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Hox Genes

Methods and Protocols

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Preface

Hox Genes: A Fertile Interplay of Concepts and Methods

Why a *Methods in Molecular Biology* issue on Hox genes? "Methods" books usually focus on a set of techniques suited to a given experimental approach. Here, it was chosen to present techniques and methods centered on a research topic: Hox genes. The study of Hox genes has been at the heart of the emerging molecular embryology, molecular genetics of development, and evolutionary-developmental biology (evo-devo). Key steps have been the identification of "A gene complex controlling segmentation in *Drosophila*" reported by Ed Lewis in 1978, the cloning of bithorax in the early 1980s, the discovery of homeobox genes in vertebrate genomes, and the discovery that Hox gene functions in patterning embryos are conserved [1–6].

Hox genes have been initially identified as crucial determinants to pattern the main anteroposterior body axis of animal embryos [1]. Their functions however rapidly emerged as extending beyond axial patterning, with involvement in multiple organogenetic processes, including but not restricted to limb, heart, gut, respiratory, urogenital, or endocrine organogenesis, as well as in later neuronal circuit connectivity or in adult hematopoiesis [7, 8]. The repertoire of Hox functions has become immensely vast, and their recognized role in a number of pathologies is likely only an under-representation of the consequences of their dysfunction [9]. Hox genes encode transcription factors [10], and their extensive structural and functional conservation allowed apprehending how variations in gene sequence and activity underlie morphological evolution [11–15].

The study of Hox genes has been paradigmatic for the unification of biology by the end of the twentieth century, when the emergence of molecular biology promoted the interconnection and cross-influence of most fields of biological sciences. Formerly, experimental embryology, genetics, biochemistry, physiology, evolutionary biology, and other life sciences were loosely cross-talking due to a lack of unifying concepts, as well as difficulties in appraising the biological reality in a holistic way, from the bottom molecular scale to the top, organismic (or ecosystemic) scale. Equally responsible for the limited cross talk between disciplines were the technological difficulties and deficit to appraise questions from a multiscale point of view. In the 1990s, these challenges had been taken up with the convergence of genome sequencing projects, reverse molecular genetics, descriptive genetics, embryology, and histology of model organisms like *Drosophila*, the mouse, the worm *Caenorhabditis elegans*, *Xenopus*, or the zebrafish. The study of Hox genes has accompanied this unifying movement, feeding and being fed by this forwards-backwards movement between methodological development and concept evolution, questions raised, and strategies to address them.

In this issue of *Methods in Molecular Biology*, we aim at reviewing techniques and methodologies which arose from or were successfully applied to the study of Hox genes and Hox proteins. This overview does not pretend, by far, to be exhaustive and only provides a sample of possible experimental approaches that have accompanied 35 years of research in

Hox biology. By essence, the techniques and strategies described in the 21 chapters constitutive of this *Methods in Molecular Biology* issue on Hox genes are also relevant beyond the Hox field.

Extracting Information from Genome and Gene Sequences

Comparative studies permit insights into the degree of freedom in molecular evolution that results from a balance between permissiveness and restrictiveness to changes. This depends on the biochemical, structural, and functional constraints acting on molecules as well as on the robustness and plasticity of the molecules and of the networks of interacting actors involved. Such comparative studies, notably focused on Hox and homeobox genes more broadly, are facilitated by the availability of fully sequenced and assembled genomes, which is the case for classical animal models but lacking for non-model organisms. With the now powerful capacity of high-throughput sequencing platforms, the perspective of sequencing new animal genomes is conceivable for most research projects. Marlétaz et al. describe the rationale and methods to proceed with genome sequencing and assembly. They provide relevant reference to bioinformatic tools, which allow homeobox gene extraction and classification, thereby enabling the reconstruction of the phylogenetic history of homeobox genes.

Comparative studies not only focus on protein-coding sequence but also on *cis*-regulatory elements governing Hox gene expression. This is essential as, in many instances, the functional specificity of Hox genes is driven by their expression levels and patterns rather than by the specificity of the corresponding Hox proteins. This has been shown to especially stand for Hox genes belonging to a same paralogy group in vertebrates [16, 17]. Furthermore, evolutionary changes in Hox gene regulation have been identified to be at the heart of body plan diversification, as it has been illustrated in chelicerates, myriapods, and insects or in snakes, lacertilians, and mammals for example [18, 19]. In their contribution, Matharu and Mishra discuss the challenging issue of identifying Hox cis-regulatory sequences and suggest strategies to mine the most plausible Hox regulatory information.

Manipulating Genomes

Once genes are identified and putative regulatory elements inferred, their functional analysis will heavily rely on genome manipulation. In the mouse, since the initial gene recombination strategies that led to the first gene knockouts in the late 1980s to early 1990s [20, 21], a tremendous number of tools have been developed relying on the use of site-specific recombination systems and on the generation of vectors allowing to carry hundreds of kilobases. Genome manipulation strategies and techniques nowadays are versatile and permit inverting, deleting, and exchanging from single genes to entire Hox clusters, thereby allowing the study of long-distance mechanisms of gene regulation. In their chapter, Tschopp and Duboule review the vast repertoire of alleles displaying lox recombination sites suited to engineer modified Hox gene clusters and provide guidance on how to design and to proceed with site-specific recombination-based genome engineering techniques.

Working with bacterial artificial chromosomes (BACs) as recombination vectors allows the manipulation of large sequences encompassing multiple genes and their associated regulatory sequences. Parrish et al. report how combining BAC transgenesis with site-specific recombination provides enough modularity to tackle the complexity of Hox gene biology by inactivating, deleting, or reassorting genes or sequence elements.

In *Drosophila*, a convenient way to manipulate the genome relies on P-element mobilization. In their chapter, de Navas et al. present a gene replacement strategy to insert P-GAL4 elements in Hox genes so as to induce UAS-responsive genes in cells where the

Hox::P-GAL4 is active, in a context mutant for the resident Hox gene. The UAS-driven gene may be chosen to promote reporter activity or to express mutant Hox variants or any other genes of interest, making the approach useful to study both protein function and *cis* -regulatory elements. While lacking the targeted precision of site-specific recombination, the methodology takes advantage of the facility to screen for phenotypic markers.

Regulatory Landscapes and Topological Organization of Regulatory Elements

Gene regulation relies on short-range as well as long-range regulatory mechanisms, highlighting the necessity to apprehend the role of regulatory landscapes and nuclear topological organization. Recently developed approaches now allow grasping the physical connection between remote regulatory elements and gene promoters. These approaches capture DNA synapses involved in enhanceosomes or silenceosomes. Generic methods include 3C, 4C, 5C, Hi-C, ChIP-loop, or ChIA-PET [22]. Tagging DNA elements involved in such distant contacts is another way to map distant chromosome interactions. Cléard et al. describe an elegant DNA tagging method in *Drosophila* based on fusing the bacterial DAM DNA methyltransferase to a DNA-binding or a chromatin-binding protein that targets the methyltransferase activity towards specific DNA elements (insulators, enhancers/silencers, promoters, …). In the method detailed here, suited for genomes poorly methylated only, the DAM methyltransferase is fused to the DNA-binding domain of GAL4, and the long-range DNA-DNA interactions are mapped from a UAS sequence recognized by GAL4 and inserted at a locus of interest. Since DAM methylate GATC sequences which cleavage by DpnI and DpnII are methylation dependent, DNA methylation can easily be located both on a loci-specific or on a genome-wide scale.

A somewhat conceptually similar approach is presented by Agelopoulos et al. and consists in cell- and gene-specific chromatin immunoprecipitation (cgChIP). It involves the tagging of a *cis*-regulatory element of interest (providing the gene specificity) with a lacO sequence that allows the recruitment of an epitope-tagged LacI expressed in a cell-specific way using the UAS-GAL4 system (providing the cell specificity). ChIPing the tagged LacI allows fishing distant DNA elements in contact with the *cis*-regulatory element specifically in cells expressing LacI. Combining cgChIP with a second immunoprecipitation step or with western blot analyses further allows the identification of proteins involved in connecting the *cis*-regulatory element with the remote DNA element.

Long-distance interactions involving regulatory sequences serve to not only connect remote regulatory sequences to gene promoters but also locate genes within the nucleus according to their transcriptional status. This highlights the importance of viewing gene regulation in correlation with the topological organization of the nucleus. Coupling DNA fluorescent in situ hybridization (FISH) with immunostaining (FISH-I) allows visualizing DNA topology and DNA relocation in nuclear sub-compartments. Bantignies and Cavalli describe how these techniques capture Hox gene nuclear relocation upon transcriptional activation and repression in *Drosophila* embryos and cultured cells.

Recognizing Expression Patterns

The function of a gene primarily depends on its temporal and spatial deployment, making the characterization of gene expression patterns an obligatory step in understanding gene functions. Hox gene studies largely relied on but also contributed to the development and refinement of in situ mRNA hybridization techniques and immunohistochemical detection of proteins [23–25]. These expression studies uncovered the collinearity relationship whereby gene order in the Hox clusters relates to the embryonic territories where they are active.

While prominent aspects of a gene expression pattern are usually rapidly identified, expression at early developmental stages often characterized by the paucity of material, size of biological samples, and weak expression levels renders grasping some aspects of the pattern quite challenging. Illustrative of this difficulty, early Hox gene expression, a way before the onset of gastrulation in vertebrates, was only recently identified. Characterizing gene expression in single oocytes, zygotes, morula stage, or blastocyst embryos raises new technical challenges. Paul et al. describe a set of techniques useful to analyze gene expression in oocytes and early bovine embryos.

Complementary to ISH or IHC techniques which identify where and when Hox genes are active, it is also extremely informative in a functional perspective to assess the destiny of cells that have expressed Hox genes at an earlier time of their ontogeny. Lineage tracing meets this objective. As a straightforward way to trace cell lineages in mouse, Laforest et al. present a binary transgenic strategy combining cell- or tissue-specific Cre-expressing transgene with the induction of reporter genes inserted in the permissive ROSA26 locus. Once the reporter has been switched on by Cre-mediated recombination, it remains activated in all their descent. Such approaches have been instructive to better understand Hox-knockout phenotypes that impact on structures which at late stages of development do not express Hox genes. Lineage tracing provided evidence that cells having expressed the gene at earlier stages of development actually contribute to these structures and that activity of the gene is critical for the proper determination, migration, and functional allocation of these sometimes distant cells, as exemplified by migratory neural crest cells [26, 27].

Addressing Gene Function in Vertebrate Model Organisms

Addressing the functions of Hox genes is at the heart of developmental biology. There are numerous approaches to invalidate gene functions. Gene inactivation by homologous recombination has been a major approach to inactivate genes in the mouse. Recombining and engineering the mouse genome have increasingly become more amenable by the use of site-specific recombinases and BAC recombination vectors, as described by Tschopp and Duboule and Parrish et al. Such approaches require massive access to the genome, as allowed by electroporation of ES cells in mouse, which is not always the case. This for example applies to the zebrafish. Ladam and Sagerström describe how Hox gene inactivation is achieved taking the advantage of accessing the living, translucid, one- or two-cell fish embryos to inject antisense morpholinos, mRNA, or TOL2-transposon-based transgenes. Studying gene function will certainly gain efficacy with new targeted recombination systems derived from CRISPR-cas9 or TALEN [28] that appear to be usable in most living organism, from *C. elegans* to human cell lines and plants.

Addressing Gene Function in Non-model Organisms

Studying non-model organisms is central to evo-devo and constitutes a major challenge for the future of Hox gene studies. It should delineate the variations as well as the constraints underlying Hox gene and protein evolution and provide a phylogenetic perspective on Hox gene function. The study of Parhyale is illustrative of a non-model organism for which transgenesis tools have recently been developed and applied to Hox biology. Parhyale is a crustacean, a sister group of hexapods showing a direct embryonic development. Kontarakis and Pavlopoulos provide a description of this organism supporting the soundness of investing tools to study its Hox genes. They also present how to carry out transposon-based transient and stable transgenesis in Parhyale.

The bovine embryo has slow one cell-to-blastocyst stage transition, favoring expression and functional studies at this transition when compared to the mouse. Hox transcripts and proteins accumulate during oocyte maturation and varying Hox transcript abundance in early bovine embryos indicates that Hox genes could play roles at the onset of embryonic development. Paul et al. have set up RNA interference to knock down mRNA levels by microinjecting siRNAs in oocytes or zygotes in the bovine embryo, which should allow addressing this possible very early Hox gene function.

Identifying the Genomic Distribution and Target Genes of Hox Proteins

Hox proteins are transcription factors. Grasping how Hox proteins fulfill their function requires apprehending genome occupancy and genome landscapes associated with Hox protein activity as well as the identification of Hox target genes [29–31].

Recognizing Hox targets has first relied on candidate gene approaches, resulting in a slowly growing list of target genes. With the entry into the post-genomic era and the development of DNA microarrays, we now have a more comprehensive view of Hox downstream target genes. Polychronidou and Lohmann describe how to proceed to run mRNA extraction and microarray hybridization from *Drosophila* embryos. An additional layer of resolution in generated datasets can be achieved by sorting cells to increase sample homogeneity. Defaye and Perrin propose a method to FACS cells based on cell-specific GFP expression in a way that is quantitatively compatible to carry out a transcriptomic characterization of tissue-specific cell populations.

Transcriptomic approaches do not discriminate between direct and indirect target genes. Crossing transcriptomic data with the genomic distribution of Hox proteins should in principle identify direct target genes. This is now routinely achieved through chromatin immunoprecipitation (ChIP) that requires a ChIP-grade antibody directed against the protein of interest and that allows the recovery of the chromatin associated to that protein. The approach carried out at a genome-wide scale is informative to map Hox-responsive enhancers but also to predict Hox protein partners that may influence Hox DNA-binding specificities. Amin and Bobola describe how to proceed with ChIP and ChIP-Seq following microdissection of mouse embryonic territories, i.e., branchial arches, and Agrawal and Shashidara describe a method to perform ChIP analysis from dissected *Drosophila* imaginal discs.

Probing Protein-DNA Interactions

Understanding the mode of action of transcription factors requires deciphering the molecular rules governing the selectivity, affinity, and specificity in DNA binding. For homeoproteins, and Hox proteins in particular, there appears to be a paradox in that their homeodomain is extensively conserved while the genetics establishes that their functions and activities are specific. This paradox can at least partly be resolved considering that Hox proteins do not act alone but along with cofactors proposed to increase Hox DNA-binding specificities.

Techniques to appraise the DNA-binding specificity of Hox proteins are diverse. Electrophoresis mobility shift assays (EMSA) have been widely used to study Hox DNA-binding properties [32–34]. Churion et al. describe how EMSA techniques allow us to quantitatively determine DNA-binding specificities and interaction affinities. Quantitative EMSA also enables tackling cooperativity and stoichiometry in DNA binding. As pointed out by Churion et al., working with Hox proteins is especially challenging since these proteins are prone to form aggregates [35]. The authors also address this issue in the context of EMSA experiments.

A major limitation of EMSA experiments is that they are not suited to handle extensive repertoires of sequences. The SELEX-Seq method described by Riley et al. circumvents this limitation. It consists in a variant of SELEX, or "Systematic Evolution of Ligands by Exponential Enrichment," combined to high-throughput next-generation sequencing. The principle of the method is to prepare an extensive library of DNA probes and to proceed with cycles of selection-amplification of protein-bound sequences to determine, qualitatively and quantitatively, the repertoire of sequences bound by a protein or a protein complex. Deep sequencing permits informative data to emerge in the very first round of the selection procedure and allows the identification of multiple preferred binding sequences.

Identifying Protein Partners

Partnerships with other DNA-binding proteins have been central to apprehend Hox protein function, with the most illustrative example being provided by the evolutionarily conserved three amino acid loop extension (TALE) homeodomain proteins [36–39]. Protein partnership also has the potential to uncover possible novel functions, including non-transcriptional processes. Two proteome-wide interaction screens provided data suggesting such functions for Hox proteins [40, 41]. Bergiers et al. present methods to screen for interactions by high-stringency yeast two-hybrid assays, to validate candidate interactions by affinity coprecipitation, and to build interaction networks. These methods have an important heuristic value, but data nevertheless need to be anchored in biological contexts. An elegant way to validate protein interactions, to probe their structural determinants, and to follow them up in vivo is reported by Duffraisse et al. who describe how to carry out bimolecular fluorescence complementation (BiFC) in live *Drosophila* embryos. The BiFC rationale is to fuse semi-GFPs (or Venus) to two validated or candidate interactors which upon interaction will reconstitute a functional fluorescent protein. The method is sensitive and allows mapping binding interfaces, following intracellular shuttling and monitoring live interactions.

From the Bench to the Clinic

There is a wealth of evidence supporting roles for Hox proteins in pathological processes. The most documented situations relate to cancers and in particular to hematological malignancies [9]. Correlating Hox gene activities to disease development is informative to refine the typology and etiology of pathologies. Further, such correlations are also valuable to formulate prognostics and to design therapeutic strategies. In that context, Kettyle et al. describe procedures to establish correlations between transcriptomic profiling of a given disease, which in the paradigm the authors describe consists in HOX-TALE-related leukemia, and transcriptomic changes elicited by exposure to a small molecule. This approach, referred to as "connectivity mapping," is based on functional correlations rather than on structural modeling and allows screening for molecules which can be envisioned as a putative drug to treat the disease.

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