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Review Article

Hox Genes: Let's Work Together

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Hox genes determine antero-posterior (A-P) axis in all the Bilaterians. Function of *Hox* genes in a collinear and non-overlapping order sets the A-P body axis. *Hox* genes achieve the non-overlapping functions by posterior prevalence phenomenon. In the posterior prevalence a posterior *Hox* gene dominates over the anterior *Hox* genes to impose its function in regions of overlapping expression. Now, a few studies have shown exceptions to this rule. This review discusses the mechanisms and exceptions to the rule of posterior prevalence. Our current understanding suggests that anterior and posterior *Hox* genes have adopted mechanisms to work together during development.

Keywords: Hox Genes; Bithorax Complex; Posterior Prevalence/Dominance, *abd-A*, *Abd-B*, *Drosophila melanogaster*

Introduction

Determination of Antero-posterior (A-P) body axis is a key process during development of animals. A set of genes called *Hox* genes determine A-P body axis in all the Bilaterians (Duncan, 1987; Fienberg *et al*., 1987; Lewis, 1978; McGinnis and Krumlauf, 1992). Hox proteins are homeo-domain containing transcription factors. This sixty amino acid long homeodomain can directly bind to DNA and regulate the expression of several hundred genes (Ekker *et al*., 1991). The combined outcome of gene expression set by different Hox proteins along A-P axis ultimately sets the final transcription profile and identity of the cells. Considering the importance of Hox proteins, their binding in the genome should be highly specific. One known way of providing specificity to Hox protein binding in the genome is through cooperative binding with cofactors (Chan *et al*., 1994; Mann and Chan, 1996; Mann *et al*., 2009; van Dijk and Murre, 1994). The best characterized cofactors of Hox proteins in *Drosophila* are Homothorax (HTH) and Extradenticle (EXD) (Ryoo *et al*., 1999; van Dijk and Murre, 1994).

Hox genes are mostly clustered in the genome (Akam, 1989; Duboule, 2007; Duboule and Dolle, 1989). The presence of Hox genes in the cluster is collinear with their spatial expression along the embryo (Beachy *et al*., 1985; Duboule and Morata, 1994; Harding *et al*., 1985; Holland and Hogan, 1988; Pearson *et al*., 2005). The Hox gene at 3′ end of the complex is expressed towards anterior part of the embryo while genes towards 5' end express towards posterior part of the embryo. The collinear expression of *Hox* genes is most crucial in determining the A-P body axis. Similar to the expression, the function of *Hox* genes is also collinear and different *Hox* genes function sequentially across the A-P axis of the embryo to determine the formation of different body organs (Duboule, 1998; Krumlauf, 1994; Lewis, 1978; McGinnis and Krumlauf, 1992; Morata and Kerridge, 1981; Scott *et al*., 1983). Apart from collinear expression, there are several instances where expression of anterior *Hox* genes overlaps with the expression of the posterior *Hox* genes. Functional studies in overlapping expression regions showed that in such regions posterior *Hox* gene dominates over anterior *Hox* gene and determines the identity in the

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overlapping region. (Gibson and Gehring, 1988; Gonzalez-Reyes *et al*., 1990; Gould *et al*., 1997; Lamka *et al*., 1992; Maeda and Karch, 2006; Miller *et al*., 2001; Schneuwly *et al*., 1987; Singh and Mishra, 2014; Struhl, 1983). This phenomenon operating among of *Hox* genes is called as posterior dominance/ prevalence or phenotypic suppression of anterior *Hox* genes (Capovilla and Botas, 1998; Macias and Morata, 1996; Struhl, 1983). Posterior prevalenc equestions the advantage of overlapping expression of *Hox* genes. If posterior prevalence rule operates without any exception, then the expression of anterior *Hox* gene in the domain of a posterior *Hox* gene is futile. The expression analysis of *Hox* genes across the animal phylum suggests that the overlapping expression of *Hox* genes is a common phenomenon and selected during the evolution. This strong evolutionary selection indicates an unknown functional relevance to overlapping expression.

Hox genes were discovered and studied in great detail in *Drosophila.* The advantage of fly genetics and resources provides a great lead to study the function of *Hox* genes in *Drosophila*. The *Hox* genes in *Drosophila* are clustered in two complexes; Antennapedia (ANT-C) and Bithorax (BX-C) (Sanchez-Herrero *et al*., 1985; Scott *et al*., 1983). Of the total eight *Hox* genes, ANT-C has five; *labial* (*lab*), *proboscipedia*(*pb*), *Deformed* (*Dfd*), *Sexcombreduced* (*Scr*), *Antennapedia* (*Antp*) while BX-C has three *Hox*genes; *Ultrabithorax (Ubx*), *abdominal-A (abd-A*) and *Abdominal-B (Abd-B*) (Akam, 1989; Duncan, 1987; McGinnis and Krumlauf, 1992). *Hox* genes show regulatory hierarchy in A-P body axis determination (Bachiller *et al*., 1994; Duboule and Morata, 1994; Gonzalez-Reyes *et al*., 1990; Gould *et al*., 1997; Lamka *et al*., 1992; Macias and Morata, 1996; Miller *et al*., 2001; Sprecher *et al*., 2004). The *Hox* genes of BX-C suppress the function of ANT-Cgenes while the *abd-A* and *Abd-B* could suppress function of *Ubx* (Beachy *et al*., 1988; Hafen *et al*., 1984; Lamka *et al*., 1992; Struhl and White, 1985). Ectopic expression of posterior *Hox* gene suppresses phenotypes of anterior *Hox* genes suggesting a posterior prevalence (Gonzalez-Reyes and Morata, 1990; Gonzalez-Reyes *et al*., 1990; Lamka *et al*., 1992; Miller *et al*., 2001). There have been many exceptions and adjustments to the posterior prevalence theory (Foronda *et al*., 2006; Gonzalez-Reyes *et al*., 1990; Lamka *et al*., 1992; Mann and Hogness, 1990; Miller *et al*., 2001; Noro *et al*., 2011; Singh and Mishra, 2008; Singh and Mishra, 2014; Yekta, *et al*., 2008). In this review, I would mainly discuss exceptions and mechanisms of posterior prevalence rule.Throughout this review the term anterior and posterior *Hox* gene has been used repeatedly. An anterior *Hox* gene represents any *Hox* gene placed towards the 3' end of the *HOX*-complex with respect to the other *Hox* genes. Similarly, a posterior *Hox* gene means the *Hox* gene is placed towards the 5' end of the *HOX* complex with respect to the other *Hox* genes.

Mechanisms of Posterior Prevalence in *Drosophila*

Posterior Hox protein can impose posterior prevalence phenomenon on an anterior Hox gene at various stages of gene regulation like transcription, post-transcription, translation and post translation. In this section, we will discuss the known mechanisms of posterior prevalence in *Drosophila*.

Transcriptional Suppression

A simple and straightforward mechanism of posterior prevalence imposed by posterior Hox proteins on an anterior *Hox* gene could by direct transcriptional repressing. *Hox* genes function in collinear order across the A-P axis of the embryo to differentiate different parasegments of the embryo into different body parts. So in the posterior embryonic parasegments where expression of *Hox* genes overlaps, posterior *Hox* genes can directly impose transcriptional suppression on the anterior Hox genes. This hypothesis is not completely true because we do see overlapping expression of *Hox* genes (Harding *et al*., 1985; Kuroiwa, 1987; McGinnis and Krumlauf, 1992; Pearson *et al*., 2005). Actually, a closer look suggests that they even express in same nuclei (Singh and Mishra, 2014). This suggests that posterior Hox proteins do not completely suppress the transcription of anterior *Hox* genes. However, initial studies have clearly shown that posteriorly expressed *Hox* genes have the ability to repress more anteriorly expressed *Hox* genes (Gonzalez-Reyes and Morata, 1990; Mann and Hogness, 1990; Miller *et al*., 2001). For example, BX-C *Hox* genes repressANT-C *Hox* genes (Gonzalez-Reyes and Morata, 1990; Miller *et al*., 2001), *abd-A* and *Abd-B* repress *Ubx* (Struhl and White, 1985) and *Abd-B* represses *abd-A* (Singh and

Mishra, 2014). Probably expression of posterior *Hox* gene in the expression domain of anterior *Hox* gene partially but not completely suppresses their expression. This is further supported by the fact that in overlapping expression domains expression of anteriorly expressed *Hox* genes is generally lower in comparison to posteriorly expressed *Hox* genes. In an *in vitro* experiment, authors showed that Ubx protein can bind near the promoter of anterior *Hox* gene *Antp* (Beachy *et al*., 1988). This suggests that, Ubx can directly repress *Antp* gene expression by binding on its promoter. Further, in genome-wide *in vivo* binding analysis of Ubx protein in the *Drosophila* imaginal discs using chromatin immuno-precipitation showed Ubx binding on the promoters of all anterior Hox genes and not posterior *Hox* genes (Slattery *et al*., 2011). This observation provides an evidence for direct Ubx-mediated transcriptional regulation of anterior *Hox* genes.

Suppression through Non-coding RNAs

Sense and anti-sense long non-coding RNAs are detected from overlapping region of the BX-C (Bender, 2008; Stark *et al*., 2008; Tyler *et al*., 2008). The sense transcript is named as *mir-iab-4* and anti-sense transcript is named as *mir-iab-8*. Further analysis of these two long transcripts showed that, they are further processed to give rise to a sense miR-iab-4-5p and an anti-sense miR-iab-8-5p miRNA respectively. These two ~22 bases long miRNAs are complementary to each other. Although, these two miRNAs are generated from the same part of the genome, they have very different expression and function. The *mir-iab-4* miRNA is expressed from parasegment (PS) 7 to 12 of embryo, while *miR-iab-8* is expressed in PS 13 and 14. This shows a completely non-overlapping expression. Further, functional studies suggested that miRNA-iab-4 has potential to knock-down *Antp* and *Ubx* mRNAs while miRNA-iab-8 can knockdown *Antp*, *Ubx* and *abd-A* mRNAs (Stark *et al*., 2008; Tyler *et al*., 2008). This intriguing observation suggests a very unique phenomenon where these miRNAs might be indirectly helping posterior *Hox* genes for imposing posterior prevalence on anteriorly expressed *Hox* genes. Further analyses of these miRNAs with *Hox* genes show that expression of *mir-iab-4* overlaps with overlapping expression domains of *Antp, Ubx* and *abd-A*, but it can target only *Antp* and *Ubx* mRNAs and not *abd-A* mRNA. So, miR-iab-4-5p would help *abd-A* to down-regulate *Ubx* and *Antp* in its expression domain. Similarly, miR-iab-8-5p has potential to target mRNAs of *Antp, Ubx* and *abd-A* but not *Abd-B*. So, *mir-iab-8* miRNA might indirectly help *Abd-B* function in *Antp, Ubx* and *abd-A* free background in PS 13 and 14. Knock-out of miRNA-iab-4 and miRNA-iab-8 miRNAs leads to higher expression of anterior *Hox* genes in the domain of posterior *Hox* genes (Bender, 2008). This suggests that these two miRNAs help posterior *Hox* genes to function in a lower or no background of anterior *Hox* genes (Singh and Mishra, 2008).

The ~22bp miR-iab-8-5p is produced from a very small region of 92kb *mir-iab-8*. Further analysis of this large ncRNAs was required to know the additional role of this transcript. Gummalla *et. al*. further dissected the transcriptional unit of this large ncRNA and observed that this long transcript uses an additional mechanism to inhibit transcription of *abd-A* in the PS 13 and 14 where *Abd-B* is exclusively expressed (Gummalla *et al*., 2012). Authors observed that the 3' end of the *mir-iab-8* transcript lies just upstream of the *abd-A* gene and transcription of miRNA-iab-8 interferes with the *abd-A* gene promoter. Their observation further suggests that the transcriptional interference imposed by *mir-iab-8* on *abd-A* transcription is stronger and wide spread than miR-iab-8-5p mediated suppression of *abd-A* transcript. This clearly suggests that down-regulation *abd-A*in PS 13 and 14 by *mir-iab-8* and miRNA-iab-8 indirectly helps *Abd-B* to impose posterior prevalence.

Co-factor Competition

Transcriptional suppression by posteriorly expressed *Hox* genes and post-transcriptional regulation by ncRNAs does not completely explain the posterior prevalence phenomenon. This is mainly because we still observe co-expression of *Hox* genes in the *Drosophila* embryos. This indicates that posterior prevalence may operate at post-translational level as well. This is further supported by the observation that, ectopic expression of anterior *Hox* genes in the domain of posterior *Hox* gene using transgenic system fails to counter act posterior prevalence. For example, expression of *Antp* and *Ubx* in the abdominal segments fails to transform abdominal segments (Gonzalez-Reyes *et al*. 1990; Mann and Hogness, 1990). This

means posteriorly expressed *Hox* genes can still block the activity of anteriorly expressed *Hox* gene even in the case of forced expression of anterior *Hox* gene using different promoter and only translated regions of the gene (Gonzalez-Reyes and Morata, 1990). This convincingly suggests that posterior Hox proteins also use post-translational mechanisms to suppress activity of an anteriorly expressed Hox protein.

In *in vitro* conditions Hox proteins are known to bind with very similar sequence and in the presence of cofactors, such as the homeo-domain proteins Extradenticle (Exd), specificity is increased (Mann *et al*., 2009). These observations suggest that competition for co-factors may cause the post-translational mechanism of posterior prevalence. This was tested by Noro *et al*. using *in vivo* reporter gene assay with Scr, an anterior Hox protein and Abd-A, a posterior Hox protein (Noro *et al*., 2011). Authors identified an evolutionary conserved motif in the Abd-A protein, which is required to dominate over Scr protein. This short motif provides selective advantage to Abd-A over Scr for co-factor dependent DNA binding (Noro *et al*., 2011). So in the case of shared binding sites in the genome posterior Hox protein would have a higher affinity than the anterior expressed Hox protein. This clearly shows molecular advantage to posterior Hox protein over the anterior Hox protein to function in the overlapping expression domains.

Exceptions to the Posterior Prevalence Rule

Posterior Hox Gene Activates the Expression of Anterior Hox gene

Till now, we have discussed that expression of many *Hox* genes is found to be overlapping in various para-segments of the embryo. Does overlapping expression have a functional consequence on development of embryo? This question has been analyzed by over-expression and knock-down of *Hox* genes in overlapping expression domains. Miller *et al.* analyzed the expression of anterior *Hox* gene in the case of ectopic expression of a posterior Hox gene in *Drosophila* embryos (Miller *et al*., 2001). Authors observed that ectopic expression of posterior Hox genes, *Scr* and *Dfd* in antennal segment of embryonic epidermis leads to ectopic expression of anterior *Hox* gene *pb*. In wild type embryos, *pb* is known to expressed in epidermal cells of the labial, maxillary and ventral mandibular mesoderm (Miller *et al*., 2001; Pultz *et al*., 1988). Over-expression of *Scr* and *Dfd* showed that only *Scr* represses *pb* expression in mandibular mesoderm while *Dfd* has no effect on the mandibular mesoderm expression of *pb*. Authors further analysed the expression of *pb* in *Scr* and *Dfd* mutants. *Scr* mutant embryos show reduced *pb* expression in posterior labial segment while *Dfd* mutant embryo show reduced *pb* expression in anterior maxillary (Miller *et al*., 2001). In addition, *Scr* and *Dfd* double knock-out embryos show severe reduction of wild type expression of *pb*. This clearly implies that *Scr* and *Dfd* activate *pb* expression in the epidermal cells of the labial, anterior maxillary respectively, while, *Scr* and *Dfd* both are required to express *pb* in mandibular mesoderm. Down-regulation of anterior *Hox* gene by posterior Hox gene follows the posterior prevalence rule, while, activation of anterior *Hox* gene expression by posterior *Hox* gene argues against this rule.

In a similar observation, Foronda *et al*. showed that the expression of *abd-A* is also regulated by *Abd-B* in female genital disc of *Drosophila* (Foronda *et al*., 2006). The *abd-A* gene expresses in A8 segment of female genital disc. Loss of *abd-A* shows defect in development of internal female genitalia, suggesting that, A8 of genital disc develops into internal organs of female genitalia. Analysis of *Abd-B* expression in female genital disc shows an overlapping expression of *abd-A* and *Abd-B* in A8 segment. Loss of *Abd-B* leads to loss of *abd-A* expression and complete loss of external and internal genitalia in females. This convincingly suggests that Abd-B protein maintains the expression of *abd-A* gene and both genes are involved in development of female genitalia. This observation breaks the posterior prevalence rule operating among the *Hox* genes and suggests that they work together for developing female genitalia.

Independent Function of Two Hox Genes in Same Cell

The three *Hox* genes of BX-C determine identity of the last thoracic and all the abdominal segments. *Ubx* expression determines identity of the third thoracic and first abdominal segment, *abd-A* determines identity of abdominal segment 2 to 4 and *Abd-B* expression determines identity of A5 to the last segment of adult. This non-overlapping function of Hox genes does not

match with their embryonic expression. During embryonic development, Ubx expresses in parasegment (PS) 5-12, *abd-A* expresses in PS 7-12 and *Abd-B* expresses in PS 10-14. This suggests that, the expression of *Ubx* and *abd-A* overlap in PS 7-12, while, *Ubx, abd-A* and *Abd-B* overlap from PS 10-12. In our recent study, we analyzed the role of anterior *Hox* gene in the domain of the posterior *Hox* genes during development of the adult abdominal epithelia (Singh and Mishra, 2014). Adult abdominal epithelium develops from histoblast nest cells (HNCs) that are embedded in between larval epithelial cells (LECs) of larvae. At the start of pupal development, HNCs proliferate and induce apoptosis in LECs to replace it with pupal epithelia that ultimately develops into adult abdominal epithelia (Madhavan and Madhavan, 1990; Madhavan and Madhavan, 1980; Ninov *et al*., 2007). We observed the expression of *Ubx* only in thoracic segment 3 and abdominal segment 1 of pupal epithelia and not in posterior segments. This is in contrast to the observed embryonic expression pattern. On the other hand, corresponding embryonic expression of *abd-A* is seen in A2 to A7 segments of pupal epithelia. The expression of *Abd-B* in pupal epithelia clearly matches with embryonic expression pattern (Singh and Mishra, 2014). Similar to embryonic expression, the expression of *Abd-B* is lowest in A5 and highest in A7. We also observed overlapping expression of *abd-A* and *Abd-B* in cells of A5 to A7. The overlapping expression is not only in same segment but also in same nuclei of LECs and HNCs.

Using HNC and LEC specific Gal4 lines we knocked down *abd-A* and *Abd-B* in these cells to understand the cell type specific role of *abd-A* and *Abd-B* during abdominal epithelia development. Knock-down of *abd-A* in HNCs result into loss of abdominal epithelia, while knock-down in LECs showed dorsal closure defect of abdominal epithelia phenotype. Further analysis suggested that in overlapping expression domain (segment 5 to 7), *abd-A* is required in HNCs for their proliferation, while in LECs it is required for their removal during development (Singh and Mishra, 2014). On the other hand, knock-down of *Abd-B* in HNCs show typical loss of *Abd-B* phenotype, where posterior segments gets transformed into anterior segment (Singh and Mishra, 2014). We observed an extra segment in male suggesting transformation of A7 into anterior segment. Interestingly, knock-down of *Abd-B* in LECs did not show any visible phenotype, indicating that expression of *Abd-B* in these cells is either dispensable or knock-down is not good enough to produce a phenotype.

The knock-down of *abd-A* in HNCs showed loss of epithelia in A2 to A6 segment, although *abd-A* is known to determine the identity of A2 to A4 segments only. This suggested that *abd-A* is also required in HNCs of A5 and A6 segments, where it overlaps with *Abd-B*. In a similar experiment, we also observed the transformation of A5 and A6 into anterior segments on knocking down of *Abd-B* in HNCs (Singh and Mishra, 2014). Ectopic expression of *Abd-B* in anterior segments where identity is determined by *abd-A* gene leads to their transformation in posterior segments (Karch *et al*., 1994). This suggests that in overlapping domains *Abd-B* dominates over *abd-A* for providing identity. On the other hand, *abd-A* expression in the overlapping expression domain is required for proliferation of epithelial cells. Loss of the *abd-A* leads to the loss of abdominal epithelia. In wild type male flies, *Abd-B* down-regulates the expression of *abd-A* gene in A7 segment to eliminate this segment from male abdomen (Foronda *et al*., 2012; Singh and Mishra, 2014; Wang *et al*., 2011). Most surprising to us, we observed that higher expression of *abd-A* in A7 segment using *Abd-B* Gal4 leads to emergence of A7 segment in the male flies (Singh and Mishra, 2014). This suggests that, for epithelia formation *abd-A* can function even in the presence of *Abd-B*. So, to conclude, *abd-A* and *Abd-B* have adapted independent functions in same cell for abdominal epithelia formation. This observation challenges the posterior prevalence rule operating among *Hox* genes and indicates a cooperative role during development.

Concluding Remarks

Thus, a close analysis for requirement of posterior prevalence and exceptions to this rule altogether suggests that, the phenomenon of posterior prevalence operates among the *Hox* genes, although, there can be exceptions with respect to time and space of development. *Hox* genes are generally considered as selector or master genes, because their mutation may lead to complete/partial transformation of the body parts (Garcia-Bellido, 1975; Lewis, 1978; Pradel and White, 1998). This suggests, that they work at the top of the gene hierarchy and determine identity of the

cell.This is not true in all the cases and tissue-specific loss of *Hox* gene function suggests that they function at various levels to work as selector as well as a realizator gene (Akam, 1998; Pradel and White, 1998). The instances where posterior *Hox* gene activates the expression of an anterior *Hox* gene, we hypothesize that, anterior *Hox* gene might be allowed to do micromanagements as realizator the presence of a posterior *Hox* gene (Akam, 1998). In all these cases, the function of an anterior *Hox* gene is still under the control of a posterior *Hox* gene suggesting their function is downstream to the posterior Hox gene. Accordingto the posterior prevalence rule, posterior *Hox* genes repress the expression of anterior *Hox* genes, but in these instances, a posterior *Hox* gene activates the expression of an anterior *Hox* gene. So, in the overlapping expression domains, a posterior *Hox* gene would still work as a master gene and anterior *Hox* genes might have adopted a downstream realizator function to carry out diverse cellular functions.

Other example of posterior prevalence shows that two *Hox* genes *abd-A* and *Abd-B* have adopted independent functions for development of abdominal epithelia (Singh and Mishra, 2014). In the overlapping expression domains (A5 to A7), the role of *Abd-B* primarily is providing identity to the epithelial cells, while *abd-A* expression is required for their proliferation. This observation clearly shows that function of *abd-A* in epithelia cell proliferation is possible even in the presence of *Abd-B*. Although there are two important points to mention here, one that the higher expression of *Abd-B* can still suppress the epithelia formation function of *abd-A*, second in non-overlapping domain (A2 to A4) with *Abd-B*, *abd-A* provides identity to the segment and work as a selector gene. From these observations, we conclude that anterior *Hox* gene *abd-A* has adopted realizator function in overlapping domains (A5 to A7) with posterior *Hox* genes, while in non-overlapping expression domains (A2 to A4) with posterior *Hox* gene it still works as a selector gene. It would be really interesting to find an exception of posterior prevalence where an anterior *Hox* gene can determine identity of a cell in the presence of a posterior *Hox* protein.

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