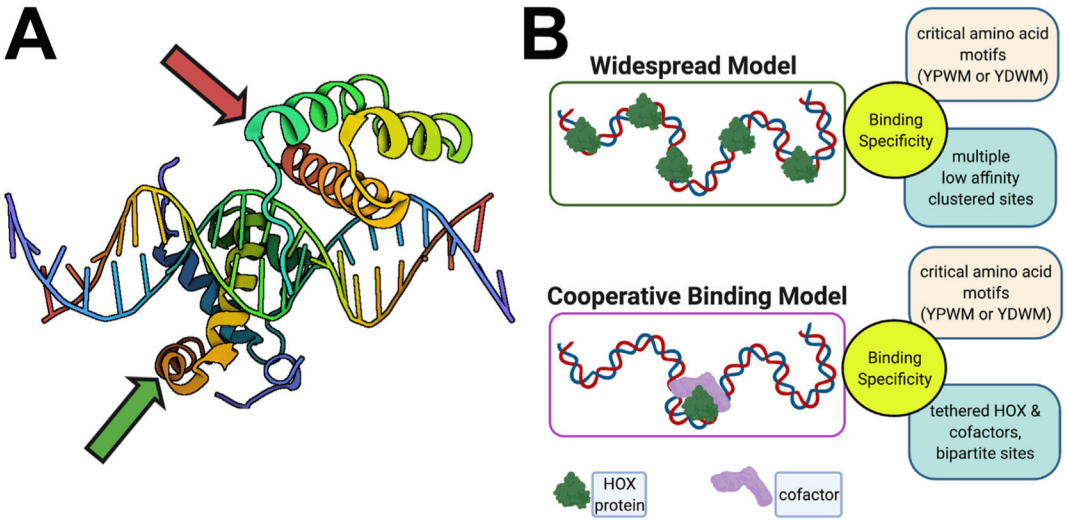

Fig. 1. Current Models for HOX-cofactor interactions on DNA.

HOX proteins have characteristic domains that interact with DNA directly and with cofactors. The image shown (A) is rendered based on X-ray diffraction data of the ternary complex formed when the HOXB1-PBX1 complex (Protein Database ID# 1B72) interacts with DNA (Piper et al., 1999). The red arrow indicates the homeodomain of HOXB1, and the green arrow indicates the binding domain for PBX1. The Widespread model proposed by Biggin and McGinnis (1997) is shown (B) in comparison with a more comprehensive model that builds on the Co-Selective model and incorporates a broader range of regulatory components in a Cooperative Binding model for HOX interactions with DNA. In the Widespread Binding model there are numerous low affinity HOX-response elements and binding sites to which Hox proteins can bind in a clustered fashion. The preponderance of clustering, rather than high affinity interactions at these DNA domains, determine specificity and outcome. In contrast, the Cooperative Binding model reflects high affinity, bipartite sites to which HOX proteins, in combination with varying cofactors (*i.e.* TALE proteins) are able to bind to discrete DNA domains to determine HOX protein binding specificity and affect gene expression outcome. Note that these models are not mutually exclusive, but rather describe potential interaction mechanisms that may lead to fine-tuning HOX protein binding specificity and tightly regulated gene expression outcomes. Images generated with BioRender.com.

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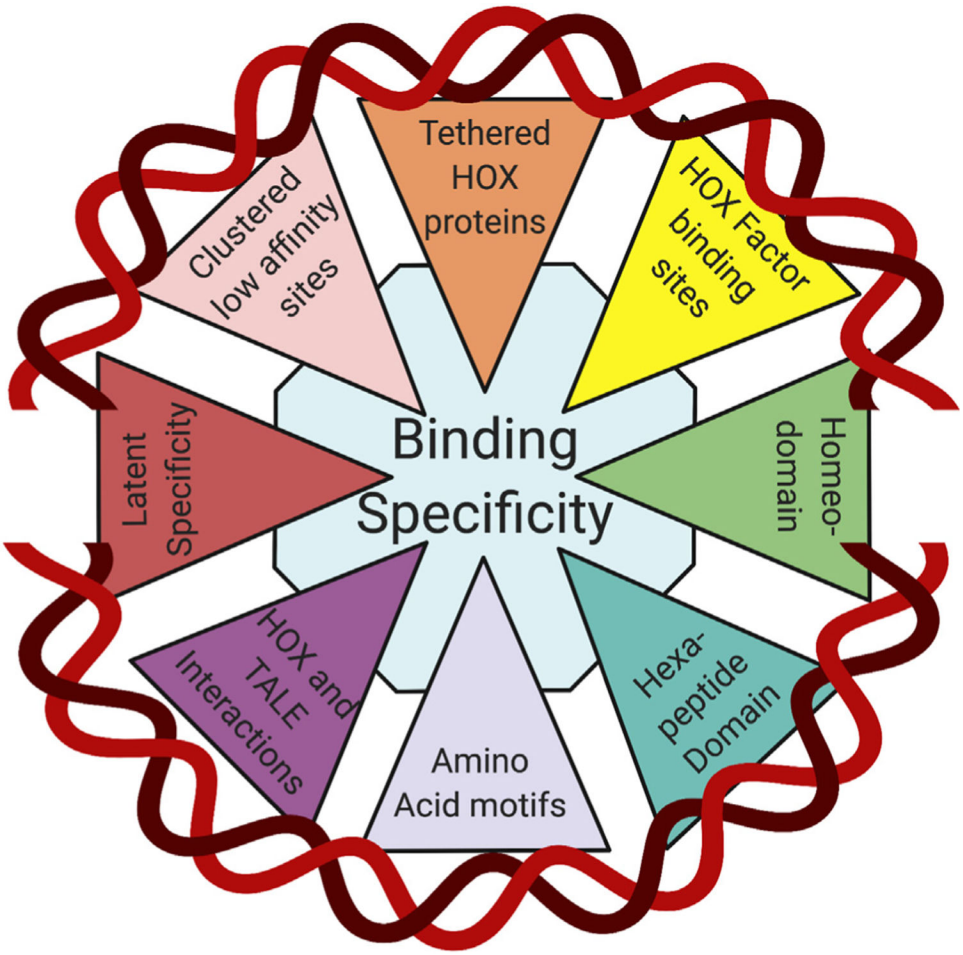

Fig. 2. Integrative Model for Hox protein binding specificity.

The power and potency of HOX proteins in regulating gene expression is founded on a complex integrative weave of regulation that contributes to the transcription factor landscape regulating cell fate decisions and segment identity. From the smallest amino acid changes in critical binding motifs to the recruitment of a wide range of co-activators/repressors, the collective modulation impacts the ability of HOX proteins to bind DNA targets at critical spatial and temporal points during development. The hexapeptide domain adjacent to homeodomain of the HOX protein is required for interaction with HOX-specific DNA sequences, but the specificity could be modified via clusters of low affinity sites, latent specificity revealed by binding partners, or protein-protein interactions with TALE proteins, for example. This multi-level perspective linked to HOX protein function may not be restricted to this pleiotropic family of gene expression regulators. It is certainly feasible to consider that mechanisms of this type may be explored in other transcription factor families in terms of laying the groundwork for an integrative and modular approach to gene expression regulation. Image generated with BioRender.com.