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# Cellular analysis of newly identified Hox downstream genes in *Drosophila*

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ABSTRACT

*Hox* genes code for conserved homeodomain transcription factors, which act as regional regulators for the specification of segmental identities along the anterior-posterior axis in all animals studied. They execute their function mainly through the activation or repression of their downstream genes. We have recently identified a large number of genes to be directly or indirectly targeted by Hox proteins through gene expression profiling in the model organism *Drosophila*. However, the cell-specific regulation of these downstream genes and the functional significance of the regulation are largely unknown. We have validated and functionally studied many of the newly identified downstream genes of the Hox proteins Deformed (Dfd) and Abdominal-B (Abd-B), and provide evidence that Hox proteins regulate a diverse group of downstream genes, from transcription factors to realisators with major and minor roles during morphogenesis.



## Introduction

It has been known for a long time that *Hox* genes, conserved throughout metazoan evolution, specify the identity of body segments along the anterior-posterior (A-P) axis in most animals (Lewis, 1978; McGinnis and Krumlauf, 1992; Mann and Morata, 2000; Pearson et al., 2005). These genes are expressed in strictly fixed and partially overlapping domains (Lewis, 1978; McGinnis and Krumlauf, 1992; Mann and Morata, 2000; Pearson et al., 2005). The activity of Hox proteins is required for the formation of specific morphological features in each segment (McGinnis and Krumlauf, 1992; Mann and Morata, 2000; Pearson et al., 2005).

More than 30 years ago, Garcia-Bellido (1975) proposed a cascade of regulatory events required for proper morphogenesis: the selector gene model. He believed that the on/off status of selector genes would define specific regions within the body and that the at that time unknown selectors would execute this function through the regulation of their downstream (including realisator) genes (Garcia-Bellido, 1975). This model was later supported by genetic studies, which also led to the identification of selector genes (Lawrence and Struhl, 1996; Tautz, 1996; Pradel and White, 1998; Guss et al., 2001), including the *Hox* genes (Lewis, 1978; Kaufman et al., 1990). Hox proteins act as master regulators of morphogenesis along the A-P axis, highlighted by the fact that the mutations in *Hox* genes often result in "homeotic transformation", the switch of one body structure to resemble in form and shape a homologous structure present in the body (Lewis, 1978).

*Hox* genes code for homeodomain-containing transcription factors, thus they function as regional regulators mainly via activation or repression of downstream genes (Hueber et al., 2007; Hueber and Lohmann, 2008). During the last years, substantial efforts have been made to identify Hox downstream genes (summarized in (Hueber and Lohmann, 2008)). In these studies, large numbers of genes were identified to be targeted by Hox proteins, and Hox downstream genes were found across diverse functional classes, ranging from regulatory molecules, like transcription factors and signaling components, to realisators (Leemans et al., 2001; Mohit et al., 2006; Hersh et al., 2007; Hueber et al., 2007; Rohrschneider et al., 2007; Hueber and Lohmann, 2008). Thus, these studies have extensively advanced our knowledge about the nature of Hox target genes, since most of the previously known Hox downstream genes coded primarily for transcription factors, which were identified from the traditional genetic approach based on phenotypic analysis (Hueber and Lohmann, 2008).

We have previously identified a large number of Hox downstream genes by a gene profiling approach (Hueber et al., 2007). To this end, we ubiquitously misexpressed different *Hox* genes, expression changes were monitored subsequently by microarray analysis (Hueber et al., 2007). The approach was considered to be successful, since most of the known Hox downstream genes were recovered by the microarray (Hueber et al., 2007). However, the cell-specific regulation of individual Hox target genes and functional significance and consequence of the regulation with respect to the Hox gene function are still poorly understood. To

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resolve these questions, we started to analyze Hox-dependent regulation and function of many of these newly identified Hox downstream genes at cellular resolution.

In our analysis, we focused on downstream genes of the head-specific *Hox* gene *Deformed* (*Dfd*) and the tail-specific *Hox* gene *Abdominal-B* (*Abd-B*). In sum, we provide evidence that Hox proteins regulate very diverse groups of downstream genes in a very precise temporal-spatial manner.

## Material and methods

### *Drosophila genetics*

*Drosophila melanogaster* stocks were maintained according to standard laboratory procedures. Embryos were collected on apple juice/agar plates supplemented with yeast paste and then allowed to age to desired stages. The *Drosophila melanogaster* strain Oregon-R was used as wild-type strain. $Dfd^{w21}$, $Dfd^{r11}$, $Adb\text{-}B^{M1}$, *arm*-Gal4, UAS-*Abd-B*, UAS-*Dfd*, UAS-*lacZ* have been described previously (Lohmann et al., 2002; Hueber et al., 2007). Other lines used are *69B*-Gal4 (BL1774); *prd*-Gal4 (BL1947); UAS-*Awh* (from J. Curtiss); *ems*-Gal4, $linG^1$, $ems^{9H}$, $ct^{db7}$, *Df(2L)5*, and *Df(1)os1A* (from J. Castelli-Gair Hombria); $qua^1$(BL3350). Trans-heterozygous *Dfd* mutants were generated by crossing the following alleles: $Dfd^{r11}$ and $Dfd^{w21}$. Homozygous mutant embryos for phenotypic analysis were identified by the absence of GFP expression. Balancer chromosomes *FM7c*[*act*::*lacZ*], *CyO*[*wg*::*lacZ*] or *TM3Sb*[*Ubx*::*lacZ*] were used to identify homozygous mutants on X, 2nd or 3rd chromosome, respectively, based on the absence of *lacZ* staining.

### *Histology*

Whole-mount in situ hybridizations were performed as described (Tautz and Pfeifle, 1989; Lohmann, 2003; Hueber et al., 2007). Antisense RNA probes for the Hox downstream genes and the *lacZ* gene were labeled with DIG-dNTP. Gene-specific probes were either created from the transcribed region of genomic DNA or cDNA clone from DGRC: *lea* (LD06565), *CG7447* (LD16414), *Awh* (RE24382), *qua* (RE36860), *CG30069* (LP06813), *skl* (RE14076), *bap* (RE13967), *pox-n* (IP01592), *sens* (IP01345), *cycE* (LD22682), *shf* (GH27042). Biotin-labeled *ems* antisense probes were prepared by PCR amplification of a transcribed genomic region of *ems* with T3 promoter added to the 5′ of antisense primer, and followed by in vitro transcription with T3 RNA polymerase. Primer sequences are available upon request. Duplex in situ hybridizations were performed according to Stöbe et al. (2009). Cuticle preparation of 1st instar larvae was according to Hu and Castelli-Gair (1999). DIC images were taken at a Zeiss AXIO ImagerM1 microscope, and all fluorescent images were taken at a Zeiss LSM510 META confocal microscope.

## Result and discussion

### *Abd-B dependent regulation of Arrowhead (Awh)*

*Arrowhead* (*Awh*) encodes a LIM-homeodomain transcription factor (Curtiss and Heilig, 1997). It has been shown to be required for the establishment of a subset of imaginal tissues: the abdominal histoblasts and the salivary gland imaginal rings (Curtiss and Heilig, 1995, 1997). By analyzing the expression of *Awh*, we found that it might play a role in the posterior spiracle morphogenesis. In stage 11 wild-type embryos, *Awh* is only expressed in the outer region of the posterior spiracle primordium located in the eighth abdominal segment (A8) (Fig. 1A). From embryonic stage 13 onwards, its expression is restricted to the anterior boundary of the posterior spiracle primordium (Fig. 1C). Thus, *Awh* seems to be normally repressed in the central region of the posterior spiracle primordium. We further analyzed *Awh* expression in *Abd-B* mutant embryos (Fig. 1B) and in embryos ubiquitously overexpressing Abd-B (Fig. 1D). Interestingly, we found that *Awh* is expressed in the whole posterior spiracle primordium in *Abd-B* mutants (Fig. 1B), which is reminiscent of its expression in other abdominal segments in wild-type embryos (Fig. 1A). On the other hand, when Abd-B was ectopically expressed, *Awh* transcription was restricted to the boundary of each trunk segment (Fig. 1D), resembling its expression in the A8 segment in wild-type embryos (Fig. 1C). Thus, we assume that Abd-B is able to activate *Awh* expression at the boundary, but is necessary to repress its expression in the central part of the posterior spiracle primordium.

We further studied *Awh* expression in embryos mutant for four known primary target genes of Abd-B: *cut* (*ct*), *empty spiracles* (*ems*), *spalt* (*sal*) and *unpaired* (*upd*) (Hu and Castelli-Gair, 1999;

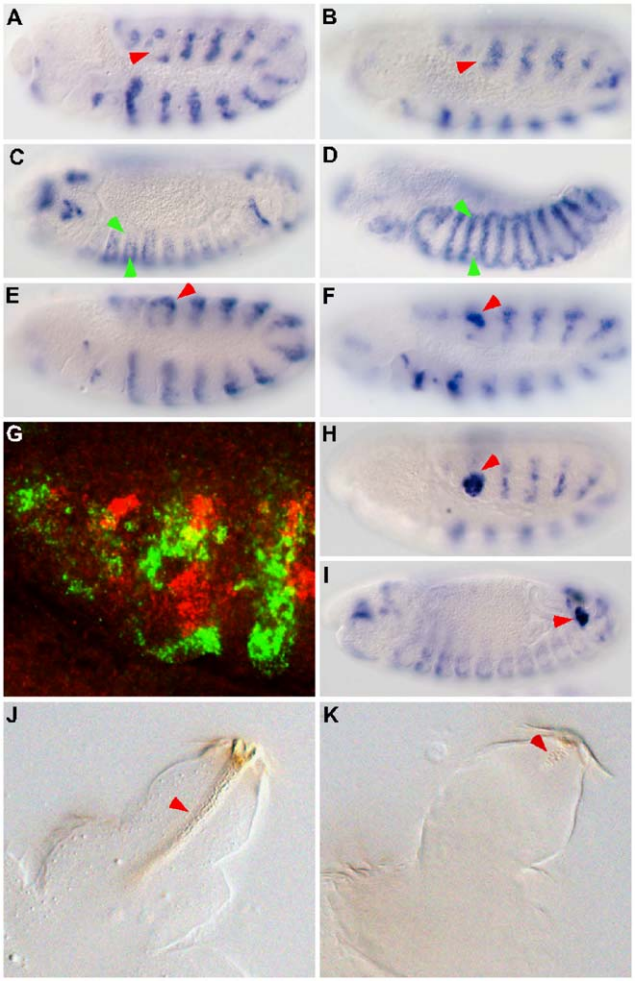


**Fig. 1.** *Awh* is repressed by Abd-B in the posterior spiracle for the survival of filzkoeper cells. (A - I) In situ hybridizations for the detection of *Awh* expression in different genetic backgrounds are shown: wild type (A), $Abd\text{-}B^{M1}$ (B), *69B*::*lacZ* (C), *69B*::*Abd-B* (D), $ems^{9H}$ (E), $lin^{G1}$ (F), *ems*::*Awh* (H, I). In (G) fluorescent duplex in situ hybridizations for the simultaneous detection of *ems* (red) and *Awh* (green) mRNA in stage 11 wild-type embryos are shown. The changes of *Awh* expression are indicated by red arrowheads when happening in the posterior spiracle primordium. Other expression changes are marked by green arrowheads. Cuticle preparations of about 22 hours old (AEL) wild-type (J) and *ems*::*Awh* (K) are shown. The filzkoerper, the lumen connecting the tracheal system to the environment, is indicated by red arrowheads.