Hyped-up for Hox in Hyderabad

Workshop on Upstream and Downstream of Hox Genes

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The EMBO Workshop on Upstream and Downstream of Hox Genes took place in Hyderabad, India, between 14 and 17 December 2005, and was organized by R. Mishra and F. Karch.

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Hox genes have had an illustrious and curious history. Perhaps one of the most famous images in biology from the past century is the four-winged mutant of the fruit fly *Drosophila melanogaster* caused by mutations in the Hox gene *Ultrabithorax (Ubx)*. This fascinating mutant fly, discovered and characterized by Edward B. Lewis, has motivated many investigators to join the field to understand how these genes are responsible for such complex changes in morphology. Another highlight of Hox history was Matthew Scott's, William McGinnis's and Walter Gehring's discovery of the homeodomain—a 60-amino-acid DNA-binding motif now known to be a key domain in hundreds of genes throughout the animal, fungal and plant kingdoms. Although a lot has been learned through experiments stemming from these important discoveries, there are still many open questions. Among the questions being debated in the field and at the meeting are: How is the regulation of the Hox genes initiated and maintained? How do Hox proteins regulate their target genes and what are these target genes? How do they orchestrate the development of entire animal structures? And how did Hox genes impinge on the evolution of life on earth?

Upstream of Hox genes

From the first talk, M. Akam (Cambridge, UK) placed the regulation and function of Hox genes centre stage. He presented evidence that the body segments—the units in which Hox genes often act—of the centipede *Strigamia maritima* are generated through a mechanism that involves repetitive cycles of gene activity. This is similar to the mechanisms that underlie vertebrate somitogenesis (Pourquie, 2003) and is in sharp contrast to a mechanism seen in *Drosophila* that involves the interpretation of unique spatial cues established by gap genes. The new and refined data from centipedes, combined with previous data from spider embryos, suggest that in several invertebrates a Notch–Delta-mediated oscillator generates serially repeated patterns that are present at the onset of the segmentation process.

Obviously, functional data in centipedes, spiders or other organisms will be needed to support this proposal. Although the use of a common molecular scenario to generate segments in certain invertebrates and somites in vertebrates would be a thrilling observation in evolutionary terms, how such oscillators control the segment-specific activation of Hox genes remains an open question. This is just one of the important and fascinating issues that needs to be addressed in future studies (Peel *et al*, 2005).

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For many years, EMBO workshops dealing with new insights into the regulation and function of Hox genes have been held in Switzerland. However, last year, the EMBO World Programme, together with the Centre for Cellular and Molecular Biology (CCMB), held a workshop entitled Upstream and Downstream of Hox Genes in the CCMB's hometown of Hyderabad, India. Imagine beautiful gardens full of butterflies and palm trees, a delightful guest house neighbouring a state-of-the-art research centre, all in a peaceful oasis surrounded by a pulsating and crowded Indian city. The question at the beginning of the meeting was whether the talks and discussions would match the stunning environment; it was answered rapidly and affirmatively.

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Regulation of gene complexes

To nobody's surprise, lively discussions on old but still intriguing questions about the organization of homeotic genes in clusters (Garcia-Fernandez, 2005) took place at the meeting. To what extent are Hox genes clustered in different species? What are the reasons for keeping clusters together during evolution? And how is gene expression in Hox clusters regulated?

D. Chourrout (Bergen, Norway) reported that in the tunicate *Oikopleura dioica*, the Hox genes are not clustered, a situation that seems to occur more frequently than initially expected. A hypothesis for why Hox gene clustering might have been lost in *Oikopleura* is that this species has a determinative mode, which might have rendered superfluous the patterning of the anteroposterior axis. P. Holland (Oxford, UK) reported on studies of a different Hox gene cluster, the ParaHox cluster, which contains three homeobox genes, *gsx*, *xlox* (*pdx*) and *cdx*. This cluster is present in amphioxus, amphibians and mammals, but is interrupted in teleost fish, most probably as a secondary consequence of a previous whole-genome duplication. Although these findings suggest that the conserved clustering of the ParaHox genes in several species is not due to a global control mechanism, much more work is required to validate such a proposal experimentally.

The idea that global control mechanisms are indeed at work in some of the Hox complexes has gained strong support from studies of the HoxD cluster in the mouse. The expression of the posterior genes of the HoxD cluster in the distal limb bud relies on a global control region (GCR) as shown in the results of an elegant series of experiments by the group of D. Duboule (Geneva, Switzerland; Kmita & Duboule, 2003). Studies presented by F. Spitz from this laboratory added yet another twist to the lunatic landscape between the posterior HoxD genes and the distant GCR. Using several genetic tricks, a reporter gene (*Hoxd9lac*) was inserted 350 kb upstream of the HoxD complex, approximately 40 kb away and on the other side of the previously characterized GCR element in a region devoid of active transcriptional units, a so-called 'gene desert'. The introduced reporter gene was expressed in limbs and in brain regions similarly to the genes that flank the GCR and in a manner indistinguishable from the expression of the same transgene when inserted close to *Hoxd13*. This result shows that the regulatory influence of the GCR extends in both directions and over at least 350 kb. Intriguingly, animals homozygous for this remote insertion have strong defects in their hands and feet, a phenocopy of the loss of *Hoxd13*. This suggests that the insertion of a transcriptional unit in this gene desert is detrimental to the organism and that the maintenance of such deserts during evolution is because they contain functional regulatory elements and maintain the appropriate control of gene expression.

S. Tümpel (Kansas City, MO, USA) from R. Krumlauf's laboratory showed that, in contrast to such deserts, certain DNA regions might be crowded with information. In an attempt to identify *cis*-regulatory regions of the *Hoxa2* gene that are responsible for expression in rhombomere 2, Tümpel identified an enhancer in the coding region of the *Hoxa2* gene, 3' from the homeodomain. This is a relatively rare case of an enhancer found in coding sequences and suggests that this particular enhancer is under strong evolutionary selection both at the protein as well as at the expression level.

The establishment of the temporal and spatial collinearity of vertebrate Hox gene clusters remains a fascinating topic. New and striking results related to this issue were reported by O. Pourquie

(Kansas City, MO, USA). The vertebral column is highly regionalized along the anteroposterior axis. During embryogenesis, the regional differentiation of the spine is controlled by Hox genes, which act early in the vertebral precursors that form the somites. Pourquie presented data from experiments on chick embryos suggesting that the progressive (collinear) activation of Hoxb genes in the territory of the epiblast that gives rise to somites regulates the flux of cells from the epiblast to the streak. This process thus contributes directly to the establishment of the characteristic nested gene expression domains in the somites. Overall, this ensures that ingressing cells that express Hox genes from consecutive paralogous groups are sorted along the anteroposterior axis. The result of this ordered ingression of epiblast cells in the streak is the establishment of the nested Hox expression domains in the mesoderm, that is, the spatial collinearity. Therefore, the establishment of the spatial collinearity in the somites of the chick embryo is in part controlled by the Hox genes themselves. In a remarkable set of fluorescence *in situ* hybridization (FISH) images, W. Bickmore (Edinburgh, UK) provided evidence that the sequential activation of the Hox genes correlates with their movement away from the main chromosomal territory, supporting the idea that Hox gene activation depends on chromosomal remodelling. Using three-dimensional FISH studies, G. Cavalli (Montpellier, France) reported that, in the inactive state, the posterior Hox genes from the Bithorax complex (BX-C) localize together with the anterior Hox gene *Antennapedia* (*Antp*) in *Drosophila*, even though these genes are separated by 10 Mbp of intervening genomic DNA. It will be fascinating to learn in future studies how these results relate to the activation of Hox gene expression during early embryogenesis, as described by Pourquie.

The Bithorax complex

A whole session of the meeting was dedicated to the BX-C of *Drosophila* (Maeda & Karch, 2006). The BX-C encodes three Hox proteins, Ubx, abdominal A (abdA) and AbdB, which are responsible for the identities of the segments that form the posterior thorax and the abdomen of the fly. All the talks focused on the *AbdB* gene. Arrays of segment-specific regulatory regions (*iab-4* to *iab-8*; Fig 1), located 3' of the gene, sequentially activate *AbdB* expression along the anteroposterior axis of the embryo. Each of these segment-specific regulatory regions is embedded in a chromosomal domain that functions autonomously owing to the presence of chromatin boundaries that flank the domains. After a regulatory domain has become activated during early embryogenesis, it remains so through the action of the trithorax-group genes (*trxG*). Conversely, the products of the Polycomb-group genes (*PcG*) keep the regulatory domains inactive in segments in which they have not been activated during early embryogenesis. The activity of the *PcG*-repressing complexes is often presented as an on/off switch. H. Gyurkovics (Szeged, Hungary) showed that it is possible to hyperactivate the *iab-7* regulatory domain in *PcG* mutant backgrounds. Thus, *PcG* products are also at work in segments in which the regulatory domains are active, an important observation that points towards a continuous or rheostatic role of the *PcG* products rather than a binary switch.

Several talks focused on the long-distance interactions between the remote regulatory domains and their target, the *AbdB* promoter. In ectopic chromosomal contexts, the BX-C boundaries (*Mcp*, *Fab-7* and *Fab-8*; Fig 1) that normally flank the regulatory domains behave as chromatin insulators, blocking enhancer–promoter interactions when placed between them. This insulator activity is

Fig 1 | *Cis*-regulatory elements in the Bithorax complex in *Drosophila melanogaster*. Regions *iab-5*, *iab-6*, *iab-7* and *iab-8* contain enhancer elements that regulate the expression of the *AbdB* gene in different parasegments. The role of the other regulatory elements is described in the text. The question marks flanking *Fab-6* indicate that this regulatory element has not yet been identified. The figure was kindly provided by F. Karch. *abdA*, abdominal A; *AbdB*, abdominal B; *Antp*, Antennapedia; BX-C, Bithorax complex; *Dfd*, Deformed; *pb*, proboscoped; PRE, Polycomb response element; PTE, promoter-tethering element; PTS, promoter-targeting sequence; *Scr*, Sex combs reduced; *Ubx*, Ultrabithorax.

paradoxical because in their native chromosomal environment the boundaries do not prevent the distally located regulatory domains from interacting with the downstream *AbdB* promoter. Explanations for this paradox came from two talks characterizing specialized regulatory elements that provide specificity to enhancer–promoter interactions. One of these elements consists of the promoter targeting sequences (PTSs) that can overcome an intervening insulator in transgenic assays. J. Zhou (Philadelphia, PA, USA) reviewed several properties of two PTSs found near the *Fab-7* and *Fab-8* boundaries, respectively (Fig 1). First, the PTSs not only allow the distal enhancers to overcome the intervening insulator and interact with the promoter, but also seem to enhance these interactions. Second, in promoter competition assays, the PTSs seem to choose and stabilize the enhancer–promoter interaction. Finally, targeting of the enhancer by the PTSs to one of the two promoters seems to exclude the interaction of another enhancer with the same promoter. R. Drewell (Reno, NV, USA) described the existence of a promoter-tethering element (PTE) located upstream from the *AbdB* promoter. In promoter competition assays, the promoter containing a PTE was regulated favourably by the *iab-5* segment-specific enhancer. Another model that accounts for the paradox of how distal enhancers overcome insulators in the BX-C suggests that boundaries interact with one another to allow distal enhancers to bypass them. Such a model involves the formation of chromatin loops. To test this hypothesis, the laboratory of F. Karch (Geneva, Switzerland) used the Dam methyltransferase to probe for long-distance interactions between *Fab-7* and other regions of the BX-C. Arrays of Gal4-binding sites were introduced in the native *Fab-7* region of the BX-C by gene conversion. A transgene fusing Dam to Gal4 was then introduced into the strain, which led to the recruitment of Dam at *Fab-7* and local DNA methylation in the proximity of the boundary. Interestingly, Karch reported that strong methylation was also observed at the *AbdB* promoter 35 kb away, indicating that the two

regions are in close proximity *in vivo* owing to a DNA loop. Moreover, this methylation pattern in the *AbdB* promoter required an intact *Fab-7*. These experiments provide the first direct evidence for long-distance physical interactions between certain regulatory elements in the BX-C. Karch also reported that replacing *Fab-7* with *Fab-8* did not alter segment specification, strongly suggesting that these two elements behave in an identical manner, but are sequentially regulated.

Epigenetic control of Hox genes

A particularly intensely discussed topic at the meeting was the molecular nature of protein complexes bound to Polycomb response elements (PREs), including their presence at the PRE in the active and the repressed state, as well as their role in chromatin remodelling. These studies are moving ahead rapidly because biochemical studies in flies and other organisms are at an advanced level and are being linked to genetic loss-of-function and gain-of-function data (Ringrose & Paro, 2004; Zhang *et al*, 2004).

Drosophila PcG proteins work in concert to maintain the transcriptional silence of their target genes, including the Hox genes of the ANTP-C and BX-C gene complexes. The trxG proteins antagonize PcG-dependent repression, acting as either anti-repressors or co-activators. Two PcG protein complexes have been purified from *Drosophila* embryos. The PRC1 complex includes the PcG proteins Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc) and Ring1 (also known as Sce), in addition to Zeste (Z) and several general transcription factors. PRC1 has been shown to inhibit chromatin remodelling by human SWI/SNF complexes and to inhibit transcription *in vitro*. The ESC–E(Z) (also known as PRC2) complex includes the PcG proteins Extra sex combs (Esc), Enhancer of zeste [E(Z)], Suppressor of zeste 12 [SU(Z)12] and Nurf55, a histone-binding protein that is present in several other chromatin complexes. E(Z) is a SET-domain protein, which has histone methyltransferase activity.

E(Z) and its human homologue, EZH2, primarily methylate histone H3 at lysine 27 (H3-K27). The chromodomain of Pc binds preferentially to H3 tails methylated at K27 *in vitro*.

R. Jones (Dallas, TX, USA) reported on studies that focused on PcG-dependent repression of the Hox gene *Ubx* in wing imaginal discs. Chromatin immunoprecipitation (ChIP) assays of wild-type and PcG-mutant wing discs have revealed the presence of several PcG complexes at a PRE in the *bxd* region, a regulatory region approximately 25 kb upstream of the *Ubx* promoter. This led Jones to propose a model for a hierarchical recruitment pathway. The sequence-specific DNA-binding PcG proteins Pho and Pho-like (Pho-L), homologues of the human YY1 protein, interact directly with subunits of the ESC–E(Z) complex and are needed for E(Z) recruitment to the *bxd* PRE. In turn, E(Z) is required for recruitment of PRC1 (or a related Pc-containing complex). Previous studies proposed that E(Z)-methylated H3-K27 (H3mK27) acts as a tag that facilitates PRC1 recruitment. Direct Pho–Pc interaction might also contribute to PRC1 binding. Recruitment to the *bxd* PRE leads to the association of PRC1 and ESC–E(Z) with sites downstream of the *Ubx* promoter.

Jones reported that heat inactivation of the temperature-sensitive E(Z)⁶¹ protein results in immediate loss of E(Z) from the *bxd* PRE, followed by loss of H3mK27 no more than one hour later. Concomitant with H3mK27 loss, transcription through the non-coding *bxd* region became detectable. Replacement of histone H3 by the H3.3 variant has been observed at actively transcribed regions throughout the *Drosophila* genome (Schwartz & Ahmad, 2005). This suggests that transcription through the PRE could provide a mechanism for the rapid replacement of histones bearing the H3mK27 epigenetic mark.

Surprisingly, Jones reported that RNA polymerase was also present at the transcriptionally silenced *Ubx* promoter in wing discs. Thus, the PcG protein complexes do not seem to repress transcription by preventing assembly of pre-initiation complexes, but rather by blocking transcription initiation and/or elongation. In haltere discs, PcG complexes were not found immediately downstream of the *Ubx* promoter, but instead ~1.5 kb downstream. Kismet (KIS) is a trxB protein that shares sequence homologies with ATP-dependent chromatin remodelling proteins and seems to be required for transcription initiation or early steps in elongation (Srinivasan *et al*, 2005). In ChIP assays, KIS was found to be located immediately downstream of the *Ubx* promoter in haltere discs, consistent with a potential role in promoter escape by RNA polymerase. These studies are consistent with a model in which the inhibition of KIS chromatin remodelling activity might be one mechanism by which the PcG represses transcription.

J. Müller (Heidelberg, Germany) reported the purification and characterization of a new PcG protein complex that contains Pho. Biochemically purified Pho complexes are distinct from PRC1 and PRC2 but genetic and molecular analyses of one of these Pho complexes revealed that it contains a novel PcG protein with a unique discriminatory binding activity for methylated lysine residues in histones H3 and H4. Müller presented evidence that Pho targets the complex to PREs, and this suggests that the modified histone-binding activity might be needed for maintaining the flanking chromatin in a Polycomb-repressed state, rather than for the initial recruitment of the complex. Further studies are required to shed more light on the different PRC complexes and their exact functions.

Downstream of Hox genes

Before the downstream target genes were even discussed, A. Prochiantz (Paris, France) reported on a new role for some Hox proteins as signalling molecules in the developing nervous system (rather than as transcription factors acting in a cell-autonomous fashion). After the fortuitous observation that an Antp protein could be taken up by cells when added to the culture medium, Prochiantz's group has studied this phenomenon in cultured cells and in developing organ systems, focusing on a non-autonomous role for Engrailed-2 (En-2) in axon guidance. Prochiantz's fascinating idea is that En-2 can be secreted and then internalized by growing axons, in which it might regulate translation of mRNAs in growth cones. The data presented were striking and seem to confirm that such a mechanism occurs *in vivo*. Whether such a role can also be attributed to the Hox proteins discussed here remains to be investigated.

Many questions remain about how Hox proteins regulate transcription and with which other proteins they interact (Pearson *et al*, 2005). Y. Graba (Marseille, France) reported on the function of conserved peptide sequences outside the homeodomain. Interestingly, different motifs conserved either in all homeotic proteins or in homologues across different species act as activity modulators and influence protein function differently at distinct target genes, suggesting that there is a complex network of protein–protein interactions that governs functional specificities. F. Prince from W. Gehring's laboratory (Basel, Switzerland) reported on an interaction between the highly conserved YPWM motif in Antp and a component of the basal transcription machinery, suggesting that the same motif might be involved in interactions with different proteins (the YPWM motif has already been shown to interact with the homeodomain of Extradenticle, a well-studied co-factor of homeotic proteins). Obviously, these studies are just beginning, and much more biochemical work needs to be done before gene regulation by Hox proteins is fully understood.

Several themes emerged for the types of gene and process that Hox genes regulate to control cellular identities and segmental morphologies. In flies, one of the hallmarks of Hox activity is their ability to convert one homologous structure into another. Lewis's four-winged *Ubx* mutant fly, which illustrates how one selector gene chooses the haltere fate over the wing fate, is one of the most famous examples of these phenotypes, but to the Hox biologist, they are commonplace. D. Cribbs (Toulouse, France) described how the Hox gene *proboscipedia* (*pb*) selects for the proboscis fate in the fly by downregulating the Hedgehog (Hh) signalling pathway, perhaps by interfering directly with the activity of the downstream Hh transcription factor, Cubitus interruptus (Ci). The result of this regulation is reduced *wingless* (*wg*) and *decapentaplegic* (*dpp*) expression in the labial imaginal disc. Continuing with the idea that Hox proteins modulate signalling pathway activities, three talks (E. Sanchez-Herrero, Madrid, Spain; R. Mann, New York, NY, USA; and K. Makhajani from L. Shashidhara's group, Hyderabad, India) presented data suggesting that *Ubx* reduces the size of the haltere in the fly by downregulating Dpp signalling. *Ubx* seems to execute this size difference (relative to the homologous wing appendage) by affecting several steps in the Dpp pathway, including *dpp* expression levels as well as steps downstream of Dpp production. Similarly, Shashidhara reported that *Ubx* also reduces the amount of signalling by the epidermal growth factor receptor pathway in the haltere.

Hox genes also have a key role in organogenesis. M. Semeriva (Marseille, France) described his laboratory's elegant efforts to describe the role of Hox genes in the development of the fly heart. Semeriva described three broad steps. First, Hox genes are required to distinguish anterior aorta—requiring no Hox input—from posterior aorta, which can be defined by any one of three Hox genes, *Antp*, *Ubx* or *abdA*. In step two, Hox genes specify particular heart structures. In the third and perhaps the most curious step, the larval heart is remodelled into the adult heart in a Hox-dependent manner, but without any cell proliferation. Also, some of the same Hox genes are used at this step as in earlier steps, raising the question of how this switch in Hox activity occurs. Semeriva's idea is that Hox collaborates with genes induced by the steroid hormone ecdysone to carry out these later remodelling events. It will be interesting to identify the target genes of this remodelling step.

Perhaps one of the most fascinating talks, by K. Vijay Raghavan (Bangalore, India), addressed how fly larvae move in a peristaltic-like manner. It seems that Hox genes are important for this process, probably to help define both the motor neurons and the musculature in the abdomen. Raghavan showed movies of crawling mutant larvae to demonstrate that either *Ubx* or *abdA* were required for the segments to generate these wave-like movements. It seems that either of these genes is sufficient to allow these movements to occur. There is also a genetic screen underway in Bangalore to identify the neuronal basis of this movement, which we hope to hear more about at the next Hox meeting.

In addition to modulating signalling pathways, Hox genes can also regulate cell death. A. Gould (London, UK) reviewed how Hox genes control neuroblast lifespan by altering the number of progeny produced by a fly neuroblast. A burst of expression of *abdA* results in the activation of proapoptotic death genes, a process that requires the competency factor, grainyhead. Using neuroblast clone size as a readout, Gould also described a fruitful (no pun intended) genetic screen for proliferation mutants that led to the identification of the fly homologue of the yeast kinase Dsk1. Although the target for Dsk1 is not known, Gould observed a decrease in AbdA protein levels, suggesting that its effects on neuroblast proliferation could be caused by an effect on *abdA*'s ability to regulate apoptosis.

One phenotype of Hox genes already described by Ed Lewis was the break of the tracheal tree in the absence of the BX-C. M. Affolter (Basel, Switzerland) presented evidence that the Hox co-factors Homothorax and Extradenticle are required for the synthesis of the fibroblast growth factor ligand Branchless in a mesodermal cell that is essential for branch fusion of adjacent metameres. Surprisingly, the same defect in the absence of the posterior Hox genes is due to an effect on tracheal fusion cells and not on mesodermal cells. Hox genes seem to act in small groups of cells or even in single cells during tracheal development, consistent with their role as micromanagers rather than global regulators (Akam, 1998).

The meeting ended with two talks highlighting the accomplishments and the personality of Ed Lewis. G. Morata (Madrid, Spain) presented the Madrid view of the BX-C. In a lucid, vivid, and at times humorous, description of the Hox field, from the isolation of the *bithorax* mutation to the cloning of the entire complex in 1995,

Morata expressed both his respect for Lewis and outlined the seminal contributions made by himself and other Spaniards to our understanding of Hox genes. In a more personal account, I. Duncan (St Louis, MO, USA) gave us a heartfelt view of Lewis as a generous friend, mentor, flautist and wonderful colleague. All in all, it was a terrific and fitting way to spend our last afternoon in Hyderabad.

Every evening throughout the week, a delicious Indian buffet, with fresh bread grilled in authentic tandoori ovens before our eyes on the roof of the research institute, gave ample time for students and lecturers to exchange ideas, plan future collaborations, and discuss adventures during excursions and shopping trips into Hyderabad. The impressions gathered from participants of the meeting were unanimous: a stimulating meeting in a thrilling setting, with the staff and the Principle Investigators of the CCMB as the perfect hosts.

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