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Hox regulation of transcription – more complex(es)

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Abstract

Hox genes encode transcription factors with important roles during embryogenesis and tissue differentiation. Genetic analyses initially demonstrated that interfering with *Hox* genes has profound effects on the specification of cell identity, suggesting that Hox proteins regulate very specific sets of target genes. However, subsequent biochemical analyses revealed that Hox proteins bind DNA with relatively low affinity and specificity. Furthermore, it became clear that a given Hox protein could activate or repress transcription depending on the context. A resolution to these paradoxes presented itself with the discovery that Hox proteins do not function in isolation, but interact with other factors in complexes. The first such “cofactors” were members of the Extradenticle/Pbx and Homothorax/Meis/Prep families. However, the list of Hox-interacting proteins has continued to grow, suggesting that Hox complexes contain many more components than initially thought. Additionally, the activities of the various components and the exact mechanisms whereby they modulate the activity of the complex remain puzzling. Here we review the various proteins known to participate in Hox complexes and discuss their likely functions. We also consider that Hox complexes of different compositions may have different activities and discuss mechanisms whereby Hox complexes may be switched between active and inactive states.

Keywords

Pbx; Meis; Prep; extradenticle; homothorax; HDAC; CBP; cofactor; gene expression

Introduction

Hox genes were originally identified as key regulators of body segment identity in the fruit fly *Drosophila melanogaster* based on the analysis of mutants where one body segment has taken the identity of another (a.k.a. homeotic transformation; reviewed in (Lewis, 1994)). Genetic and molecular analyses revealed that genes affected by these mutations (homeotic genes) are arranged in genomic clusters that have undergone duplications, such that most vertebrates have four clusters - although teleost fish have as many as seven clusters (reviewed in (Lemons and McGinnis, 2006)). As a result, the mouse and human genomes have 39 Hox proteins, while the zebrafish has 48. Cloning of homeotic genes further revealed that they share a helix-turn-helix DNA binding motif – the homeobox (reviewed in (Gehring et al., 1994)). The presence of this DNA binding domain suggested that Hox proteins function as transcription factors (reviewed in (Levine and Hoey, 1988)). Thus, a straightforward model emerged where Hox proteins regulate the expression of genes involved in the formation of body-segment specific features. However, it soon became apparent that Hox proteins have poor affinity and specificity for DNA sequences, with most Hox proteins binding AT rich sequences (Levine and Hoey, 1988). This poor biochemical specificity is in sharp contrast to the exacting genetic specificity revealed by the distinct

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phenotypes observed in *Hox* mutants. Furthermore, it was found that the same Hox protein can either activate or repress transcription depending on the precise circumstances (Krasnow et al., 1989), thereby raising questions as to what activities are actually mediated by Hox proteins. A resolution to these enigmas presented itself when it became apparent that Hox proteins do not act in isolation, but cooperate with other factors to carry out their functions (reviewed in (Mann et al., 2009)). While the list of factors acting together with Hox proteins was initially relatively short, it has grown considerably over the last several years, suggesting that Hox proteins function in large multi-protein complexes. Here we will review the types of proteins found in Hox complexes as well as the likely effect of each component on the overall activity of the complex. We will also consider the possibility that complexes with different subunit composition may have unique activities.

Composition of Hox Transcription Complexes

While Hox proteins may function in many settings – indeed, they interact directly with factors involved in processes as diverse as vesicular trafficking and cell migration (Lambert et al., 2012) – this review focuses on the role of Hox proteins in transcriptional regulation. In this context, the current view is that Hox proteins act in multi-component regulatory complexes. For the purpose of this review, we will focus on factors that have been shown to act as part of Hox complexes by biochemical assays. However, it should be noted that genetic screens have identified factors that cooperate with Hox proteins and it is likely that some such factors also bind Hox complexes, perhaps in a context-specific manner (for a recent review, see (Mann et al., 2009)). Given these criteria, the components of Hox complexes can, somewhat simplistically, be divided into three groups: 1. The Hox proteins themselves; 2. Cofactors with DNA binding domains that interact with Hox proteins and bind adjacent DNA elements; 3. General factors that do not bind DNA directly, but are recruited by Hox proteins and/or cofactors (Fig. 1A).

Hox proteins

In the context of multi-component complexes, Hox proteins provide several key functions. First, Hox proteins are responsible for targeting the complex to the appropriate gene regulatory elements. The DNA binding specificities of homeodomain-containing proteins have recently been characterized in some detail (Berger et al., 2008; Noyes et al., 2008), identifying at least 65 different specificities for murine homeodomain proteins and 11 for homeodomain proteins in *D. melanogaster*. Since there are many types of homeodomain proteins in addition to Hox proteins, the first level of classification parsed Hox proteins into only two groups – an Abd-B group (corresponding to paralog groups 9-13 in chordates) and an Antp group containing Hox proteins from the remaining paralog groups (reviewed in (Affolter et al., 2008)). However, closer examination of DNA-binding specificities revealed that Hox proteins can be further subdivided based on their preferences for lower affinity target sites. Consequently, Hox proteins as a family bind a relatively broad range of DNA sequences – thereby allowing individual Hox proteins to selectively target transcription complexes to distinct regulatory elements – although it is not fully clear how the lower affinity sites are selected in vivo (see next section for a potential role for cofactors in this process). Second, Hox proteins provide interaction surfaces that recruit additional factors to the complex. For instance, Hox proteins interact with Extradenticle (Exd)/Pbx family proteins via a short motif (YPWM) found N-terminal to the homeodomain (Fig. 1B; reviewed in (Mann and Chan, 1996)). This motif is present in many Hox proteins and the central tryptophan residue makes contact directly with a loop in the Exd/Pbx homeodomain. Some Hox proteins that lack the YPWM motif nevertheless contain a tryptophan in a similar position that may be involved in contacting Exd/Pbx proteins (Shen et al., 1997b). Notably, regions in Hox proteins other than the YPWM motif (such as the homeodomain and C-

terminal residues; (Chan et al., 1994)) are also likely to be required for binding to Exd/Pbx, particularly in the presence of Homothorax (Hth)/Meis/Prep factors (Hudry et al., 2012). In addition, some Hox proteins interact directly with Hth/Meis/Prep factors (Fig. 1B). This interaction appears to be restricted primarily, but not exclusively, to the Abd-B paralogs (Shen et al., 1997a; Williams et al., 2005) and may be independent of the conserved tryptophan motif – instead requiring N-terminal sequences in the Hox protein. Hox proteins also contain domains able to interact with components of the general transcription machinery, as well as regions that recruit chromatin-modifying enzymes (Fig. 1B). For instance, the N-terminus of several Hox proteins binds the CBP/p300 acetyltransferases (Chariot et al., 1999; Saleh et al., 2000; Shen et al., 2001; Choe et al., 2009), that function as coactivators by acetylating histones (Bannister and Kouzarides, 1996; Ogryzko et al., 1996) and other transcription components (e.g. p53 (Gu and Roeder, 1997)). Lastly, a recent search for Hoxa1-binding proteins identified more than forty interacting factors, suggesting that numerous additional components are recruited to Hox complexes (Lambert et al., 2012). Hence, Hox proteins play a central role in the targeting of transcription complexes to specific genomic regulatory elements, as well as in the recruitment of key components to such complexes.

Notably, Hox proteins are expressed in a tissue restricted fashion such that any given cell expresses only a subset of Hox proteins. This arrangement is reinforced by Hox proteins activating and repressing each other's expression in cross-regulatory patterns, as well as by the action of microRNAs targeting *Hox* transcripts (reviewed in (Yekta et al., 2008)). The tissue restricted expression indicates that Hox function is partially regulated at the level of transcription to ensure that Hox complexes of a particular specificity are only present in a subpopulation of cells, although there is also evidence that Hox activity may be modulated post-translationally (e.g. by phosphorylation; see below). The restricted expression of Hox proteins is particularly apparent during embryogenesis when Hox proteins are found in restricted domains along the anteroposterior, dorsoventral and proximodistal axes (reviewed in (Kmita and Duboule, 2003)). For instance, Hox proteins show collinear expression along the anteroposterior axis – in the sense that *Hox* genes located at the 3' end of the *Hox* clusters are expressed earlier and further anteriorly than genes located further 5'. These *Hox* expression domains overlap, causing several *Hox* genes to be expressed in the same region. In these cases, it appears that there is a functional hierarchy where the activity of posterior Hox proteins dominates over the activity of anterior ones. This phenomenon is known as phenotypic suppression, or posterior dominance (reviewed in (Duboule and Morata, 1994)), and the mechanism is unclear, but may involve posterior Hox proteins competing with anterior ones for Exd/Pbx factors (Noro et al., 2011). Hence the tissue restricted expression of Hox proteins, coupled with processes such as posterior dominance, serve to limit Hox function in vivo.

Cofactors

As discussed, factors that interact with Hox proteins can be divided into two classes – cofactors and general factors (Fig. 1A). The first class, discussed in this section, are cofactors that bind DNA – indeed, these cofactors contain homeodomains, albeit of a slightly different type than that found in Hox proteins – and have important roles in the modulation of Hox function. There are two types of Hox cofactors– Extradenticle/Pbx (herein referred to as PBC proteins) and Homothorax/Meis/Prep (herein referred to as HMP proteins).

PBC proteins—*extradenticle* (*exd*) was identified in *Drosophila* as a mutation that causes homeotic phenotypes without affecting the expression of *Hox* genes, suggesting that it is required for Hox function (reviewed in (Mann and Chan, 1996)). Pbx proteins were initially

discovered as fusion proteins resulting from chromosome translocations that cause pre-B cell leukemia in humans (reviewed in (Korsmeyer, 1992)). Subsequently, Pbx proteins were shown to be required for Hox function in several biological systems (Pöpperl et al., 2000; DiMartino et al., 2001; Selleri et al., 2001; Waskiewicz et al., 2002; Manley et al., 2004). While there is only one *exd* gene in *Drosophila*, there are at least four *Pbx* genes in vertebrates. Sequence analyses demonstrated that Exd and Pbx proteins are orthologous. Indeed PBC genes appear broadly conserved and have been found also in *C. elegans* (Burglin and Ruvkun, 1992; Burglin, 1997; Van Aiken et al., 2002). Sequence analyses also revealed the presence of a homeodomain in PBC proteins. The PBC homeodomain is unique in that it contains an additional three amino acids in the loop between helix one and helix two. This arrangement is emblematic of the “three amino acid loop extension” (TALE) subclass of homeodomains (Bertolino et al., 1995). Notably, these additional amino acids form a pocket that constitutes the contact for the YPWM motif in Hox proteins (Passner et al., 1999; Piper et al., 1999) and are therefore essential for the interaction between PBC and Hox proteins that possess this motif – primarily Antp class Hox proteins (Shen et al., 1997b). Pbx also makes contact with Hox proteins via an additional helix immediately C-terminal to the PBC homeodomain and via residues in the linker between the homeodomain and the YPWM motif (Piper et al., 1999; Saadaoui et al., 2011). Indeed, different Hox:PBC interaction modes may be used in different contexts (Saadaoui et al., 2011).

The interaction with PBC proteins is critical for Hox function (reviewed in (Mann and Chan, 1996)). First, several studies have demonstrated that Hox proteins bind DNA with greater affinity in complexes with PBC proteins than alone. In fact, in many instances this effect appears to be cooperative such that the affinity of the complex is greater than the summed affinities of the two monomers (Chan et al., 1994; Chang et al., 1995; Popperl et al., 1995). Second, PBC proteins affect DNA target selection by Hox proteins (Chan et al., 1994; Chang et al., 1996; Lu and Kamps, 1997; Joshi et al., 2007; Slattery et al., 2011). This increased selectivity is mediated, at least in part, by the N-terminal arm of the homeodomain and the adjacent linker region making contacts in the minor groove of DNA. These interactions are thought to be favored in the dimer due to PBC binding to the YPWM motif and fixing the region N-terminal to the Hox homeodomain in a conformation permissive for minor groove contacts. In contrast, there are relatively minor changes in PBC DNA binding preference upon dimerization with Hox proteins (Chang et al., 1995; Slattery et al., 2011), consistent with Hox proteins contributing target selectivity while PBC proteins are required to fully reveal that selectivity. Third, PBC proteins bind factors other than Hox proteins and play important roles in recruiting such factors to Hox transcription complexes (Fig. 1B). For instance, Pbx binds both histone deacetylases (HDACs) and histone acetyltransferases (HATs) (Saleh et al., 2000; Choe et al., 2009), as well as N-CoR/SMRT co-repressors (Asahara et al., 1999), suggesting a role for Pbx in regulating the activity of Hox transcription complexes. Additional Pbx interacting factors, such as the zinc finger Pbx1-interacting protein (ZFPIP; (Laurent et al., 2007)) and hematopoietic Pbx-interacting protein (HPIP; (Abramovich et al., 2000)), continue to be identified, but their function is poorly understood. Lastly, there have been some reports of Pbx homodimers (Neuteboom and Murre, 1997; Calvo et al., 1999), but their potential roles in vivo are not clear. Hence, PBC proteins bind Hox proteins to affect affinity and selectivity for DNA target sequences, as well as to recruit additional factors to the Hox transcription complex. Importantly, PBC proteins fulfill this function not only for Hox proteins, but also for other transcription factors, such as Engrailed, Pdx1, Emx2, Smads and MyoD (Peers et al., 1995; Serrano and Maschat, 1998; Knoepfler et al., 1999; Kobayashi et al., 2003; Bailey et al., 2004; Capellini et al., 2010).

Notably, some functions appear to be conserved within the PBC family - for instance, *Drosophila exd* can rescue embryonic development of a zebrafish *pbx4* mutant (Pöpperl et

al., 2000). However, all functions may not be conserved. In particular, several *Pbx* genes are alternatively spliced, leading to the formation of distinct isoforms (Nourse et al., 1990; Monica et al., 1991; Milech et al., 2001). While such isoforms do not differ in DNA binding specificity, they may contribute different activities to transcription complexes. For instance, the Pbx1a isoform binds the N-CoR/SMRT corepressors, while the Pbx1b isoform, that lacks a C-terminal extension, does not (Fig. 1B; (Asahara et al., 1999)).

In contrast to *Hox* genes, whose expression is tightly controlled both spatially and temporally during development and differentiation, *exd* is broadly expressed in *Drosophila* and *Pbx* genes are expressed in broad, often overlapping, domains in vertebrates (Monica et al., 1991; Rauskolb et al., 1993; Roberts et al., 1995; Pöpperl et al., 2000; Vlachakis et al., 2000; Maeda et al., 2002). Hence, it is unlikely that restricted *PBC* expression represents a regulatory step in controlling their availability as Hox cofactors. However, there is evidence that both nuclear localization and protein stability of PBC factors is controlled via interactions with other factors, such as Hth/Meis/Prep (Aspland and White, 1997; Rieckhof et al., 1997; Pai et al., 1998; Abu-Shaar et al., 1999; Berthelsen et al., 1999; Vlachakis et al., 2001; Waskiewicz et al., 2001), and Pbx nuclear localization is also regulated by PKA-mediated phosphorylation (Kilstrup-Nielsen et al., 2003), suggesting that PBC availability may be controlled post-translationally.

HMP proteins—The second family of Hox cofactors consists of the Homothorax, Meis and Prep proteins. These proteins also belong to the TALE family, but form a subgroup distinct from the PBC proteins. The *Drosophila* ortholog is known as *homothorax* (*hth*; (Rieckhof et al., 1997)) and orthologous genes are found also in *C. elegans* (Burglin, 1997; Van Auken et al., 2002). As in the case of *exd*, disrupting *hth* produces a homeotic phenotype without affecting *Hox* gene expression (Rieckhof et al., 1997). *Meis* genes were originally identified as proto-oncogenes co-activated with *Hox* genes in leukemias (Moskowitz et al., 1995; Nakamura et al., 1996b), while Prep proteins were discovered as transcription factors regulating expression of the urokinase plasminogen activator gene (Berthelsen et al., 1998b). Subsequent analyses revealed the existence of three vertebrate *Meis* genes (*Meis1*, 2, 3; (Nakamura et al., 1996a); four in zebrafish (Waskiewicz et al., 2001)) and two vertebrate *Prep* genes (*Prep1*, 2; (Imoto et al., 2001)). Many groups have demonstrated that HMP proteins form complexes with PBC and Hox proteins (reviewed in (Mann and Affolter, 1998)). In particular, HMP proteins interact with PBC proteins via conserved domains in the N-termini of HMP and PBC family proteins (Fig. 1B; (Chang et al., 1997; Knoepfler et al., 1997; Berthelsen et al., 1998a; Abu-Shaar et al., 1999; Berthelsen et al., 1999; Jaw et al., 2000; Vlachakis et al., 2001)). While PBC:HMP dimers have been observed to form on regulatory elements in vitro (e.g. (Berthelsen et al., 1998b; Bischof et al., 1998)) and such dimers may have functions in vivo, they will not be considered further here since they would act independently of Hox complexes. Meis/Prep proteins are also able to bind some, but not all, Hox proteins. Specifically, Meis proteins interact directly with Hox proteins of the Abd-B class (paralog groups 9-13) via a C-terminal domain containing the Meis homeodomain (Shen et al., 1997a). In contrast, Meis proteins do not interact with Antp class Hox proteins (paralog groups 1-8), at least not when tested in gel shift assays (Shen et al., 1997a; Williams et al., 2005). As discussed above, Pbx proteins appear to have a complementary pattern of Hox interactions such that Pbx binds Antp, but not Abd-B, class Hox proteins (Shen et al., 1997b). Since PBC and HMP proteins interact with each other via N-terminal domains, while both families interact with Hox proteins via residues within and near their homeodomains, it is plausible that HMP:PBC:Hox complexes might form. Indeed, numerous studies have demonstrated the existence of such trimeric complexes (Berthelsen et al., 1998a; Jacobs et al., 1999; Ryoo et al., 1999; Shanmugam et al., 1999; Shen et al., 1999; Ferretti et al., 2000; Vlachakis et al., 2000) and ChIP experiments have identified the presence of all three protein families at *Hox*-regulated enhancers in developing embryos

(Choe et al., 2009; Penkov et al., 2013). Since all Hox proteins appear to interact with either PBC or HMP proteins, it is formally possible that Hox proteins from all paralog groups act in such trimeric complexes, but this has not been tested exhaustively.

HMP proteins are required for Hox function in several contexts (Rieckhof et al., 1997; Jacobs et al., 1999; Ferretti et al., 2000; Waskiewicz et al., 2001; Choe et al., 2002). However, HMP proteins do not appear to modify Hox DNA binding specificity the way PBC proteins do. Instead, HMP may modulate Hox function indirectly by regulating the stability and nuclear localization of PBC, as discussed above (Aspland and White, 1997; Rieckhof et al., 1997; Pai et al., 1998; Abu-Shaar et al., 1999; Berthelsen et al., 1999; Vlachakis et al., 2001; Waskiewicz et al., 2001), as well as by modulating the interaction between PBC and Hox proteins (Hudry et al., 2012). As for PBC proteins, HMP proteins may also regulate the recruitment of transcriptional co-regulators (Fig. 1B). In particular, Pbx:Hox complexes bind HDACs and repress transcription, at least under some conditions (Saleh et al., 2000). This repression can be overcome by blocking HDAC activity (with TSA) or by inclusion of Meis proteins (Saleh et al., 2000; Huang et al., 2005; Choe et al., 2009), suggesting that Meis proteins function by counteracting HDAC activity. Indeed, Meis proteins compete with HDAC for binding to Pbx such that in the presence of Meis, HDACs are displaced from the complex (Choe et al., 2009). In addition, the Meis1 protein contains a C-terminal activation domain that is activated by PKA and/or GSK-3 signaling to interact with TORC and CBP (Huang et al., 2005; Goh et al., 2009; Wang et al., 2010). Hence, HMP proteins may function by controlling co-regulator recruitment to Hox complexes, thereby modulating the function of these complexes.

The two *Prep* genes are essentially ubiquitously expressed (Ferretti et al., 1999; Imoto et al., 2001; Waskiewicz et al., 2001) – similar to *Pbx* genes – and *hth* is broadly expressed in *Drosophila* (Rieckhof et al., 1997). In addition, while expression of the *Meis* genes is spatially and temporally restricted (Nakamura et al., 1996a; Waskiewicz et al., 2001), there are multiple *Meis* genes (3 in vertebrates, except 4 in zebrafish), suggesting that most cells express one or several Meis/Prep proteins. There have been a few reports of functional differences between Meis and Prep proteins – for instance, Meis1 accelerates *HoxA9*-dependent leukemia (Thorsteinsdottir et al., 2001) and responds to PKA signaling (Huang et al., 2005), but Prep1 does neither. However, in other assays Meis and Prep proteins appear interchangeable (Choe et al., 2002). There may also be functional differences among the Meis proteins. In particular, the Meis C-terminus harbors an activation domain (Huang et al., 2005; Goh et al., 2009). C-terminal sequences vary considerably among Meis family members, but zebrafish Meis3 (that has a truncated C-terminus) is nevertheless capable of functioning as a Hox cofactor to activate transcription (Vlachakis et al., 2001; Choe et al., 2009). Hence, Meis/Prep family members may differ in some functional features, but also share several key features. Hox complexes that contain different Meis/Prep family members may therefore share many activities, but may also have some distinct functions, as supported by recent genome wide ChIP data (Penkov et al., 2013).

Lastly, as in the case of PBC proteins, HMP proteins act as cofactors not only to Hox proteins, but also to other transcription factors such as MyoD and Engrailed (Kobayashi et al., 2003; Berkes et al., 2004).

General factors

The second class of factors that associates with Hox complexes has more general roles in transcription. Most of these factors differ from the PBC and HMP cofactors in that they lack DNA-binding domains and must therefore be recruited to Hox complexes exclusively via protein-protein interactions. In general, these factors are ubiquitously expressed and, accordingly, are available for recruitment to Hox complexes in most cell types. This group

of factors includes chromatin-modifying enzymes mentioned previously. Specifically, Hox and Pbx proteins interact with CBP (Chariot et al., 1999; Saleh et al., 2000; Shen et al., 2001; Choe et al., 2009), as does Meis1 (Huang et al., 2005; Wang et al., 2010), but apparently not Meis3 (Choe et al., 2009). CBP acts as a histone acetyltransferase (HAT) that acetylates nucleosomes, thereby promoting open chromatin and facilitating transcription (Goodman and Smolik, 2000). CBP can also acetylate non-histone proteins, but, to date there is no evidence that acetylation of Hox complex components affects the activity of the complex. In addition, Pbx proteins interact with HDAC1 and HDAC3 co-repressors, either directly or via NCoR/SMRT, (Asahara et al., 1999; Saleh et al., 2000; Choe et al., 2009). HDACs function as histone deacetylases to oppose the activity of CBP, thereby repressing transcription. The recruitment of co-repressors appears to be mediated via both N- and C-terminal domains in Pbx (Asahara et al., 1999; Saleh et al., 2000), while the CBP co-activator interacts with N-terminal activation domains in Hox proteins and a C-terminal domain in Meis1 (Chariot et al., 1999; Saleh et al., 2000; Huang et al., 2005; Goh et al., 2009).

Importantly, these co-activators and co-repressors have themselves been found to be part of larger regulatory complexes. For instance, HDAC1 is part of the Sin3, NuRD and CoREST complexes, while HDAC3 is part of the NCoR/SMRT complex (reviewed in (Hayakawa and Nakayama, 2011)). Hence, while experiments to date have examined only recruitment of individual co-activators/co-repressors, it is likely that they are recruited as components of larger complexes that contribute additional activities. For instance, both the NuRD and CoREST complexes contain enzymes that regulate nucleosome positioning and modification (Tong et al., 1998; Xue et al., 1998; Shi et al., 2004)).

Ultimately RNA Polymerase II must be recruited to Hox-regulated promoters and activated to drive transcription. As in other systems, this is likely to be accomplished by recruitment of the Mediator complex (reviewed in (Conaway and Conaway, 2011)). Mediator is commonly recruited to enhancers by sequence specific transcription factors and Mediator then bridges the distance to the transcription start site where it facilitates RNA Polymerase II loading. Hence, it is likely that Hox, PBC and/or HMP proteins recruit Mediator. However, interactions between these factors and Mediator have not been well characterized and it is unclear how recruitment and activity of Mediator is regulated by Hox transcription complexes.

Are there Multiple Types of Hox Transcription Complexes with Unique Subunit Composition?

Comparisons among Hox proteins reveal that sequences are highly variable outside the homeodomain (reviewed in (Sharkey et al., 1997)), suggesting that each Hox protein, or at least each Hox paralog group, may interact with a unique set of factors. Furthermore, the number of known Hox-interacting factors is large – and ever increasing, making it unlikely that all these factors interact with Hox complexes simultaneously. Indeed, as discussed, there are cases where two factors use the same interaction site in the Hox complex, such that only one of the factors can be bound at a time (Choe et al., 2009). Hence, diversity in Hox protein sequence and competition among interacting factors suggest that there is likely to exist multiple Hox-containing complexes that vary in their subunit composition.

Variations in Hox complex composition

By definition, every Hox transcription complex will contain a Hox protein. As outlined above, Hox proteins provide DNA-binding specificity and are therefore responsible for targeting the correct regulatory element. Since Hox proteins bind DNA poorly as monomers,

it is likely that at least one cofactor will also be part of every Hox transcription complex. PBC proteins are the most likely candidates in this regard since their role in improving the affinity and specificity of Hox binding to DNA is well documented (Mann and Chan, 1996; Slattery et al., 2011). It is possible that complexes containing a Hox protein together with a cofactor of the HMP family, but lacking a PBC cofactor, exist – particularly since some Hox proteins appear to lack interaction sites for PBC proteins (Shen et al., 1997b) – but there is little direct evidence that such complexes have functional roles in vivo. Hence, a Hox protein together with a PBC cofactor may represent a minimal set of core components shared among all Hox transcription complexes. Notably, there is likely to be some variability among complexes already at this level, since there are multiple Pbx family members that also can be alternatively spliced (as discussed above).

Trimeric complexes consisting of Hox, PBC and HMP proteins have been reported to form in several contexts and to be required for transcription of several Hox-regulated genes (Berthelsen et al., 1998a; Jacobs et al., 1999; Ryoo et al., 1999; Shanmugam et al., 1999; Shen et al., 1999; Ferretti et al., 2000; Vlachakis et al., 2000). Hence, it is possible that one difference between Hox transcription complexes stems from the presence of one (PBC) or two (PBC and HMP) cofactors in the complex. Since PBC and HMP cofactors interact with distinct general factors, complexes that differ in cofactor composition would be further differentiated by recruitment of distinct general factors. In fact, complex composition can become very complicated when one considers that one cofactor may interact with multiple general factors (e.g. Pbx proteins bind both HDAC and CBP; (Saleh et al., 2000; Choe et al., 2009)), or when complex components are present that recruit general factors with opposing activities – e.g. Pbx cofactors recruit HDACs (Saleh et al., 2000) while Hox proteins recruit CBP (Chariot et al., 1999; Saleh et al., 2000; Shen et al., 2001; Choe et al., 2009).

Regulation of Hox complex composition

Given that various types of Hox complexes are likely to occur, it seems likely that regulatory mechanisms exist to ensure the presence, or activity, of only a subset of all possible interacting factors. At present there are no reported cases where a factor is present in a Hox complex, but its enzymatic activity is suppressed, although this remains a plausible mechanism for control of Hox complex activity. In contrast, there is evidence for competition among factors for inclusion in Hox complexes. As discussed, Meis proteins compete with HDACs for binding to Pbx proteins, as demonstrated both in cell lines and in zebrafish embryos (Choe et al., 2009), suggesting that Hox complexes containing Meis proteins lack HDAC activity. Furthermore, when anterior and posterior Hox proteins are co-expressed, posterior ones are more likely to form complexes with Exd on target sequences in *Drosophila*, leading to posterior Hox functions dominating over more anterior ones (Noro et al., 2011). Such examples of competition among Hox complex components indicate that the relative concentrations and binding affinities of the various factors may dictate the exact composition of each complex, but neither of these parameters is known for any Hox complex components.

At its simplest level, therefore, complex formation could be regulated by controlling the presence of each factor. However, except for Hox proteins, most Hox complex components are expressed very broadly, or, as in the case of Meis proteins, there are multiple family members with comparable activities. As a result, any given cell is likely to express most potential Hox complex components. For that reason, it seems unlikely that tissue specific gene expression is an important mechanism for regulation of Hox complex composition. In contrast, there is evidence for regulation at the level of subcellular localization, at least in the case of the cofactors (Fig. 2). In particular, PBC proteins are found in the cytoplasm in some cell types (e.g. *Drosophila* embryos and Schneider cells), apparently as a result of active

nuclear export via an exportin-1 dependent mechanism (Rieckhof et al., 1997; Pai et al., 1998; Abu-Shaar et al., 1999; Berthelsen et al., 1999), although it is also possible that PBC proteins are retained in the cytoplasm via interactions with non-muscle myosin (Huang et al., 2003). PBC proteins localize to the nucleus upon interaction with HMP factors, most likely as a result of this interaction blocking the PBC nuclear export signal (NES), but PBC localization is also affected by PKA-mediated phosphorylation (Kilstrup-Nielsen et al., 2003). Similarly, HMP proteins are located in the cytoplasm of many cell types (e.g. NIH3T3, COS7, HeLa cells and zebrafish embryos) and enter the nucleus upon interaction with PBC factors (Berthelsen et al., 1999; Vlachakis et al., 2001). Hence, both PBC and HMP proteins are predicted to be located to the nucleus in cells that co-express cofactors from both families. The mutual dependence of PBC and HMP proteins for nuclear localization would seem to exclude the formation of nuclear Hox complexes containing only one cofactor. However, Exd is nuclear in some *Drosophila* tissues and Pbx is nuclear in several mammalian cell lines, as well as in zebrafish embryos, apparently independently of HMP interactions (Rieckhof et al., 1997; Berthelsen et al., 1999; Vlachakis et al., 2001; Choe et al., 2002), indicating that PBC factors may enter the nucleus by multiple mechanisms and suggesting the existence of Hox transcription complexes containing PBC, but not HMP, proteins. Accordingly, nuclear localization is a plausible mechanism for regulating the incorporation of cofactors into Hox complexes. While such regulation might have some advantages – e.g. rapid control of complex formation without the need to translate protein – it nevertheless represents a relatively blunt regulatory approach. In particular, there are likely to be many different Hox complexes acting in a cell at any given time and all these complexes would be affected if a cofactor were retained in the cytoplasm. Hence, regulation of nuclear localization might be used at key developmental steps, such as when differentiation of a tissue is initiated, but it seems unlikely to provide fine control of the composition of individual Hox complexes.

It is also possible that complex composition is regulated at the level of assembly (Fig. 2). For instance, the Antennapedia (Antp) Hox protein can be phosphorylated by casein kinase II (CKII; (Jaffe et al., 1997)). When the consensus CKII target sites are mutated to glutamic acid (in order to mimic phosphorylation), Antp is significantly impaired in its ability to interact with Exd (Jaffe et al., 1997), suggesting that phosphorylation affects Hox complex assembly. In addition, the association between CREB and Meis is regulated by GSK-3 mediated phosphorylation of CREB (Wang et al., 2010). While there is little evidence for other covalent modifications of Hox complex components, it is plausible that such modifications take place – e.g. Hox and Pbx proteins interact with acetyl transferases – and that they modify Hox complex assembly.

Complex formation might also be dictated by target site composition (Fig. 2). For instance, a given regulatory DNA element may lack the binding site for one of the cofactors, thereby forcing the formation of specific complexes, or the orientation of binding sites may put constraints on the Hox complex such that certain subunits cannot be incorporated. However, it is problematic to infer the type of complex formed on a particular regulatory element from the sites present in that element. First, it is sometimes difficult to reliably define binding sites by sequence analysis. Indeed some elements originally reported as containing only Hox and Pbx sites were subsequently found to also contain Meis/Prep sites (e.g. (Popperl et al., 1995; Ferretti et al., 2000)). Second, HMP cofactors may participate in Hox complexes without contacting DNA. For instance, HMP-containing complexes can form on sites that lack Hth, Meis or Prep binding sites and HMP constructs lacking their homeodomains can still be incorporated into complexes, although formation of such complexes is sometimes less efficient (Berthelsen et al., 1998a; Ryoo et al., 1999; Shanmugam et al., 1999; Vlachakis et al., 2000; Vlachakis et al., 2001).

Hence, it is likely that there are multiple types of Hox transcription complexes with unique subunit composition, but the mechanisms whereby subunit composition is regulated are only beginning to be understood.

Do Different Complexes Have Different Activities?

The possible existence of Hox transcription complexes with unique subunit composition raises the likelihood that such complexes may differ in terms of their activity and that complex activity may be dynamically regulated by alterations in subunit composition. It is interesting to note that early functional analyses reported that Hox proteins could either activate or repress transcription (reviewed in (Hayashi and Scott, 1990)). In fact, in some cases the same Hox protein functioned as an activator or repressor depending on the context of the experiment – as exemplified by *ultrabithorax* (Krasnow et al., 1989; Vachon et al., 1992; Capovilla et al., 1994; Galant and Carroll, 2002). A potential explanation for these observations began to emerge as cofactors were discovered. In particular, investigators found that in some cases Hox activity is altered when Hox proteins interact with PBC proteins (Pinsonneault et al., 1997). This effect persisted under circumstances where PBC proteins were not required to facilitate binding of Hox proteins to DNA (Li et al., 1999), suggesting that complex formation can alter Hox activity independently of DNA binding.

While there are likely multiple ways to switch between repressive and activating Hox transcription complexes, data from several recent reports appear to be converging on a general outline for one possible mechanism (Fig. 3). In particular, several reports indicate that complexes containing Hox and Pbx are unable to activate transcription in some contexts. For instance, co-transfection experiments in cell lines revealed that Hox and Pbx cannot activate expression of a reporter construct containing a Hox-regulated element from the *hoxb1* promoter (Saleh et al., 2000; Choe et al., 2009). Similarly, misexpression of Hoxb1b and Pbx does not activate ectopic *hoxb1a* expression outside the hindbrain in zebrafish (Vlachakis et al., 2001). At least in this context, it appears that Hox:Pbx complexes are not merely inactive, but actively repress transcription by recruiting co-repressors (Saleh et al., 2000; Choe et al., 2009). Note that not all Hox:Pbx complexes may act as repressors – the fact that Pbx proteins exist in multiple splice forms, where some forms interact with co-repressors while others do not (Asahara et al., 1999; Saleh et al., 2000), together with the fact that Pbx can bind CBP co-activators (Choe et al., 2009), suggests that some Hox:Pbx complexes may be activators. Importantly however, the repressive Hox:Pbx complexes can be converted to activating complexes by treating cells with trichostatin A (TSA; a HDAC inhibitor) or retinoic acid (RA), or by stimulating the PKA or GSK-3 pathways (Saleh et al., 2000; Huang et al., 2005; Goh et al., 2009; Wang et al., 2010). As discussed above, co-expression of Meis proteins also converts Hox:Pbx complexes to activators by displacing HDACs (Choe et al., 2009). These observations can be combined into a model for conversion between repressive and activating complexes (Fig. 3). A Hox:Pbx complex would be repressive due to recruitment of NCoR/SMRT and HDAC co-repressors. Treatment with TSA would repress the HDAC activity, allowing the complex to support transcription – possibly in conjunction with other factors, such as CBP. In contrast, the RA, GSK-3 and/or PKA pathways might act via Meis proteins to create an activating complex. In particular, RA induces *Meis* expression (Oulad-Abdelghani et al., 1997), while both PKA and GSK-3 signaling promotes CBP-mediated transcriptional activation via the Meis C-terminus (Huang et al., 2005; Goh et al., 2009; Wang et al., 2010). Hence, the end result of the conversion process would be displacement of co-repressors and recruitment of co-activators. Notably, it is not clear that all three signaling pathways would be required simultaneously. For instance, cells that already express Meis might not require RA signaling. Depending on the cell type, it is also possible that other pathways than PKA or GSK-3 might act to regulate Meis function. Lastly, it is not clear how broadly this model

can be extended and it is possible that different target genes are regulated in different manners, but it is noteworthy that HMP proteins are required for the expression of several well-studied Hox-regulated promoters (Jacobs et al., 1999; Ryoo et al., 1999; Ferretti et al., 2000; Vlachakis et al., 2001), suggesting that HMP factors may be generally associated with activating Hox complexes. Consequently, it appears that subunit composition has a direct effect on the function of Hox transcription complexes, although the relationship between composition and function is not yet fully defined.

These findings also raise the question as to how changes in subunit composition can alter complex function. In the case of the Hox:PBC complexes discussed here, the functional switch is caused by Meis proteins competing with HDACs for binding to the Pbx N-terminus (Choe et al., 2009). In the absence of Meis cofactors, HDAC is bound to Pbx and the complex is repressive, but in the presence of Meis proteins, Meis associates with Pbx and HDAC is displaced. Furthermore, when HDAC is displaced, CBP (a histone acetyltransferase) can associate with the Hox:Pbx:Meis complex and promote transcription. A similar displacement process has been shown for the Otx2 homeodomain protein, where Meis2 displaces the Tle4 repressor (Agoston and Schulte, 2009), suggesting that this may be a general mechanism for controlling activity of a transcription complex. However, it is easy to envision other mechanisms to achieve the same end – e.g. modification of subunits by covalent modifications such as phosphorylation (Galant and Carroll, 2002; Ronshaugen et al., 2002) or acetylation may affect their activity – and it is likely that such alternative mechanisms will emerge.

Lastly, it is important to keep in mind that most genes are controlled by multiple regulatory elements, each of which receives distinct inputs – often via distinct signaling pathways. Thus, the effect of a Hox complex acting at one such regulatory element will be integrated with the effects of other factors acting at adjacent regulatory elements to control the overall activity of a gene. In fact there are numerous instances where Hox-regulated genes receive inputs from several signaling pathways (for a recent review, see (Mann et al., 2009)).

Perspectives

Several important questions remain with regards to the composition and function of Hox transcription complexes. First, we still do not know the exact composition of even one Hox-transcription complex and it is therefore difficult to study the function and regulation of such complexes. It would be useful to identify all components of a complex under well-defined conditions, perhaps by purification from cell lines followed by mass spectrometry analysis. Second, it would be informative to compare the composition of two closely related complexes that have different activity – e.g. two complexes that contain the same Hox protein, but that function either as an activator or a repressor – in order to understand which components are responsible for imparting a specific activity. Third, a system for reconstitution of Hox complex function would allow the identification of a minimal set of factors required for each function. While these approaches rely on biochemical purification strategies to identify novel complex components, candidates can also be identified by protein interaction screens (as recently reported for Hoxa1, (Lambert et al., 2012)), or genetic screens, followed by functionally characterization to determine if they act in Hox-complexes.

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Bullet points

- Hox proteins function in multi-subunit complexes
- Hox complexes contain cofactors and general transcription regulatory factors
- Hox complexes of different composition have different functions
- Hox complexes may switch from one function to another by changes in composition

Figure 1.

A. Diagram of a generalized Hox transcription complex. The Hox protein (brown) is shown binding to DNA, as are cofactors (blue) that interact both with the Hox protein and adjacent DNA elements, while general factors (grey) are recruited solely via protein-protein interactions. **B.** Diagram of Hox, PBC and HMP family proteins. Hox, PBC and HMP proteins are shown schematically with N-termini to the left and C-termini to the right. The diagram is intended to represent one generic member of each family and is not drawn to scale. Interacting factors are listed above each protein family at the approximate location reported to contain the binding site for that factor. The dashed line in PBC indicates the longer C-terminus present in Pbx1A. Note that all members of a family may not engage in all interactions shown. For instance, Prep proteins may not interact with CBP or TORC. Also, interacting proteins for which binding sites have not been mapped are not shown – for instance, PBC proteins are reported to bind CBP, but the exact binding site in PBC has not been delineated.

Figure 2.

Diagram of potential steps regulating Hox complex assembly. The formation of Hox complexes may be regulated at several steps. Step 1 represents translocation to the nucleus. Note that individual HMP and PBC proteins are cytoplasmic – as result of tethering in the cytoplasm, phosphorylation status, nuclear export and/or lack of a functional nuclear import signal – and enter the nucleus as HMP:PBC dimers, while Hox proteins appear to enter the nucleus on their own. In addition, interaction between HMP and PBC proteins promotes the stability of these proteins. Step 2 indicates modifications of complex components affecting complex assembly – in this case exemplified by phosphorylation of Hox proteins. Step 3 refers to the assembly of complexes on DNA, where the specific arrangement of binding sites may affect the type of complexes that can form. See text for further details.

Figure 3.

Diagram of repressive and activating Hox transcription complexes. Hox transcription complexes may be converted from a repressive form (left side) to an activating form (right side) by signaling via the retinoic acid (RA), protein kinase A (PKA) or glycogen synthase kinase-3 (GSK-3) pathways. See text for details.